Division of

Cancer Etiology

1988 Annual Report
Intramural Activities
Volume I

October 1, 1987
September 30, 1988

U.S. DEPARTMENT
OF HEALTH
AND HUMAN SERVICES

National
Institutes of
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National
Cancer
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Bethesda,
Maryland 20892

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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

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ANNUAL REPORT
OF THE
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

Richard H. Adamson, Ph.D., Director

October 1, 1987 through September 30, 1988

OVERVIEW

The Division of Cancer Etiology (DCE) is comprised of three major programs: the Biological Carcinogenesis Program, the Chemical and Physical Carcinogenesis Program and the Epidemiology and Biostatistics Program. The Biological Carcinogenesis Program consists of one extramural component (the Biological Carcinogenesis Branch) and six intramural laboratories (the Laboratory of Cellular and Molecular Biology, the Laboratory of Molecular Oncology, the Laboratory of Molecular Virology, the Laboratory of Tumor Virus Biology, the Laboratory of Viral Carcinogenesis and the Laboratory of Tumor Cell Biology). The Chemical and Physical Carcinogenesis Program consists of two extramural components (the Chemical and Physical Carcinogenesis Branch and the Radiation Effects Branch) and eight intramural laboratories (the Laboratory of Biology, the Laboratory of Cellular Carcinogenesis and Tumor Promotion, the Laboratory of Chemoprevention, the Laboratory of Comparative Carcinogenesis, the Laboratory of Experimental Carcinogenesis, the Laboratory of Experimental Pathology, the Laboratory of Human Carcinogenesis and the Laboratory of Molecular Carcinogenesis). The Epidemiology and Biostatistics Program consists of one extramural component (the Extramural Programs Branch) and four intramural branches (the Biostatistics Branch, the Clinical Epidemiology Branch, the Environmental Epidemiology Branch and the Radiation Epidemiology Branch).

The Division has been structured in such a way as to maximize interaction between intramural scientists and the extramural community, facilitate the sharing of scarce resources and generally improve the management of research in cancer etiology. Efforts continue to further increase interaction between the biological carcinogenesis and chemical carcinogenesis laboratories and to enhance their interaction with the components of the Epidemiology and Biostatistics Program. The current organizational chart for DCE is shown in Figure 1. During the past year an Associate Director for Biological Carcinogenesis has joined the DCE staff. Included in his activities is the direction of DCE's vaccine research and development efforts.

The distribution of funds among the intramural and extramural components of the Chemical and Physical Carcinogenesis Program, the Biological Carcinogenesis Program and the Epidemiology and Biostatistics Program is shown in Table 1 and Figure 2.

The past year has seen a continued reduction in overall contract support. This has been realized by reducing activities which provide materials and services, and by initiating and continuing various cost-recovery mechanisms. For example, in the Biological Carcinogenesis Branch five

mechanisms. For example, in the Biological Carcinogenesis Branch five resource contracts are functioning in the cost-recovery, or "payback" mode. These include one for production of viral reagents, one for animal resources, two for specialized testing services and one for storage and distribution of stored frozen biological reagents. In the Chemical and Physical Carcinogenesis Branch, payback systems have been established for the Radiochemical Repository and for the Chemical Carcinogen Reference Standard Repository. All samples distributed under the chemical research resource program are now under this cost-recovery system. Reimbursement for full or partial costs of services has led to a more careful use of costly resource reagents and chemicals, with a subsequent reduced level of effort in several resource contracts or the termination of unnecessary activities. As support for research contracts has decreased, support for investigator-initiated research grants has continued to increase, and the Cooperative Agreement is now being utilized as an additional instrument of support. In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Facility (FCRF)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRF has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens. During the past year, the position of Associate director for FCRF has been created, and the position filled by Dr. Werner Kirsten. FCRF is the focus of NCI research on AIDS, with particular emphasis on the development of an AIDS vaccine. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research. At the present time the following DCE laboratories are located at FCRF: the Laboratory of Comparative Carcinogenesis, the Laboratory of Molecular Oncology, and the Laboratory of Viral Carcinogenesis.

2. The DCE Board of Scientific Counselors

   Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, chemoprevention, epidemiology, immunology, pathology and genetics, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs. One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories and branches. These visits have been conducted by teams which, as a rule, are comprised of two to four members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the

scientists in the Laboratory or Branch being reviewed. The site visit reports, which reflect a consensus of the members of the team, are critically examined by the entire Board of Scientific Counselors and, after discussion, recommendations based on the reports are submitted to the Division Director. Approximately one year later the Division Director reports back to the Board on the changes made as a result of the site visit.

The third cycle of site visits to the Division's intramural operation began with a site visit to the Biostatistics Branch which was held October 6-7, 1987. Also site visited during the past year were the Laboratory of Tumor Virus Biology, the Laborotory of Viral Carcinogenesis, the Enviromental Epidemiology Branch and the Laboratory of Cellular and Molecular Biology. Site visits will also be made to the Clinical Epidemiology Branch and the Laboratory of Molecular Carcinogenesis in the coming year.

Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board continued to examine new concepts this year as new directions in extramural research activities developed.

Several workshops, involving Board members as well as participants from the scientific community outside NIH, were held this year. As a consequence, new initiatives resulted in issuing and/or funding Requests for Applications (RFAs) in the areas of papillomavirus-host interactions; functional anti-sense RNA in oncogenic viral systems; animal models for human papillomavirus-associated neoplastic disease (in conjunction with NIAID); retrovirus animal models and HIV pathogenesis; biochemical markers of human exposure for use in epidemiologic studies; and epidemiologic studies of HIV-associated malignancies.

Workshops held during the past year included one on mechanisms of pathogenic diversity of Epstein-Barr virus; identification and characterization of new primate retroviruses; and protease inhibitors as cancer chemopreventive agents. In 1985, in response to requests from members of the DCE Board of Scientific Counselors, a workshop to explore ways to strengthen extramural epidemiologic research was held. Several recommendations emerged from the workshop which were presented to, and endorsed by, the Board of Scientific Counselors. Attempts have since been made by program staff to implement these recommendations. As a result, a Small Grants program for the epidemiology and biostatistics area has been created, with the first round of applications received during the 1986 fiscal year. Awards under this mechanism are intended to support initiatives which focus on 1) planning of a complex epidemiologic investigation, 2) developing or validating a laboratory procedure for the ultimate purpose of applying it to cancer epidemiologic research, or 3) carrying out an epidemiologic research project for which rapid funding is

justified. The Small Grants program is an important innovation and the quality of applications continues to improve.

The objectives of the extramural research programs are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), First Independent Research Support and Transition (FIRST) awards (R29), conference grants (R13), Cooperative Agreements (U01), contracts (N01), small business innovative research (SBIR) contracts (N43/44) and grants (R43/44), academic research enhancement awards (R15), outstanding investigator awards (R35) and the new Method to Extend Research In Time (MERIT) award (R37).

Continuing modifications of funding mechanisms approved by the Board include a gradual transfer of current resources to a cost-reimbursement system (payback system), increased availability of funding to support new resource activities, the phasing out of contract-supported research in areas adequately covered by grant applications, use of RFAs to stimulate research activity in high priority areas, and the use of the Cooperative Agreement as an additional mechanism of support for investigator-initiated research involving significant involvement of NCI staff.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost outside advisory group.

FIGURE 1

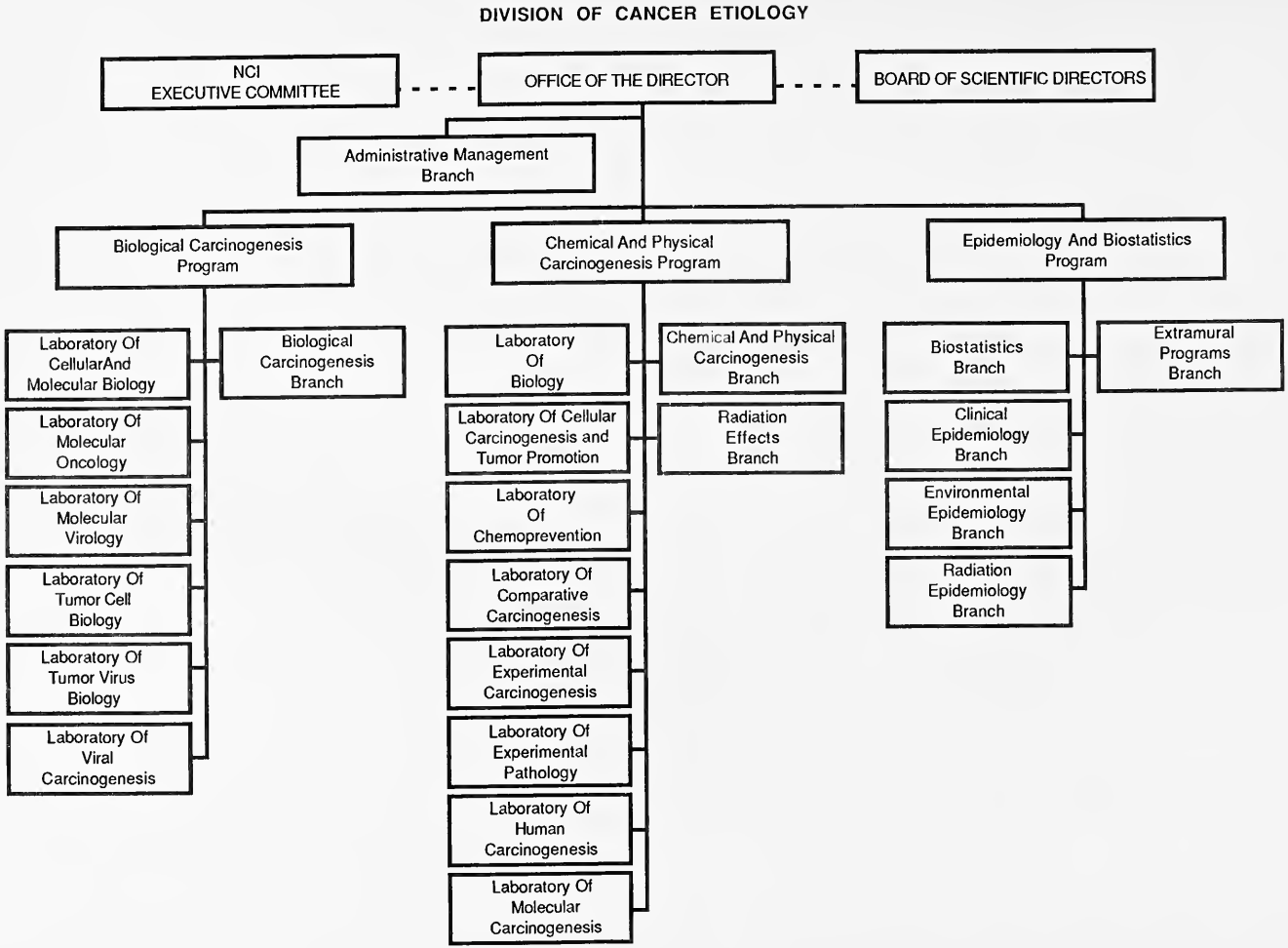

DIVISION OF CANCER ETIOLOGY
NCI EXECUTIVE COMMITTEE
OFFICE OF THE DIRECTOR
BOARD OF SCIENTIFIC DIRECTORS
Administrative Management Branch
Biological Carcinogenesis Program
Chemical And Physical Carcinogenesis Program
Epidemiology And Biostatistics Program
Laboratory Of CellularAnd Molecular Biology
Laboratory Of Molecular Oncology
Laboratory Of Molecular Virology
Laboratory Of Tumor Cell Biology
Laboratory Of Tumor Virus Biology
Laboratory Of Viral Carcinogenesis
Biological Carcinogenesis Branch
Laboratory Of Biology
Laboratory Of Cellular Carcinogenesis and Tumor Promotion
Laboratory Of Chemoprevention
Laboratory Of Comparative Carcinogenesis
Laboratory Of Experimental Carcinogenesis
Laboratory Of Experimental Pathology
Laboratory Of Human Carcinogenesis
Laboratory Of Molecular Carcinogenesis
Chemical And Physical Carcinogenesis Branch
Radiation Effects Branch
Biostatistics Branch
Clinical Epidemiology Branch
Environmental Epidemiology Branch
Radiation Epidemiology Branch
Extramural Programs Branch

TABLE I

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

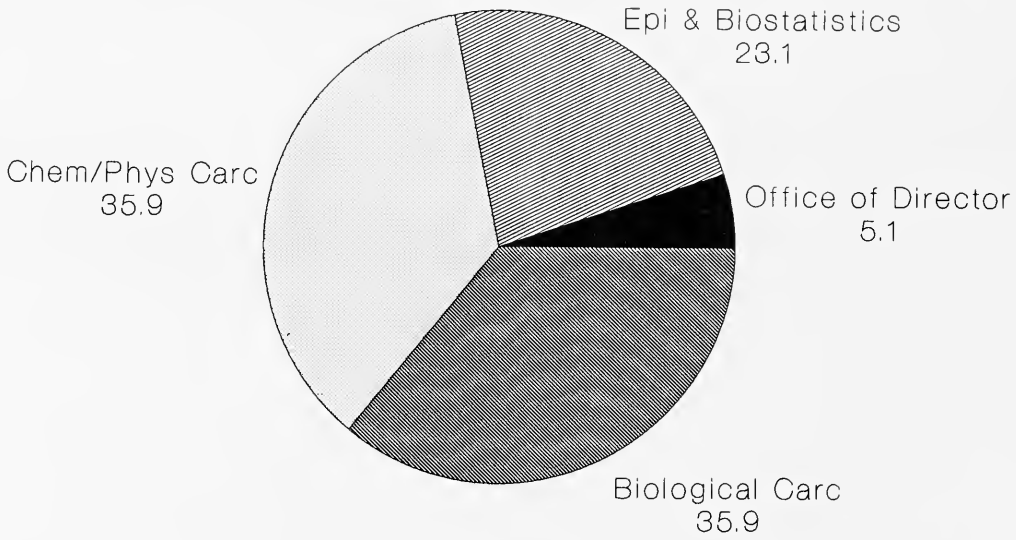
Table of Mechanisms by Organizational Unit Based On
Estimated Current Level of Expenditures
(Dollars in Thousands)

FY 1988 Estimate

	Office of the Division Director	Chemical and Physical Carcinogenesis	Biological Carcinogenesis	Epidemiology and Biostatistics	Total
In House	11,233	17,820	17,600	7,809	54,462
Contracts	3,102	6,722	5,946	24,877	40,647
RFA	0	2,065	3,912	2,377	8,354
Cooperative Agreements	763	3,958	113	1,665	6,499
Research Project Grants	0	74,853	78,044	31,012	183,909
TOTAL	15,098	105,418	105,615	67,740	293,871

FIGURE 2

NATIONAL CANCER INSTITUTE
DIVISION OF CANCER ETIOLOGY



Distribution of Funds, FY 1988 Estimate

SCIENTIFIC HIGHLIGHTS

Introduction: The Division of Cancer Etiology is responsible for planning and conducting the Institute's program of coordinated research on cancer causation and basic research on prevention. It is also responsible for directing and coordinating AIDS vaccine research and development efforts for NCI. The Division supports both intramural laboratories and branches and extramural programs which seek to elucidate the mechanisms of cancer induction at each step of the cellular process from initiation to transformation of normal cells into malignant cells. The overall purpose of these studies is to provide information for reversing, interrupting or preventing this process before the development of clinical disease.

To accomplish these goals, investigators pursue fundamental studies on normal and malignant cells and on carcinogens or procarcinogens, such as viruses and chemicals, using the disciplines of cellular and molecular biology, immunology, biochemistry, and microbiology. Epidemiologic studies of human populations are performed to identify risk factors predisposing to various cancers using the disciplines of clinical medicine, genetics, mathematics, biometry, and biostatistics.

Fundamental information on biological carcinogenesis has been acquired by studying human and animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may themselves be directly responsible for some malignant transformations in man. Investigations of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur through either the direct effects of viral transforming genes, through the influence of viral enhancing elements (promotors or long terminal repeats) on cellular oncogenes, or through the interaction of viruses with a variety of environmental factors, such as hormones, chemicals, radiation, and the like.

Similarly, chemical carcinogens, both naturally occurring and synthetic, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, systems that repair DNA damage and on the possible activation of oncogenes by chemical carcinogens. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state.

Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of intrinsic and extrinsic risk factors with various cancers. In view of the increasing importance of nutrition and life-style in the causation or prevention of cancer, in particular the role of macro- and micronutrients in the diet, alcohol consumption and tobacco use, special emphasis has been given to projects that have immediate health implications. Many studies deal with the presence of mutagens, carcinogens and natural anticarcinogens in foods; other studies assess the carcinogenic effects of smokeless tobacco and passive smoking. In addition, major studies on the viral etiology of cancer, cancer incidence in the workplace, effects of low-level radiation, and environmental pollutants in air, water, and soil are under investigation. Studies on the pathogenesis, natural history, transmission and cofactors for HIV (human immunodeficiency virus) infection have received major emphasis during the past year.

BIOLOGICAL CARCINOGENESIS PROGRAM

Oncogenes

Certain RNA viruses have been shown to derive their ability to cause cancer in animals, and perhaps in humans, due to genes found in normal cells. These "oncogenes" develop by recombination with proto-oncogenes in normal cells, genes whose gene products play roles in cellular growth. In some cases, the gene products of the oncogenes have been shown to be necessary for transformation to take place. There are about 40 proto-oncogenes described to date. Virtually all of the human oncogene homologs have now been chromosomally mapped to specific positions on the human gene map.

Studies of the human gene map permit an understanding of the role of oncogenes in the cause of cancer and ultimately may permit interventions to prevent or treat virus-induced cancers. DCE laboratories have collaborated extensively with other laboratories around the world to move toward complete mapping of the human genes. Thirty-five distinct human structural genes have been mapped by DCE scientists.

Studies of the raf oncogene family in man have revealed that one of these, c-raf-1, may play a role in all histologic types of human lung carcinoma; the gene is expressed at very high levels in 60-80% of all such tumors. To aid in this evaluation, an animal model system was developed for rapid (5-13 weeks) high frequency induction of lung adenocarcinoma in mice, in which transforming DNA and uniformly high levels of c-raf RNA and protein were shown to be present. Other experiments resulted in the functional mapping of c-raf-1 and A-raf-1 products in the signal transduction pathway of growth factors which showed that both genes appear to act downstream of certain other oncogenes. These results indicate that raf protein kinases and their regulation might be ideal targets for designing modulating drugs.

v-raf and v-myc oncogenes were shown to act synergistically in the mouse for the induction of carcinoma and lymphoid/hematopoietic tumors, and in vitro in the transformation of various hematologic and lymphoid cells. It was also shown that high level expression of v-myc can abrogate the requirement of some of these cells for a variety of c-myc or other growth factors, including IL-3, IL-2, and platelet-derived growth factor. Thus, myc appears to serve as a second messenger for competence-inducing growth factors.

The human ets-1 and ets-2 proto-oncogene loci have been found, consisting of sequences analogous to portions of the E26 avian leukemia virus. Restriction mapping and sequence analysis of ets-2-containing clones revealed the presence of a novel human gene named erg (for ets-related gene). The ets-2 gene was found to have mitogenic activity in transfected cells. The ets and erg genes are localized on chromosome 21, and thus a possible relation to Down's syndrome and its associated high incidence of leukemia was suggested and studied. Comparative studies of ets-2 genes from different species revealed that these are nearly identical in man and mouse, and are over 90% homologous to the chicken ets-2 gene. The ets-1 gene is highly conserved from Drosophila to man. In addition, monoclonal antibodies to ets-2 protein appeared to react in yeast cells, indicating a possible presence of these proto-oncogenes in primitive eukaryotic cells.

The ets-2 mRNA has been shown to be induced in regenerating mouse liver, suggesting that the ets-2 gene expression may be involved in cellular proliferation and the development of tumors. Transfection of NIH/3T3 cells conferred upon them the ability to grow well and to grow in low-serum or serum-free medium. These properties provide an assay system to study the functions of ets-2.

Comparative studies have been carried out between man and various cat species, a project of particular importance to extrapolating studies of leukemia due to the retrovirus feline leukemia virus (FeLV) in cats to retrovirus-induced leukemias in humans. Comparative genetic studies between man and certain animal species are being conducted to permit the development of new animal models for cancer studies. Studies have revealed that the South African cheetah is extremely inbred. It is monomorphic at 52 isoenzyme loci. Unrelated cheetahs accept skin grafts, a characteristic not found in other outbred mammals.

A 56kd protein containing phosphotyrosine had been found in human colon carcinoma cell membranes. It has been shown to be the gene product of the lck gene, which is closely related to the c-src oncogene. Interestingly, high levels of lck expression were found in cell lines derived from metastatic sites compared to cell lines derived from the primary tumors, indicating a possible role of this gene in tumor progression.

Human epidermal keratinocytes were immortalized by Ad12-simian virus 40 (SV40) virus and were transformed by a variety of retroviruses containing bas, H-ras, fes, fms, erbB, and src oncogenes. Transformants induced carcinomas when transplanted into nude mice. These findings support a multistep process for neoplastic conversion of human epithelial cells.

Virus Studies: HIV

Viruses related to HIV, specifically three distinct primate lentiviruses (simian immunodeficiency viruses, SIV), have been isolated from a macaque, a mangabey, and an African green monkey. The virus isolated from the macaque, SIV/Mne, was studied by amino acid sequence analysis of gag proteins. Of 125 residues sequenced, 92% were identical to another SIV isolate (SIV/mac), 83% were identical to HIV-2, and 41% to HIV-1. SIV/Mne has been inoculated into six macaques and two baboons, resulting in viremia, decrease in CD4+ counts, and death in the macaques, and no evidence of infection was noted in the baboons.

HIV-1 mutants have been found that are defective in gag gene processing. No mature gag proteins are produced by them, although large amounts of a gag precursor are present. Electron microscopy shows aberrant "immature" virus particles present. Addition of a purified protease that is deficient results in maturation of the gag proteins in these preparations. This maturation is being used as a test system to look for protease inhibitors that may have therapeutic use.

Segments of the HIV gene have been cloned and used to produce high levels of HIV-1 proteins. Using these systems, nine HIV-envelope polypeptides (comprising 96% of the entire gp160 glycoprotein complex) have been produced at high concentrations and purified. Some of these proteins are highly immunogenic. Antibodies to gp160 and gp41 react with some of these polypeptides, indicating that the antigenic determinants of gp160 are conserved on some of the polypeptides.

The cloned sor protein was used to screen sera from individuals who were at high risk for HIV infection. Five of the nine individuals tested positive by this method prior to seroconversion in the Western blot assay.

Transgenic mice have been produced that carry the HIV tat-3 gene; these mice develop Kaposi's sarcoma-like disease.

To study the influence of the HIV tat gene, this gene was placed into the retroviral vector pGV1 under the transcriptional control of HIV long terminal repeat (LTR). Cells were transfected with this construct, and the virus products are being purified and tested. These cell lines will be used for the identification of cellular genes whose expression is controlled by tat.

Studies of the tat gene have shown that it is an absolute requirement for HIV replication. Mutants in which this gene was removed or the splice acceptor for it removed were unable to generate virus or produced only low levels of virus. These defects could be corrected by providing the gene or its product. Studies were also conducted to analyze the functions of the nef and vip genes of HIV.

Studies of neutralizing antibodies to HIV have been conducted to provide a basis for vaccine development. A major type-specific neutralization epitope was mapped to 24 amino acids within the envelope protein gp120. The mouse monoclonal antibody used in this mapping can inhibit HIV

infection by both cell-free and cell-contained virus, indicating that neutralizing antibodies raised by vaccination may be protective against virus exposure in either form. Other studies showed that neutralizing antibodies can be raised to gp160 in goats. Studies to evaluate the implications of HIV heterogeneity in different populations showed that a single amino acid change in the virus envelope can result in profound changes in recognition by neutralizing antibodies. This implies the need for generating suitable type-specific antibodies as well as group determinants in vaccine development.

Virus Studies: Human T-Cell Lymphotropic Virus Type I (HTLV-I)

Transgenic mice that carry and express the HTLV-I tat gene have been developed. These mice develop neurofibromatosis that resembles the human disease. "Trans-activation" directed by the HTLV-I LTR has been studied using the protein in HTLV-I that regulates this, p40tax.

A candidate HTLV-I vaccine, consisting of virus-free antigen produced in a persistently infected cell line, was used to immunize rabbits. Antibodies to surface and core components of the virus were induced; these inhibited syncytia formation in the syncytia inhibition assay. Similar antibodies were induced in two pig-tailed macaques immunized with the same candidate vaccine.

Virus Studies: Papillomaviruses

Studies have been conducted to evaluate the mechanisms by which human papillomaviruses (HPV) interact with cells, in particular to form cervical dysplasia and cervical cancer. One such mechanism involves the E2 open reading frame (ORF). The functional domains and gene products of E2 have been studied. Deletion of the first 15 amino acids of E2 knocks out the transcriptional transactivation function. Another area of study has been the E5 oncoprotein, in which there are two domains with different functions.

Transgenic mice carrying the complete bovine papillomavirus (BPV) genome have been created and found to develop two types of skin pathology, one of which is a dermal tumor of the fibrosarcoma type. The benign skin lesions and the tumors contain extrachromosomal BPV DNA and express BPV RNA; normal skin and organs from the same mice contain only integrated viral DNA. Chromosomal abnormalities have been found in the fibrosarcomas but not in the benign skin lesions, and this fact may be useful in studying tumorigenesis in this model.

Extensive studies have been conducted with HPV-16, which is found in over 60% of biopsies of cervical carcinoma. Most abundant transcriptions of the virus in these carcinomas map to the E6 or E7 open reading frames. Studies in DCE have shown that the E7 open reading frame encodes transcriptional transactivation and cellular transformation functions analogous to those of the adenovirus E1a proteins; thus E7 appears extremely important in carcinogenesis of the cervix.

Virus Studies: Human B-lymphotropic Virus (HBLV)

Studies have been conducted to evaluate the possible synergistic role of HIV and HBLV, a new herpesvirus found in 70% of HIV-positive persons. (HBLV is also known as human herpesvirus-6, HHV-6.) It has been shown that HBLV can infect other cells than the originally described infections of B-cells. CD-4 positive T-cells, for instance, can be infected in vitro by both HIV and HBLV, and these cells are killed at an accelerated rate compared to those infected by either of the viruses alone.

HBLV DNA was also found in seven of ten Burkitt's lymphoma patients; all ten had Epstein-Barr virus DNA (in higher titer than the HBLV DNA). This raises important questions about the possible interaction of these two viruses in this cancer.

Virus Studies: Hepatitis B Virus

Hepatocellular adenomas and carcinomas were produced in a transgenic mouse lineage that contains the entire envelope ORF of the hepatitis B virus (HBV). HBsAg is present in hepatocytes and alpha-fetoprotein levels are markedly increased prior to tumor development. This model is a significant breakthrough for the study of a virus that has not been consistently grown in cell culture.

Virus Studies: JC virus

JC virus has been used to study the role of enhancer genetic sequences that control the rate at which genes are transcribed, and thus may be important in the loss of control of cell growth in cancers. JC virus is a papovavirus associated with progressive multifocal leukoencephalopathy. Oligonucleotides that span the JC virus enhancer have been sequenced and three of the protein products identified.

Other AIDS Studies

Studies have been initiated to evaluate the possibility that genetic variations in humans are responsible for differences in responses to the AIDS virus (i.e., some infections result in rapid death; others are mild). Approximately 400 gene probes will be applied to cells from 5,000 patients to attempt to characterize their genetic polymorphisms and correlate these with disease status. To date, 392 probes and 730 AIDS patients have been collected.

Long-term cell cultures were established from lung tissues and pleural effusions from AIDS patients with Kaposi's sarcoma (KS), using recently described endothelial growth factors. When inoculated into nude mice, these cells induced murine cell tumors similar to KS. The growth factors elaborated by these cells, and the similarity of the cells to KS, will provide important models for the study of the pathogenesis of KS.

Identification of a protein in HIV-2 and SIV isolates that is not present in HIV-1 (vpx or X-ORF) provides a possible test to differentiate HIV-1 from HIV-2 more easily.

CHEMICAL AND PHYSICAL CARCINOGENESIS

As increasingly more sophisticated techniques and approaches are applied to the study of chemical and physical carcinogenesis, the distinctions between that discipline and the discipline of biological carcinogenesis and molecular biology begin to blur. The identification of activated oncogenes in chemically induced tumors, the recognition of the pivotal role that growth factors and cytokines play in the basic economy of the cell, and elucidation of the complex of controls which regulate cellular growth and differentiation all argue for an integrated theory for the mechanism of cancer induction regardless of whether the causative agent is chemical, physical, or biological in origin. Nevertheless, damage to the genome, or inadequate repair of that damage, appears to be an important determinant of an individual's risk for cancer. Metabolic phenotype, the ability of an individual to activate and detoxify chemicals, is also a major factor. Many studies have focused on these important areas during the past year.

Oncogenes in Chemical Carcinogenesis

In order to better understand the molecular mechanisms of chromosome alteration and its relation to cellular transformation, a number of proto-oncogenes have been localized by *in situ* hybridization. A high proportion of proto-oncogenes exist at breakpoints of chromosomes. The *Fyn* gene, a novel human gene structurally related to the *Svc* gene, was localized to 6q21 within the same region as *ros* and *myb* proto-oncogenes, suggesting that they may be closely linked at the molecular level. Furthermore, the existence of a fragile site at 6q21 suggests that these alterations may be important to specific hematologic malignancies and solid tumors. On the X chromosome *dbl* was localized to q27, a site associated with mental retardation and anti-hemophilia factor IX. From a thyroid binding protein DNA library the key enzyme, P55, responsible for post-translational modification of collagen synthesis has been localized. The gene for P55 is at q25 of chromosome 17; other nuclear thyroid hormones are not nearby. Because HPV-18 of HeLa cells is found on several chromosomes, the flanking sequences associated with HPV-18 and chromosome 8 were isolated. This flanking sequence was found only on chromosome 8, indicating that integration on the various chromosomes was independent of chromosome 8. The integration was also accompanied by amplification of viral sequences and induced *myc* RNA expression.

Studies on tumor suppression activities of specific human chromosomes are underway. Genetic changes related to carcinogenesis are being studied using hybrids from fusion of human lung carcinoma cells with normal human bronchial epithelial cells and of microcells of individual marked human chromosomes with human lung tumor cells. Initial studies suggest that a limited population doubling potential (mortality) is the dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubing potential, karyotype and tumorigenicity in athymic nude mice. When specific human chromosomes have been transferred by microcell methodology into HuT 292 cells, chromosome 11, but not chromosome 13, has altered the tumorigenicity of the HuT 292. The location of the putative tumor supressor gene on chromosome 11 will be determined.

The expression of activated cellular oncogenes in chemically induced rat tumors and in comparable human neoplasms and the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are being studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. The consistent activation of K-ras in rat renal mesenchymal tumors has been shown to occur by a G → A transition mutation in the second position of codon 12, which has been demonstrated in 3T3 transformants and in DNA from primary tumors whether the DNA transformed 3T3 cells or not. Further studies on the neu oncogene in the pathogenesis of schwannomas, in a different strain of rat than was previously used, confirm the consistent presence in an additional 30 such tumors (100% of those tested) of neu activated by a T → A transversion mutation in that segment of the neu locus encoding the putative transmembrane region of the protein encoded by the gene, a growth factor receptor-tyrosine kinase-type molecule. Further studies are in progress to determine whether neu is comparably activated by point mutation in chemically induced schwannomas of other species of rodents, and in human tumors including schwannomas.

An amplification of the c-myc, N-myc or L-myc gene and overexpression of the c-raf sequence are common features of small cell lung carcinomas. The role of these genes in lung carcinogenesis is being assessed by overexpressing c-raf and/or c-myc genes in human lung cells, and by reversion of the malignant phenotype by plasmids capable of anti-sense RNA transcription. Retroviral recombinants pZip-raf and pZip-raf were constructed to examine the role of the c-raf-1 and c-myc proto-oncogene in lung carcinogenesis. Immortalized human bronchial epithelial cells (BEAS-2B) transfected with pZip-raf DNA and pZipmyc DNA gave rise to undifferentiated carcinomas (raf/myc tumors) when tested in athymic nude mice, whereas c-myc- or c-raf-transfected cells are non-tumorigenic. The raf/myc tumors expressed markers of small cell lung carcinomas, i.e., neuron-specific enolase and neurosecretory granules. In addition, BEAS-2B cells transformed with the c-raf and c-myc proto-oncogenes, as well as derived tumor cell lines, acquired HLA class II antigen expression.

The c-raf-1 gene has been identified as the predominant transforming gene of three radiation-resistant head and neck cancer cell lines in the NIH 3T3 transfection assay (SQ-20B, JSQ-3, SCC-35). NIH 3T3 cells transformed with SQ-20B DNA also became radiation-resistant, suggesting a correlation between the presence of c-raf sequences and the radiation resistant phenotype. Inhibition of the c-raf function by introduction of anti-sense raf-transcribing plasmids into SQ-20B cell line reverted not only the tumorigenic phenotype but also reduced the radiation resistance. As a consequence of these experiments, the construction of an inducible promoter system for anti-sense sequences in human cells has been undertaken.

Experimental hepatocarcinogenesis in the rat is being used as a model to study the mechanism of neoplastic development with particular emphasis on defining the possible role of a set of oncogenes that are commonly associated with the process of hepatocarcinogenesis in vivo. Previous results had consistently shown up-regulation of the expression of myc and raf oncogenes during chemical hepatocarcinogenesis. In order to examine

the transforming potential of these and other oncogenes in the liver system an in vitro transformation system was established which consists of a retroviral vector containing relevant oncogenes and a rat liver-derived epithelial (RLE) cell line as the reporter cell. The main findings include: (1) v-raf, v-H-ras and a combination of v-raf and v-myc are potent transforming agents in the RLE cells; (2) Different tumor types were observed following transplantation of the infected cells. The most undifferentiated tumors originated from the v-raf infected cells, whereas transformation with v-raf/v-myc combination resulted in hepatocellular carcinoma; (3) Transformation of RLE cells with v-raf and v-H-ras resulted in increased expression of the multidrug resistance-1 (MDR-1) gene and the development of multidrug resistance; and (4) Transforming growth factor-beta (TGF-beta) is capable of differentiating the RLE cells towards the adult hepatocyte phenotype. Furthermore, transformation of the RLE cells blocks the TGF-beta-induced differentiation of these cells.

The H-ras oncogene protein product p21 was characterized as to its cellular and tissue localization and possible role in carcinogenesis in rodents and humans. Ten different antibody preparations were used. Western blotting with antibodies to H-ras p21 from E. coli expression vectors revealed that 9 of 10 antibodies immunoreacted with H-ras p21. The pattern of specific immunohistochemical staining was always characteristically on the cell membrane of Harvey virus-induced sarcomas. From 20-50% of the tumor cells were immunoreactive. In contrast, less than 1% of cells transformed by chemicals with an activated H-ras oncogene were immunoreactive on the cell membrane. Some antibodies reacted with cytoplasmic components in normal cells but never on the cell membrane. These findings provide information on the true localization of p21 immunoreactive cellular antigens. Much of the results are in contrast to those previously published. Much of the previously published studies apppear to have reported nonspecific staining rather than actual localization of H-ras p21.

Growth Factors and Cytokines

A peptide growth factor called transforming growth factor-beta (TGF-beta) has been characterized and purified to homogeneity. Although this peptide was named for its ability to cooperate with members of the epidermal growth factor family to induce phenotypic transformation and anchorage-independent growth of non-neoplastic fibroblasts, it has been shown that TGF-beta can inhibit the anchorage-independent growth of certain tumor cells. This bifunctional character of TGF-beta is best shown in experiments using fibroblasts transfected with the myc gene; in these cells, TGF-beta can either stimulate or inhibit the anchorage-independent growth of the cells depending on the complete set of other growth factors operant on the cells.

TGF-beta stimulates matrix production by mesenchymal cells. Many cell types including cells of the immune system, fibroblasts, and osteoblasts are known to secrete TGF-beta, suggesting that both fibrosis that accompanies chronic inflammation and matrix formation by bone-forming cells may be dependent on TGF-beta. It has been postulated that excessive levels of TGF-beta might contribute to fibrotic diseases. It

was shown that excessive levels of TGF-beta are found in vitreous aspirates of eyes of patients with a disease called proliferative vitreoretinopathy and that levels of the protein increase with the severity of the fibrosis. Importantly, it has also been shown that the retinal pigment epithelial cell, previously postulated to play a central role in the disease process, can serve as a source of TGF-beta. These studies will be extended to include other fibrotic diseases.

To study the role of TGF-beta in embryology, attepts have been made to clone genes related to mammalian TGF-betas from cDNA libraries made from embryonic chicken and frog tissues. This has resulted in the identification of two novel forms of TGF-beta in the chicken and one in the frog. Comparison of the deduced amino acid sequences of these new TGF-betas with those of TGF-betas 1 and 2 shows areas of conservation which suggest specific functions. The expression patterns of these new genes in both embryonic and adult tissues is being studied and attempts made to determine whether they might have specialized developmental roles. Antibodies are also being generated to these new TGF-betas to detect the protein products of these genes.

Extensive analysis of the distribution and modulation of the cellular receptor for TGF-beta has shown that binding of TGF-beta to its receptor is not a major control point in TGF-beta action. However, normal and transformed cells have been shown to secrete TGF-beta in a biologically inactive form that is unable to bind to the receptor, and it is anticipated that activation of this latent form will be a critical regulatory step in TGF-beta action. Using immunohistochemical techniques, the latent form of TGF-beta secreted by human platelets has been covalently associated with precursor sequences and a further unidentified component; this probably represents a delivery complex. A second latent form of TGF-beta, found in serum, has been identified as TGF-beta bound to alpha-2-macroglobulin; this probably represents a clearance complex. The latent form of TGF-beta made by recombinant constructs has been purified to homogeneity and this material will be used to study the biological activities of latent TGF-beta, the in vivo activation mechanism and the in vivo pharmacokinetics of the latent form. It is anticipated that this will be the clinically useful form of the molecule.

Studies with the cytokine known as leukoregulin have progressed during the past year. Leukoregulin interaction with tumor cells up-regulates their sensitivity to LAK cytotoxicity similar to the previously established ability of the lymphokine to increase the sensitivity of carcinoma, leukemia, and sarcoma cells to natural killer lymphocyte cytotoxicity. The up-regulation of target cell sensitivity by leukoregulin is unique; it does not occur in target cells exposed alone or in combination to tumor necrosis factor, interferons, or colony stimulating factors. The up-regulation of target cell sensitivity to natural cytotoxicity also occurs in T lymphocyte-directed cytotoxicity. Up-regulation of tumor cell sensitivity by T cytotoxic lymphocytes, however, is directed against allogeneic, not autologous, cells in investigations with melanoma target cells and effector lymphocytes derived from an individual with melanoma. These observations suggest that cytotoxic T cells have the capacity to react with more than one target cell receptor

configuration or that the configuration of the target cell recognition site is rapidly regulated by leukoregulin. The molecular events involved in this aspect of leukoregulin-target cell regulation are under investigation as is the question of whether they extend to antibody-dependent cellular cytotoxicity (ADCC), another major physiologic mechanism for the elimination of abnormal target cells.

The up-regulation of target cell sensitivity to natural lymphocyte cytotoxicity occurs concurrently with an increase in target cell plasma membrane permeability and increased uptake of pharmacologically active macromolecules. Leukoregulin facilitates tumor cell uptake of insulin and rapidly modulates growth factor receptors as indicated by down-regulation of transferrin uptake in K562 leukemia cells. Modulation of target cell drug uptake and growth factor binding in combination with lymphocyte cytotoxicity provides new and potentially powerful methods for controlling target cell function, and in particular for more specific and potent tumor cell destruction.

Cervical epithelial cells immortalized by human papillomavirus [HPV] and HPV-positive cervical carcinomas are being evaluated as a model of lymphokine modulation of epithelial cell sensitivity to natural immunologic cytotoxicity. Epithelial cells, immortalized by transfection with HPV-16 DNA, are treated with leukoregulin and mixed with natural killer (NK) cells or interleuken-2 (IL-2)-induced lymphokrin-activated (LAK) cells. Several HPV-16 DNA immortalized HCX lines have now been examined as to their sensitivity to NK and LAK cell cytotoxicity and modulation of NK and LAK sensitivity by leukoregulin. HPV-16 DNA HCX lines are resistant to NK and sensitivity to LAK cell cytotoxicity. Leukoregulin treatment of these target cells induces and/or up-regulates their sensitivity to NK and LAK cell cytotoxicity, with LAK cell cytotoxicity being up-regulated more than NK cell cytotoxicity. When the HPV-16 DNA immortalized cell lines are evaluated according to their length of time in culture, early passage cells are less sensitive to the enhancing effect of leukoregulin than are late passage cells. The early passage cells react similarly to the control non-transfected cervical cells. These observations are the first demonstration that leukoregulin up-regulates the sensitivity of papilloma viral DNA immortalized human epithelial cells to different forms of natural lymphocyte cytotoxicity.

Epidermal Carcinogenesis: Control of Epidermal Growth and Differentiation

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are being studied in mouse epidermis by in vivo and in vitro techniques. The initiation event in skin carcinogenesis is highly correlated to an alteration in the program of terminal differentiation of epidermal cells. In cell culture, epidermal differentiation is regulated by the concentration of extracellular calcium. Induction of terminal differentiation by increasing the calcium concentration in the culture medium causes a two-to threefold increase in the level of intracellular free calcium. Cells of initiated lines which survive in medium with high calcium showed an altered response to increased external calcium, with a sharp four-to ninefold peak of intracellular free calcium in all cells within 2 minutes. These differences in intracellular calcium between

normal and initiated keratinocytes may be related to alterations in phosphoinositide metabolism. The ras oncogene is highly correlated to the initiated phenotype in epidermis. Introduction of an activated ras gene into normal keratinocytes alters their phenotype to that of papilloma cells. Chemically induced papillomas yield an activated ras oncogene with a mutation at codon 61. Papilloma cells and initiated cells are resistant to the differentiation-inducing effects of phorbol ester tumor promoters. Since phorbol esters induce differentiation in normal cells, papilloma cells can be selected among an excess of normal cells in culture by their ability to continue to proliferate in culture medium containing phorbol esters. In culture, introduction of the v-fos oncogene into initiated cells with an activated ras results in their conversion to malignancy. TGF-beta is elaborated by normal keratinocytes induced to differentiate by 12-O-tetradecanoylphorbol-13-acetate (TPA) or by increasing external calcium. This secretion is not altered by introduction of v-ras into normal keratinocytes. In vivo, several classes of benign tumors can be induced by initiation and promotion. Papillomas with a high risk for spontaneous conversion to carcinoma are also most responsive to chemical converting agents. Malignant conversion can be accomplished by a single injection of cisplatin.

cDNA clones corresponding to the major proteins expressed in mouse epidermis have been isolated and characterized. Using a combination of in situ hybridization with RNA probes and indirect immunofluorescence with monospecific antisera, it was shown that these genes belong to at least four subsets: those expressed predominantly in the proliferating basal layer of the epidermis; those expressed predominantly in the differentiated suprabasal spinous layers and, to a lesser extent, in the granular layer; those only expressed in the granular layer; and those only expressed under hyperproliferative conditions. Genes representing each subset have been isolated and sequenced. Various strategies have been employed to identify sequences regulating the expression of these genes, including vector constructs using different regions of the genomic clones to drive expression of the chloramphenicol acetyltransferase gene and the production of transgenic mice which express a human differentiation-associated keratin gene in a tissue- and developmental-specific pattern. A gene encoding a cysteine-rich protein, which is a major component of the cornified envelope, has been isolated and shown by in situ hybridization experiments to be expressed in the granular layer of the epidermis. A monospecific antiserum has been used to demonstrate that the C-terminal portion of this protein is only detectable on the inner surface of mature envelopes. Monospecific antisera that have been produced against mouse and human keratins and other epidermal-specific differentiation products have been used to study various stages of carcinogenesis, gene expression in mutant mice exhibiting developmental defects in epidermal differentiation, the induction of terminal differentiation in malignant cell lines by pharmacological agents, the in vivo kinetics of expression of the differentiation-associated keratins with respect to cell division, and requirements for the induction of terminal differentiation products in vitro.

Vitamin A and the retinoids are of interest because they play an essential role in the maintenance of normal differentiation in most epithelial tissues under normal physiological conditions and modulate

growth by inducing differentiation of certain neoplastic cells. Vitamin A deficiency causes loosening of adhesive strength between the tracheal epithelium and the underlying connective tissue. Conversely, it has recently been found that retinoic acid (RA) and other retinoids with biological activity in maintaining normal epithelial differentiation enhance the adhesiveness of cultured fibroblastic cells (NIH-3T3) to plastic dishes coated with specific extracellular matrix proteins, such as laminin and type IV collagen. Thus the study of fibroblast cell adhesion induced by RA has become relevant to an understanding of the mechanism by which retinoids maintain epithelial cell differentiation.

In attempts to investigate the possibility that RA may act by second messenger systems involving phosphatidylinositol turnover, it was discovered that the enhanced NIH-3T3 cell adhesiveness is accompanied by a marked (70%) reduction in the carrier-mediated accumulation of inositol in these cells. The effect of RA was relatively rapid (6 to 14 hr), cell density-dependent, reversible and it involved a reduction in Vmax with no change in affinity. It also was specific for inositol in that the transport of other monosaccharides such as glucose, mannose, fucose and galactose was not affected in an appreciable manner. The possibility that this effect is the result of RA-induced structural modifications of cell surface carriers is presently being investigated.

In a parallel study intended to study the effect of the tumor promoting agent phorbol-12-myristate, 13-acetate (PMA) on cell adhesiveness, it was discovered that PMA causes a dose-dependent enhancement in 3T3 cell attachment to plastic dishes coated with laminin and type IV collagen. This effect was very rapid (30 to 60 min) and correlated with structures known to exert tumor promoting activity. This effect of PMA differs from the RA effect in time course and appears to involve activation of the receptors for laminin and type IV collagen, possibly through phosphorylation.

Studies on the early events in the interaction of phorbol ester tumor promoters with cells and tissues are underway, with particular attention to an analysis of the major phorbol ester receptor, protein kinase C (PKC). The bryostatins, macrocylic lactones, activate PKC and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. In Friend erythroleukemia cells, they restore differentiation inhibited by the phorbol esters. In primary mouse epidermal cells, they induce markers of the proliferative response but block phorbol ester induction of markers of differentiation. Part of the difference in response pattern can be explained by the bryostatins functioning to activate PKC transiently followed by supression of the pathway. Thus, both for cell-cell communication and epidermal growth factor binding, the bryostatins initially act like the phorbol esters but subsequently block phorbol ester responsiveness. In addition, the bryostatins intrinsically differ from the phorbol esters in their stimulatory activity for some responses; for example, they fail to induce arachidonic acid release even at very early times. The biochemical mechanisms for these differences are being explored through immunoblotting of PKC, comparison of phosphorylating patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated PKC isozymes. Structure-activity analysis

suggests that bryostatin derivatives differ in the degree to which they are bryostatin-like in their actions rather than phorbol ester-like. Computer modeling indicates excellent fit to the previously derived phorbol ester pharmacophore and is consistent with the structure-activity relations.

Genetic Polymorphism and DNA Damage in Chemical Carcinogenesis

There is a clear association between smoking and lung cancer, but it is still not known why some individuals, who are heavily exposed to large concentrations of chemical carcinogens, do not develop tumors, whereas others do. These observations provide circumstantial evidence for the involvement of a genetic factor that predisposes for tumor formation. Recent restriction fragment length polymorphism (RFLP) studies have shown that the loss of normal cellular sequences from chromosome 13 (in the case of retinoblastoma), chromosome 11 (in the case of Wilm's tumor and bladder cancer and breast cancer), chromosome 1 (in the case of melanoma), chromosome 22 (in the case of acoustic neurinoma) and chromosome 3 (in the case of small cell carcinoma of the lung) have been associated with malignancy. It appears that this method might be generally applied to the analysis of inherited susceptibility to cancer and therefore be informative in risk assessment for lung cancer. Tumor and normal tissue from high molecular weight DNA samples have been collected from more than 60 cancer patients for restriction enzyme digestion and Southern analysis. Initial experiments have centered on examination of genes located on the short arm of chromosome 11; loss of allelic fragments during tumorigenesis has been detected at the cellular Harvey ras locus, the insulin locus, the calcitonin locus, the beta-globin locus, the catalase locus and the Int-2 locus (homologous to the mouse mammary tumor virus (MMTV) locus). Loss of allelic heterozygosity was commonly found on chromosomes 3p, 11p and 17p. Experiments that examine additional loci throughout the human genome for these DNA samples are in progress.

Newly developed shuttle plasmids are being used as sensitive probes to measure DNA repair, DNA ligation and mutagenesis at the molecular level in human cells. These methods provide the potential for understanding the nature of DNA damage and repair processes. Using the newly developed methodology, the molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. A shuttle vector plasmid, pZ189, was used to determine that there is a restricted spectrum of mutations induced in ultraviolet (UV)-treated DNA replicating in XP cells of complementation groups A and D. The generation of plasmids with multiple base substitution mutations was related to an error-prone polymerase activity acting on nicked DNA which may be relevant to generation of immunoglobulin diversity. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV photoproduct, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. Photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. BS cell lines were reported by others to have diminished ligase activity in vitro. Utilizing a linearized replicating plasmid,

reduced ability of BS cells to ligate plasmids in vivo was demonstrated. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. A Registry of XP patients has been established. Utilizing an assay of G2 phase chromosomal hypersensitivity, collaborative studies have, for the first time, detected XP heterozygotes.

Although binding of carcinogens to DNA and its repair have been traditionally determined in whole DNA, thereby providing only average distribution data in relation to DNA structure, recent investigations in several laboratories have revealed highly selective, non-random sites both for preferential binding and for repair, depending, for example, on the form of DNA packaging and on the state of actively transcribed genes versus inactive genes. Present investigations with the carcinogen benzo[a]pyrene in liver cells in vivo have identified highly selective early preferential binding to DNAase I-hypersensitive sites, accounting for about 80% of the DNA-bound carcinogen at early times. Binding at these sites, which are only a small fraction of the genome, therefore represents "hot spots," which were found to be rapidly repaired, so that persisting adducts were located outside the DNAase I-hypersensitive sites. DNAase I-hypersensitive sites were identified in the c-Ha-*ras*-1 protooncogene and it was found that benzo[a]pyrene produces selective DNA damage at these sites in this gene.

The ability to detect low levels of carcinogen-DNA adducts in the tissues of people environmentally exposed to chemical carcinogens is invaluable to epidemiological studies of the incidence of cancer in selective populations. A number of selective methodologies have been developed to quantitate carcinogen DNA adducts. The Randerath 32P-postlabelling technique provides a fingerprint analysis of only polycyclic aromatic hydrocarbon type DNA adducts. However, the basic Randerath methodology can be adapted for sensitive quantitative detection of aryl type DNA adducts in human samples. In addition, the 32P-postlabelling method has been adapted to enable the detection of small alkylation-type carcinogen DNA adducts. The detection and quantitation of O^6-MeGua adducts in DNA has been shown, by the use of standards, to be accurate as low as one adduct in at least 1 million guanine residues. The N7-MeGua and the 8-OH Gua adducts also appear to be detectable by this methodology. The presence of unidentified 32P-labelled spots has also been observed from the analysis of DNA from cells treated with acrolein and DNA treated with fecapentaene.

Cytochrome P-450s and Metabolism of Carcinogens

Xenobiotics such as drugs and carcinogens, as well as endobiotics such as steroids and fatty acids, are metabolized by the mixed-function oxidase system. Cytochrome P-450s are the key components of mixed function oxidases; they are a family of enzymes responsible for carcinogen and drug activation and detoxification, as well as the metabolism of endogenous steroids and other endobiotics. Understanding their role in population sensitivity to carcinogens and drugs requires knowledge of their metabolic specificity.

To this end, monoclonal antibodies were successfully prepared to a number of different cytochrome P-450s. These include a constitutive form that metabolizes steroid hormones, a form inducible by ethanol and involved in the metabolism of nitrosamines, compounds suspected of being human carcinogens, and a form of P-450 induced by pregnenolone 16 alpha-carbonitrile. Monoclonal antibodies (MAbs) to various forms of rat liver cytochrome P-450 have been used as specific probes for the cytochrome P-450s in human liver. Western blot analysis with MAbs to rat ethanol-induced P-450, a form with high nitrosamine-metabolizing activity, detected a related P-450 in human liver microsomes. Individual variation in the level of this P-450 was observed. A radioimmunoassay (RIA) to this P-450 was devcloped with MAb 1-98-1. Using this assay it was possible to detect and quantitate the MAb-specific cytochrome P-450 in human liver microsomes. This human P-450 was immunopurified and analyzed by peptide maps and amino acid sequencing.

The nature of the common human drug oxidation defect, characterized by the lack of ability to metabolize debrisoquine and other drugs, has been elucidated. By analysis of human liver specimens it was discovered that individuals who cannot metabolize debrisoquine do not express a specific form of P-450 designated P-450dbl. This lack of expression was found to be due to mutant P-450dbl genes. These defective genes can be detected by assays of human lymphocyte DNAs providing the basis for a potential clinical test for this drug oxidation defect.

To evaluate the enzymology of human P-450s, full-length P-450 cDNAs from human liver cDNA libraries are being isolated. P-450s are being produced using a variety of cDNA expression systems, including yeast, COS-cell-SV40, vaccinia virus, retrovirus and baculovirus systems. Several human and rat CDNAs have been expressed in these systems and the P-450s are being evaluated for their enzymatic specificities toward drugs and carcinogens. These cDNA-expressed enzymes are also being evaluated for their ability to activate carcinogens and to produce mutagenic metabolites. By these studies it is hoped to enzymatically catalog all human P-450s. This data, in conjunction with studies on P-450 polymorphisms and variability in the human population, may make it possible to determine if P-450 expression is associated with susceptibility or resistence to chemically induced cancer.

The levels of mRNA for both multidrug resistance (MDR-1) and selective cytochrome P-450 genes were determined in adult rat liver following administration of various natural and synthetic xenobiotics. MDR-1 was induced following administration of aflatoxin B_1, 2-acetylaminofluorene (AAF), N-hydroxy-2-acetylaminofluorene (N-OH-AAF), isosafrole, phenothiazine, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), but not after phenobarbital (PB) or 7-hydroxy-2-acetylaminofluorene (7-OH-AAF) treatment. Cytochrome P-450 isoform d was induced by TCDD, isosafrole, phenothiazine and AAF, while cytochrome P-450 isoform b was induced by PB and to a lesser extent by isosafrole. Furthermore, aflatoxin, AAF and TCDD induced MDR in the B6 mouse but not in the D2 (Ah receptor-deficient) mouse, which suggests that MDR induction via aflatoxin may be by a ligand-responsive transcription factor, possibly the Ah receptor or another cytosolic receptor. Taken together, the above data have placed MDR into a set of programmed "alarm response" genes such as glutathione-

S-transferase and UDP-glucuronyl-transferase aimed at protecting the organism against the harmful effects of xenobiotics.

In another study, structure-tumor promoting activity relationships of various barbiturates, hydantoins, oxazolidinediones and benzodiazepine tranquilizers were found. Long-acting compounds were usually active as tumor promoters and enzyme inducers in target organs. A close relationship was found to exist between induction of specific cytochrome P-450 species and tumor promoting abilities of barbiturates and hydantoins. Although phenobarbital induced hepatic enzymes in rats, mice and patas monkeys, species susceptible to phenobarbital liver tumor promotion, it failed to have similar effects in the resistant species, hamsters and cynomolgus monkeys. A liver tumor-promoting index based on these findings was developed.

Hepatocarcinogenesis

Hepatocellular carcinoma is one of the most frequent worldwide causes of cancer mortality. Hepatitis B virus and certain chemical carcinogens have been implicated as etiological agents. An in vitro model system of replicative normal human hepatocytes is needed to better define the mechanistic roles of these etiological agents. Serum-free medium composed of a modified Ham's F-12 medium was found to support the long-term multiplication of human liver epithelial cells. These epithelial cells were positive for general cytokeratin expression as well as positive for cytokeratin 18 expression through four passages. In addition, human hepatocytes in primary culture transfected with the SV40 large T antigen gene formed foci within 6-8 weeks that were positive for both keratin and large T antigen expression. Conditioned medium from cultures of the transfected human liver cells was shown to cause a 30% increase in DNA synthesis of a malignant human liver cell line (HepG2). These cells should prove to be useful in studying the molecular mechanisms of hepatocellular carcinogenesis.

Studies on the fate and the possible role of oval cells in both normal liver biology and hepatocarcinogenesis are being conducted. After the administration of AAF for two weeks combined with partial hepatectomy (PH), only oval cells are able to proliferate and incorporate radiolabeled thymidine at day 7 after PH. However, at the later time points (9 to 13 days after PH) the label was present in the newly formed basophilic hepatocytes, which is an indication that oval cells are precursor cells for hepatocytes. At a high dose level of AAF, differentiation of oval cells to hepatocytes was delayed and metaplastic differentiation to intestinal and biliary epithelial cells was frequently observed. No reutilization of labeled thymidine from dying cells by the regenerating hepatocytes was observed. Lack of glucose-6-phosphatase activity and presence of alpha-fetoprotein (AFP) gene message in oval cells and early basophilic hepatocytes is a further indication that oval cells are precursor cells for hepatocytes. Differentiation of oval cells to hepatocytes after AAF administration follows a pattern similar to that observed in the embryonic liver. After AAF administration TGF-beta was present in the mesenchymal cells of the liver and in the oval cell compartment. In hepatocellular carcinoma TGF-beta was present only in stromal cells. In the fibrotic liver produced by carbon tetrachloride

administration, collagen genes and TGF-beta were expressed in the cell population lining the peripheral hepatocytes of the pseudolobules.

Previous investigations of glycoproteins isolated from the plasma membranes of normal and neoplastic rat livers revealed many qualitative and quantitative differences when analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Current efforts involve purification and characterization of the specific glycoproteins whose expression is markedly altered during chemically induced hepatocarcinogenesis in order to understand their role either as markers or causal agents during cell transformation. An earlier isolation procedure was modified to more efficiently purify the glycoproteins of interest. The new procedure provides a fraction that is enriched in the various glycoproteins of interest and enabled the final purification to be achieved by a single 2D-PAGE experiment. In addition, the best procedure for amino acid sequencing (gas phase) from these membranes was established. Using these techniques, it was possible to determine the N-terminal amino acid sequence for 4 of the 9 glycoproteins analyzed. The remaining 5 components of interest were not sequencable in this manner, presumably because of blocked N-termini. A peptide, comprised of the first 13 N-terminal residues of RLMP-1, was synthesized, purified, and used to produce rabbit antibodies for use in the larger scale purification of the whole protein and in various biological studies. The glycoproteins from normal and transformed rat liver epithelial (RLE) cells are currently being characterized by the 2D-PAGE system.

Significant progress has been made over the past year in developing the analytical technology required for the elution and subsequent microsequencing of proteins from 2D gels. A number of specific protein spots have been defined whose regulation is markedly altered during the multistep process of neoplastic transformation. Initially, microscale procedures aimed at the recovery and sequence analysis of these proteins from one-dimensional SDS-PAGE were investigated. Electroelution and passive extraction techniques were found to be suitable only when large amounts (> 500 pmoles) of protein were available. Electroblotting techniques in which the proteins are transferred to quaternary ammonium-derivatized glass fiber paper were modified such that 50 pmoles of soybean trypsin inhibitor applied to a 1D-SDS polyacrylamide gel could subsequently be correctly sequenced to 17 cycles. Other supports for protein electroblotting and subsequent microsequence analysis were investigated and Immobilon-P was found to be the support of choice. Using this support electrophoretic conditions were optimized to allow the N-terminal sequence analysis of up to 20 residues from 50-100 pmoles of protein applied to a 2D gel. With 1D-PAGE it was possible to obtain some sequence information from as little as 5 pmoles protein. This procedure was successfully applied to the sequence analysis of a number of previously uncharacterized membrane glycoproteins. Work is currently underway to apply this technique to the analysis of other unknown proteins whose expression is altered during carcinogenesis and also to improve the sequencing yields of proteins isolated from 2D gels.

Dietary Mutagens

Higher levels of mutagens in the feces of certain populations eating a Western diet have been shown to correlate with an increased risk of colon cancer. Ninety percent of this mutagenicity can be accounted for by a group of compounds called fecapentaenes, which are potent direct-acting mutagens in the Ames Salmonella assay. Fecapentaene-12 and diacetylfecapentaene-12 were found to be potent bacterial mutagens, but weaker mammalian mutagens. Despite the mutagenic activity of these chemicals, no biological activity has been found for skin tumor initiation, promotion or complete carcinogenesis in mice, colon carcinogenesis (by intrarectal administration), or carcinogenesis by subcutaneous administration in rats or mice. The total doses administered have been limited by solubility, stability and cost.

2-Amino-3-methyl-imidazole(4,5-f)quinoline (IQ) is one of several heterocyclic arylamines found in cooked foods that have been shown to be potent carcinogens in rodent bioassays. Ongoing work in this area involves synthesis of N-OH-IQ, and N-acetoxy-IQ and IQ-N-sulfate, the reactive metabolites of IQ; synthesis and characterization of the major DNA-IQ adduct; examination of DNA-IQ adducts in monkeys by the 32P-postlabeling method; study of IQ metabolism in monkeys; and the role of specific cytochrome P-450s in the metabolic activation of IQ. N-OH-IQ was a direct mutagen in Salmonella TA98 and capable of covalently binding to DNA without further activation. N-OH-IQ is also metabolized by mammalian O-acetyltransferase and sulfotransferase to N-acetoxy-IQ and IQ-N-sulfate, respectively. The C8-guanine-IQ adduct was synthesized and shown to be formed in vitro from either N-OH-IQ or N-acetoxy-IQ reacting with DNA. Using the 32P-postlabeling assay, eight DNA-IQ adducts, including C8-guanine-IQ, were found in liver of cynomolgus monkeys fed IQ. These adducts were identical to those found in the liver of rats fed IQ. Analysis of DNA modified in vitro with N-OH-IQ showed seven adducts, including the C8-guanine-IQ adduct, that were identical to those found in vivo. Thus, N-OH-IQ appears to be responsible for all adducts found in vivo except one. DNA-IQ adducts were also detected in kidney, colon, stomach and bladder, and these adducts were identical in all organs examined. Recently, one monkey of a group of 20 receiving daily oral doses of IQ at 20 mg/kg was diagnosed with hepatocellular carcinoma. The latent period for tumor induction (23 months) is similar to the latent period for liver tumor induction by diethylnitrosourea, the most effective hepatocarcinogen in nonhuman primates.

The carcinogenic heterocyclic arylamines from cooked foods were examined by employing a novel test system combining human cells individually expressing either recombinant cytochrome P1-450 or P3-450 as the bioactivation system with Salmonella typhimurium to score mutants. Early results show that cytochrome P3-450, a single isoform of the cytochrome P-450 super-gene family, is responsible for the bioactivation of the heterocyclic arylamine food mutagens to N-hydroxylamines.

1,2-dicarbonyl compounds are produced in heat processed foods in microgram to milligram amounts as a result of the Maillard "browning reaction." These compounds are known to react with DNA bases and to cause base-substitution in the Ames assay. Since 1,2-dicarbonyls are

present in roasted beverages such as coffee, a recent study investigated the effect of five different coffee preparation methods on the mutation rate for a specific marker in Chinese hamster ovary cells, for induction of sister chromatid exchanges and for abnormal DNA replication cycles. After 22 hours of exposure, there was a 15- to 20-fold greater incidence of mutations, two- to threefold increase in SCE frequency and 3-11% incidence of abnormal DNA replication and cytokinesis. These results were dose-dependent and were inhibited by the addition of sodium bisulfite.

Mechanisms of Cadmium Carcinogenesis

The mechanisms of cadmium carcinogenesis are under active investigation. Repeated subcutaneous injections of cadmium resulted in a rapid onset and high incidence of highly malignant tumors at the injection site and had an apparent association with malignant testicular tumors. The Syrian hamster was shown to be susceptible to cadmium-induced testicular carcinogenesis, while mouse strains resistant to the acute effects of cadmium on the testes proved resistant also to its carcinogenic effects. Chronic oral exposure to cadmium in rats was associated with prostatic tumors, providing further evidence for a link between cadmium and prostatic carcinogenesis. Zinc was shown to inhibit cadmium carcinogenesis in a route- and site-dependent manner. Rare testicular tumors were also observed in rats in association with cadmium treatment including Sertoli cell tumors, rete testis adenocarcinoma and seminomas, indicating a general susceptibility within the cells of the testes. A marked enhancement of cadmium cellular efflux, a reduction in levels of nuclear cadmium and a reduction in DNA cadmium content were observed in cells isolated from the testes of rats made resistant to cadmium carcinogenesis by zinc treatment. The DNA hypomethylating agent, deoxyazacytidine, caused both a marked increase in the synthesis of metallothionein, the protein most frequently associated with cadmium tolerance, and cellular tolerance to cadmium. Analysis of restriction enzyme digests of deoxyazacytidine-treated DNA indicated that this tolerance coincided with a hypomethylation of the MT gene, a condition clearly associated with enhanced expressibility. It was shown that, like the rat, mouse and monkey testes, the hamster ovaries are deficient in metallothionein. Hamster ovaries undergo an acute phase response to cadmium similar to that of the testes. An absence of metallothionein in the rat prostate was also established. Thus, a consistent absence of metallothionein in targets of cadmium toxicity and carcinogenesis is observed and the inability to express the metallotheionein gene is probably a very important factor in susceptibility of a given tissue to cadmium carcinogenesis.

Studies on AIDS

Acquired immunodeficiency syndrome (AIDS) is characterized by the profound loss of ability to respond to environmental antigens as well as the development of Kaposi's sarcoma. HIV-1 was identified by immunocytochemistry in skin macrophages (Langerhans' cells). Furthermore, cultured Langerhans' cells were shown to express viral particles and to infect HIV-I negative monocytes. MHC class 11 antigens were found to be involved in the HIV receptor site on cells expressing

these molecules. This involvement appears to be related to the proximity of HLA-DR molecules on the cell surface to the CD4 viral ligand. Monocytes were isolated from peripheral blood lymphocytes and infected with HIV-1. HIV-1-infected monocytes do not appear to be altered in their ability to present antigens (tetanus toxoid) to autologous T-cells. When an antigen is presented together with HIV-1, infection of T-cells is increased. The presence of cytotoxic lymphocytes to the HIV-transfected and -infected target cells was studied and showed low level activity to transfected fibroblast and higher levels to HIV infected T-cell lines.

HLA typing was performed in a cohort of individuals with AIDS and either Kaposi's sarcoma or opportunistic infections, or individuals at risk for this disease. A total of 250 individuals have been HLA typed. One hundred of these patients have been followed over a 4-5 year period. The objectives of these studies are to examine possible genetic susceptibility to the development of AIDS or AIDS-related complex that is related to expression of histocompatibility antigens. The HLA-DR1 phenotype is increased in frequency in all AIDS patients compared to HIV-seropositive controls. HLA-DR3 is significantly decreased in the patients with Kaposi's sarcoma. In the HIV-seropositive individuals followed for 65 months, HLA-DR1 appears to be a specific risk factor for disease development. Sera from 30 hemophiliacs (10 developed AIDS, 10 were HIV-seropositive but AIDS-free and 10 were HIV-seronegative) obtained prior to seroconversion and subsequent yearly samples were tested for antibodies to HLA antigens. Individuals developing AIDS demonstated increased reactivity to a range of HLA antigens while losing total CD4+ cells.

Contributions to a better understanding of the pathogenesis of simian aquired immunodeficiency syndrome (SAIDS) in monkeys and AIDS in humans have been made by immunohistochemical localization of lentiviral antigens in cells and tissues of the affected species. HIV gag proteins have been shown to have cross-reactive epitopes with the cytomegalovirus (CMV) major capsid protein (153 KD). The potential implications of this include the roles of viral activation, latency and disease progression since a high proportion of AIDS patients are infected with CMV. HIV and SIV antigens, usually gag proteins, were found in endothelial cells of brain, lymph nodes and bone marrow and bone marrow stem cells for the first time. Chimpanzees with experimental HIV infection had abundant viral antigens in blood and bone marrow lymphocytes but little or none in lymph node lymphoid cells. The refinement of immunohistochemical techniques has allowed for direct comparison with in situ hybridization studies for lentiviral RNA in studies which are currently underway.

EPIDEMIOLOGY AND BIOSTATISTICS

Continued emphasis was given to case-control and cohort studies evaluating key hypotheses in cancer etiology. Case-control studies of selected cancers were undertaken when high-risk communities were identified on the cancer mortality maps, major hypotheses were testable, or special resources became available. Laboratory methods were often incorporated into studies to help clarify exposures, preclinical

responses, and mechanisms of carcinogenesis. Some descriptive surveys were also undertaken. In particular, an updated atlas was published to illustrate the geographic patterns of cancer mortality by state economic area in the 1970s. When comparisons were made to patterns in the two later decades, the most striking changes were the emergence of elevated mortality from lung cancer among women in areas of Florida and along the West Coast, and from non-Hodgkin's lymphoma among residents in the central states, especially Kansas. Most other cancer sites in the 1970s showed similar patterns as before, although there was a general trend towards geographic uniformity over time. A similar trend is seen for non-whites in a companion atlas that is nearing completion. The most striking change among non-whites was the recent emergence of elevated rates for prostate cancer along the South Atlantic coast. A detailed report on time trends in cancer incidence and mortality rates at the national level was published, revealing remarkable increases in lung cancer, skin melanoma, multiple myeloma, and non-Hodkin's lymphoma.

Diet, Nutrition, and Cancer

Epidemiologic and experimental evidence continues to support the idea that dietary factors contribute to a substantial proportion of human cancer. International comparisons of mortality from cancer, showing large variations in rates among countries clearly indicate the importance of dietary factors. Even more compelling evidence comes from epidemiologic studies on migrant populations. However, the specific dietary risk factors involved have not been well established, and efforts to generate and test hypotheses in this area will be expanded during the next year.

Using the follow-up of the First National Health and Examination Study (NHANES) cohort, studies were conducted to evaluate the effects of serum cholesterol and height and weight, in collaboration with the Division of Cancer Prevention and Control (DCPC). Men in the lowest cholesterol quintile had nearly twice the risk of those in the highest quintile for cancer incidence and mortality. Among women, a similar relation was seen for cancer mortality, but cancer incidence in the lowest quintile was only 1.2 times that of women in the highest quintile. The inverse cholesterol-cancer relationship in men was present for determinations made 6 or more years before diagnosis of cancer, which suggests that lowered serum cholesterol may not simply result from preclinical disease. Analysis by site revealed inverse associations for cancers of the lung, bladder, pancreas, and cervix. The low levels of cholesterol appeared to be a risk factor mainly for cancer sites related to smoking, a provocative finding that persisted even after adjustment for smoking. The relationship of anthropometric variables to risk of breast cancer was also examined in this cohort. Women who developed breast cancer were taller and had greater frame size (elbow width) than non-disease women. However, body size, defined by weight, relative weight, or skin-fold measurements was not associated with increased risk. The positive association of stature and frame size to breast cancer risk suggests that early nutrition may play an etiologic role.

Opportunities to study nutritional hypotheses exist in other countries, where several collaborative case-control and intervention studies are

underway. In Shandong, China, an area at high stomach cancer risk, elevated risks were associated with intake of salted food and sour pancakes, while protective effects were seen with a number of vegetables, including ones containing vitamin C and ones of the Allium class (e.g., garlic). The protective effects of Allium vegetables were striking, with a 50% or greater reduction in stomach cancer risk among heavy consumers, an intriguing finding in view of recent reports of tumor inhibition among experimental animals administered Allium compounds. In collaboration with DCPC, a nutrition intervention study in Linxian, China, an endemic area for esophageal cancer, is underway to determine specific micronutrients and trace minerals that may reduce the level of risk. Trials involving nearly 34,000 participants in this high-risk area are midway through a 5-year nutrient supplementation program.

Infectious Agents

A major commitment to investigate the epidemic of AIDS began in 1981, and continues today. A series of five cohorts, followed since the earliest phase of the epidemic, has provided a data base for understanding the natural history of HIV and its pleiotropic effects. Among seropositive individuals, the risk of AIDS has approached 50% after 6 years of follow-up, with no significant cofactors identified among a variety of life-style variables. However, HLA-Dr-1 appears associated with an increased risk for progression to AIDS. A multicenter hemophiliac cohort has allowed more precise quantification of predictors of AIDS risk, particularly a decline in T4 lymphocyte count, appearance of HIV antigen, and increased levels of acid-labile alpha-interferon. This decline in immunity is also associated with increased viremia and heightened infectivity of female spouses, and thus represents a major risk factor for sexual transmission of HIV.

Advances in laboratory techniques, such as viral isolation and molecular detection approaches, have opened new avenues for exploring the role of viruses in cancer etiology. Since 1980, a total of five human retroviruses have been discovered, including human T-cell lymphotropic virus type 1 (HTLV-I) which has been linked to a spectrum of T-cell malignancies, and HIV-I which causes AIDS. Beginning in 1982, a series of long-range epidemiologic studies has been undertaken by Program staff in several HTLV-I viral-endemic areas. In Jamaica, a case-control study involving 100 incident adult T-cell leukemias (ATL) revealed a 35-fold increased risk among HTLV-I seropositives, a population incidence of 3-5 per 100,000 in the age group at high risk, and an unexpectedly high proportion of cases with HTLV-I antibodies. Modeling ATL incidence as a function of the number of seropositives suggests that infection at a young age is likely to confer the greatest risk for disease.

Preliminary results from a case-control study in Latin America showed that human papillomaviruses (HPV) types 16 or 18 were important risk factors for invasive cervical cancer, although the association was of lesser magnitude than hypothesized on the basis of laboratory findings. The relationship of HPV infections to risk of cervical dysplasia was demonstrated in a Washington, D.C. study, with some evidence that smoking may act synergistically with the virus. Methodological studies revealed that some caution must be exercised in interpreting results of HPV-DNA

hybridization assays, particularly since there was considerable variation in typing even when the "gold standard" Southern blot assay was utilized.

Tobacco and Alcohol

Tobacco habits and lung cancer risks vary geographically in the United States. A previous case-control study of lung cancer in a high-risk area of southern Louisiana implicated the heavy use by Cajuns of local brands and hand-rolled cigarettes containing high-tar levels. This year, a case-control study of pancreas cancer revealed an excess risk among Cajuns, which was partly associated with smoking habits but not with alcohol intake. Several case-control studies evaluated the possible association of lung cancer with passive smoking. In Shanghai, the risks among nonsmoking women tend to rise with increasing years lived with a smoking husband. These findings resemble those of earlier Program studies of lung cancer among nonsmoking women in the United States and Japan, whose risk increased in proportion to the number of cigarettes their husbands smoked.

Tobacco and alcohol were identified as the primary risk factors in case-control studies of esophageal, oral, and laryngeal cancers. In a study of esophageal cancer in coastal South Carolina, nearly 90% of the Black male cases reported the consumption of moonshine whiskey, which seemed to contribute to the longstanding elevated rates of this cancer among Blacks in this area. In a large multicenter study of oral and pharyngeal cancer, it was possible to show alcohol consumption as an independent risk factor among lifelong nonsmokers. Tobacco and alcohol appeared to combine in a multiplicative fashion in enhancing oral and pharyngeal cancer risk, with over a 35-fold excess risk among heavy smokers and drinkers. In a study of laryngeal cancer in coastal Texas, smoking was the major risk factor, combining with alcohol intake in a manner intermediate between additive and multiplicative models.

Occupational/Environmental Carcinogens

Last year, an NCI study of farmers in Kansas noted a provocative association between non-Hodgkin's lymphoma and exposure to pesticides, especially phenoxyacetic acid (2,4-D). This year, a study of agricultural extension agents, exposed to pesticides while conducting demonstration projects for farmers, revealed an excess of leukemia. Preliminary data from a case-control study in Iowa and Minnesota showed elevated risks for chronic lymphatic leukemia and non-Hodgkin's lymphoma among farmers using pesticides such as DDT, malathion, dieldrin, lindane, carbaryl, chloramben, and 2,4,5-T, particularly if use occurred 20 or more years before interview. Cohort mortality studies of pesticide applicators employed by a national lawn care company and by county noxious weed departments, and a case-control study of lymphatic and hematopoietic cancer are underway to clarify associations between pesticide exposure and cancer.

In China, cohort investigations were begun to evaluate cancer risks among 100,000 workers exposed to varying levels of benzene; 80,000 workers with potential for silica exposure, including over 5,000 with silicosis; and over 20,000 tin miners, many of whom are highly exposed to radon and

inorganic arsenic beginning at very young ages. The large size of these studies will enable improved assessment of carcinogenic risks, including dose-response patterns.

Epidemiologic studies utilize relevant environmental measurements to evaluate the effects of pollutants in the general environment. To test the hypothesis raised by previous Program research that environmental arsenical air pollution in the community is related to lung cancer, a case-control study is underway in Shenyang, China, where that country's largest copper smelter is located in the center of a densely populated residential area. The risk of lung cancer from indoor air pollution is also being investigated in Shenyang, with initial data suggesting a rising lung cancer risk associated with longer duration of exposure to coal-burning stoves, which generate high levels of polycyclic hydrocarbons. Exposures to volatiles from high-temperature wok cooking were implicated in the high risk of lung cancer among nonsmoking women in Shanghai. Fumes from rapeseed and soybean oils were mutagenic in the Ames test, with rapeseed oil volatiles the more potent, further suggesting that indoor pollution from cooking oil vapors may contribute to the elevated rates of lung adenocarcinoma among Chinese women. In addition, alpha track and thermoluminescence dosimeters were evaluated for measuring radon daughter products in residential dwellings. To account for seasonal variations, whole year measurements are being carried out. Collaborative studies to evaluate the relation of indoor radon exposures to lung cancer are underway in New Jersey, Missouri, Sweden, and China.

Using data from a national case-control study of bladder cancer, an elevated risk was found to be associated with the intake of tap water from chlorinated sources of surface water, especially among long-term users. A case-control study is underway in Iowa to clarify the effects of water contaminants, with particular attention to chlorination by-products and agricultural chemicals on the risk of bladder, colon, rectum, kidney, pancreas, and brain cancers. An expanded study of bladder cancer will evaluate risk among population subgroups, such as smokers and nonsmokers.

Studies continued to investigate the relationship between cancer risk and ionizing radiation, and especially to improve estimates of risk associated with low doses. Better data on low level effects are needed to base regulatory and other decisions about the potential hazard from medical, occupational and environmental exposures, and to asses the value of exposure avoidance as a means of cancer prevention.

Special emphasis is being placed on clarifying the role of indoor radon exposure, which may contribute to 9% of all lung cancers in the U.S. based on newly developed risk models utilizing data from earlier NCI studies of uranium miners. More reliable data should come from ongoing case-control studies of lung cancer that involve careful measurements of indoor radon.

Exploratory studies are underway to utilize data on occupational exposure and cancer mortality from several agencies, including the Social Security Administration (SSA), the Internal Revenue Service (IRS), the Bureau of

the Census, and the National Center for Health Statistics (NCHS). Collaboration with the National Institute for Occupational Safety and Health (NIOSH) and with NCHS continued in an effort to develop a national reporting system for occupational mortality on the basis of state coding of death certificate entries of usual industry and occupation. Staff members provided advice on modifying the Internal Revenue Code in order to increase opportunities for epidemiologic studies of occupational groups, to broaden access to the IRS address file, and to explore ways to exchange data and ease limitations on the research uses of individually identifiable records. The change in the IRS agreement with NIOSH under which current addresses are furnished to investigators involved in occupational studies, a change that freed SSA to furnish NCI staff with Social Security numbers needed for the IRS search, proved valuable to NCI staff seeking current addresses of study subjects. Methodological studies evaluated the utility of the one-percent Continuous Work History Sample (CWHS) of the Social Security Administration (SSA) in screening industry-of-employment cohorts for evidence of differential cancer mortality. Other studies were concerned with the feasibility and usefulness of adding to the CWHS file information on specific occupations obtained from the IRS Form 1040, and with the agreement between death certificate information on usual industry of employment and occupation and parallel information obtained by SSA and IRA.

Genetic Susceptibility

In collaboration with laboratory investigators, epidemiologic and clinical observations have resulted in the delineation of familial cancer syndromes and several leads to mechanisms of host susceptibility. In collaboration with the Massachusetts General Hospital and other research groups, the gene for neurofibromatosis (NF-1) was localized to the long arm of chromosome 17, near the gene for the receptor of the nerve growth factor. Interest was extended this year to the study of NF-2, featuring bilateral neuromas and recently mapped to chromosome 22.

Studies of the sarcoma-breast cancer syndrome (Li-Fraumeni syndrome) have been extended to 24 families, and show that the constellation of tumors also includes acute leukemia, brain tumors, and adrenocortical carcinoma. Laboratory studies of the cardinal feature, soft tissue sarcoma, have revealed specific non-random chromosomal translocations, and homozygous deletion of the candidate retinoblastoma gene on 13q14 in 3 of 16 sarcoma specimens. A large family with Wilms' tumor in seven cousins has provided data for exclusion of linkage with chromosome 11p13 and 11p15 probes, raising the possibility that a familial Wilms' tumor gene is located elsewhere. A multinational registry of the polyposis-hepatoblastoma syndrome has been established and has enrolled five hepatoblastoma survivors with polyposis coli, as compared with 0.2 cases expected in the U.S. and Western Europe over the past 3 decades. In the family with a constitutional 3;8 chromosomal translocation and renal carcinoma, the breakpoint at 3p14.2 has been found to be frequently deleted in renal cancer cells of sporadic (non-familial) cases examined cytogenetically and with molecular probes, suggesting inactivation or

loss of a recessive oncogene in this region. Examination of 198 offspring of Wilms' tumor patients showed that the hereditary fraction of the neoplasm is lower than predicted previously, and that premature delivery is associated with a history of abdominal radiotherapy for Wilms' tumor in the mother.

ACTIVITIES IN THE OFFICE OF THE DIRECTOR

The Division of Cancer Etiology is responsible for planning and directing a national program of basic research including laboratory and epidemiologic studies on the causes and natural history of cancer, on the molecular biology, natural history, and transmission of HIV, and on the development of a vaccine for AIDS. Basic research on methods and approaches to cancer prevention is also within the Division's sphere of activities. These research efforts are carried out in the intramural laboratories and branches of the Division as well as extramurally, utilizing research grants, cooperative agreements, interagency agreements and contracts. The Office of the Director coordinates, plans and directs a program of national and international research in cancer etiology and also serves as a focal point for the Federal government for the synthesis and dissemination of clinical, epidemiological and experimental data related to cancer etiology and cancer prevention.

Activities in the area of environmental carcinogenesis are located in the Office of the Director. A number of cooperative projects and collaborations with other Federal agencies have been carried out under interagency agreements with the U.S. Environmental Protection Agency (EPA), National Institute for Occupational Safety and Health (NIOSH), and National Oceanic and Atmospheric Administration (NOAA). In addition to managing and serving as Project Officers on these interagency agreements, staff from the Office of the Director interface with State agencies, industrial and trade organizations, academic institutions and professional societies, serving a primary role in dissemination of information on environmental problems and industrial exposures in carcinogenesis.

The Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), organized within the Office of the Director 15 years ago, also serves as a vehicle for information exchange. The ICGEC was originally constituted to provide a mechanism for interagency contacts to secure access to data bases; it has provided, indirectly, a stimulus for development of projects in the area of environmental and occupational carcinogenesis. It consists of representatives from 28 agencies or subagencies, and meets every few months. By October 1988 there will have been 88 meetings. Topics of meetings held this year are "Laboratory Studies of Feral Marine Fish," "Oncogenes in Animal Models" and "Radon Exposure."

Staff in the Office of the Director participate on the Task Force on Environmental Cancer and Heart and Lung Disease, for which EPA is the lead agency. This Task Force was formed some years ago in response to Congressional stimulation as a result of mandates under the Clean Air Act. A Congressionally mandated DHHS report entitled "Research Activities of Relevance to the Clean Air Act: Biennial Report to Congress" is prepared by the Office of the Director every 2 years.

The Registry of Experimental Cancers is directed and maintained by staff of the Office of the Director. The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily

rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 7,337 (4411 since last year) single or group accessions from investigators outside the NCI, and approximately 68,741 records have been coded. Forty-five investigators have come to the Registry for study and consultation on single or multiple visits. The Director General of the World Health Organization (WHO) designated the Registry of Experimental Cancers as the WHO Collaborating Center for Reference on Tumors of Laboratory Animals on October 26, 1976 and the Pan American Health Organization (PAHO) renewed this collaboration on July 19, 1983. This is the only such repository in the world to be so designated by the WHO. The Registry facilitates communication between U.S. scientists and those of other countries, now numbering 153, which are members of WHO.

The Office of the Director supports, by staff and by contractor, the NCI Chemical Selection Working Group (CSWG) for NCI nominations of chemicals to the National Toxicology Program (NTP). It also maintains a Chemical Selection Planning Group (CSPG) which works with a contractor to develop nominations and make decisions on chemicals to be submitted to the CSWG. Another information dissemination activity involves preparation, under contract, of the "Survey of Compounds Which Have Been Tested for Carcinogenic Activity." Previous contracts provided for the preparation of volumes for 1974-75, 1976-77, and 1979-80, which have been distributed. The present contract, with Technical Resources, Inc., provides for the preparation of volumes for 1981-86. The 1985-86 volume of the survey was printed by the Government Printing Office and placed on sale through the Superintendant of Documents. In addition, DCE distributed copies to over 600 regulatory and public health agencies and research institutes throughout the world. The latest volume contains entries on 771 chemicals extracted from 963 articles selected from 613 journals published during 1985-86. Also distributed were new cumulative indices containing the chemical names, Chemical Abstract Service (CAS) registry numbers and accession numbers for all chemicals included in PHS-149 up to and including 1986. Work is progressing on the completion of the 1987-1988 volume. The contractor's journal screening of the literature from January 1, 1987 to December 31, 1987 has resulted in the identification of 450 articles. Data extraction, chemical, biomedical and copy editing has been completed. Screening efforts of the 1988 literature is progressing on schedule.

International Agency for Research on Cancer (IARC) Monograph Series "Evaluation of the Carcinogenic Risk of Chemicals to Humans"

The Division supports a Cooperative Agreement with IARC which is managed by staff of the Office of the Director. IARC is located in Lyon, France and the title of the project is "Evaluation of Carcinogenic Risks to Humans." IARC established this program in 1970 and monographs have been published in volumes so entitled for a large number of chemicals. Thus far 44 volumes have been published and several are in production; the volumes contain monographs in which the carcinogenic risk to man of chemicals, groups of chemicals and, more recently, of industrial and occupational exposures, as well as life-style factors, is evaluated on the basis of results in experimental animals, studies in in vitro systems and epidemiologic studies. The monographs also contain background

information on the chemicals under consideration such as chemical and physical properties, analysis, occurrence, production, use and estimated human exposures from all sources. This information is provided to IARC by NCI through a resource contract currently held by Tracor-Jitco. The IARC monograpohs have become a highly respected and authoritative reference source for countries around the world. Another IARC activity supported under this agreement is the compilation of a listing of laboratories around the world into a compendium entitled "Survey of Chemicals Being Tested for Carcinogenicity." The IARC initiated this survey in 1973 on a worldwide basis; thus far 11 surveys have been published and the twelfth survey is in preparation. These surveys are made available so that laboratories involved in carcinogenesis research can coordinate their testing and research, thus avoiding unnecessary duplication.

Registry of Tumors in Lower Animals

The Division continues to support the Registry of Tumors in Lower Animals (RTLA) which is located at the Smithsonian Institution in Washington, DC The RTLA is the focal point through which information on neoplasms in lower animals is channelled and maintained. Neoplasms and tumor-bearing animals of invertebrate or cold-blooded vertebrate species are collected, studied, classified and preserved at the Registry, which maintains the largest collection of lower animals in the world. In addition to maintaining a specimen depository, the RTLA provides a diagnostic service to biologists in many fields and consequently assists in the identification of clusters of neoplasms in animals that may have been feral exposed to environmental carcinogens in their habitat. Another ongoing activity of the RTLA is the collection and indexing of all scientific literature pertinent to neoplasia in lower animals, including experimentally induced, genetically influenced and "spontaneous" tumors. Together with a computerized listing of the Registry's specimen accessions, this constitutes virtually all the information available on neoplasms in lower animals.

Special Projects on Environmental Carcinogenesis

Centers for Disease Control (CDC): Studies on the Human Health Consequences of Polybrominated Biphenyls (PBB's) Contamination of Farms in Michigan

The Center for Environmental Health Science, Michigan Department of Public Helath (MDPH) continues to maintain and expand a 3900 individual cohort of Michigan farmers and consumers contaminated by the animal feed error of 1973. Enrollment of infants born to cohort members and the enrollment of others who previously declined overbalances the minimal "drop-out" rate. Each issue of the newsletter increases the state-wide interest in the project and is a definite aid in increasing the cohort members. The four sponsoring agencies have made a one-time investment of additional monies to provide contractual expert support for designing a master computer program that will facilitate the manipulation and retrieval of the data archived in the "Osiris" files.

Environmental Protection Agency (EPA): Performance of Collaborative Studies in the Area of Environmental Cancer

No studies were funded during this reporting period under this interagency agreement. However, dialog between NCI and EPA staff continued and members from both agencies reaffirmed interest in continuing and broadening collaboration in areas of mutual interest. Therefore, a four-year renewal of this interagency agreement was presented to and approved by the Division of Cancer Etiology Board of Scientific Counselors and executed by NCI and EPA staff. As projects of interest to both agencies are developed, they will be considered for funding under this mechanism.

National Institute for Occupational Safety and Health (NIOSH): Conduct of Research on Occupational Carcinogenesis

The major epidemiological study "Occupational Cancer in Workers Exposed to Silica and Asbestos in the North Carolina Dusty Trade Industries" is generally satisfactory. The silica cohort study (by the Univ. of North Carolina) is on schedule. Because of a one-year delay in assembling the asbestos cohort, NIOSH will probably require a one-year no-cost extension to complete the analysis. No further funding is anticipated. Likewise, the "Ethylene Oxide Mortality Study" and the industrial hygiene component of the NCI Acrylonitrile study by NIOSH are on schedule and no further funding is required. The "Industry and Occupation Coding of Death Certificates" is progressing very well technically. Some 20 states are coding routinely in this effort that is now five years old. Manuscripts and tapes are now available. There is a question as to whether or not the National Center for Health Statistics will be able to assume funding responsibility as of FY'89.

National Oceanic and Atmospheric Administration (NOAA): Etiology of Tumors in Bottom-Dwelling Marine Fish

Scientific advances that are reported include the development of a predictive modelling that correlates quantities of specific sediment contaminants with hepatic lesions in benthic species. The complete histogenesis (by light and electron microscopy) of hepatic neoplasia in the English sole has been defined.

Gulf Coast Research Laboratory; Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Animal

This contract is completing year six of the original four-year contract that was extended an additional two years because of the facility damage caused by a hurricane. With one exception (tributyltin) that was caused by chemistry difficulties, all bioassays will be completed on time and milestones met. A publication in the open literature will assure that the tributyltin bioassay will also be available. The initial sponsorship of this pioneering effort by the NCI was recognized by the Department of Defense when they awarded a contract to continue and expand this project. In addition, the EPA laboratory in Duluth, Minnesota now has an ongoing

effort to study some 60 different chemicals in aquatic animals. Also, the equipment identified and developed under this contract is being used.

Microbiological Associates Inc.: In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay and Salmonella Typhimurium Assay

After a thorough competitive process dictated by the small business set-aside procedure, the incumbent was awarded the two mutagenicity contracts. In addition to supporting nominations for chronic testing to the National Toxicology Program and supplying data to the Chemical Carcinogenesis Research Information System (CCRIS) data base of the National Library of Medicine's TOXNET, this activity provided several manuscripts on groups of compounds for the open literature. Colony sizing of revertants occurring in the lymphoma assay has been added to the protocol to provide a more mechanistic appreciation of the positive results.

Tracor Jitco, Inc.: A Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to provide Chemical Process, Production and Economic Information to the International Agency for Research on Cancer (IARC)

During this reporting period, the contractor prepared summary sheets on 11 chemicals which were considered for nomination for carcinogenicity testing by the National Toxicology Program (NTP) at two meetings of the Chemical Selection Working Group. In addition, information data sheets were prepared on 11 candidate chemicals for consideration by the Chemical Selection Planning Group. NCI, as in the past, has been the primary source for nominations of candidate chemicals to the NTP. The contractor also provided support for three IARC working group meetings. Data for Sections 1 and 2 (Chemical and Physical Data and Production, Use, Occurrence and Analysis) were prepared for a total of 34 monographs. A contractor representative attended each one of the meetings. The CCRIS data base is maintained current and is accessible to the public through the National Library of Medicine's TOXNET System. Periodic updates were provided to the National Technical Information Service for use by commercially available data services. Work continued on the extraction of data for the inclusion of a tumor inhibitor file into CCRIS. A demonstration meeting between DCE and Division of Cancer Prevention and Control staff was held to present the CCRIS data base system and discuss the feasibility of including tumor inhibitor data. The Bioassay Report Summary Handbook has been updated by the addition of summary reports on 22 new chemials.

Chemical Carcinogenesis in Nonhuman Primates

Staff of the Office of the Director direct a large project on chemical carcinogenesis in nonhuman primates. A wide variety of substances, including antitumor and antineoplastic agents; food aditives, food components and environmental contaminants; "model" rodent carcinogens; and nitroso- compounds have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Of the 29 test compounds, 16 have not as yet

demonstrated carcinogenic activity, although some have been on test for less than 4 years. Ten of the compounds are carcinogenic in nonhuman primates, producing tumors in 10-100% of the treated animals. 1-Methyl-1-nitrosurea (MNU) induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine produced malignant neoplasms, one-half of which were acute nonlymphocytic leukemia, and monkeys receiving melphalan developed fibrosarcomas of the endocervix. The effects of seven of the compounds (diethylnitrosamine [DENA], dipropylnitrosamine [DPNA], 1-nitrosopiperidine, aflatoxin B-1, MAM-acetate, urethane and sterigmatocystin) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin (acute myeloblastic leukemia), butter yellow (bronchioaveolar carcinoma), cyclophosphamide (transitional cell carcinoma of the urinary bladder), 3-methyl-DAB (hepatocellular carcinoma), 2-acetylaminofluorene (mammary adenocarcinoma), and 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (hepatocellular carcinoma).

OFFICE OF THE DIRECTOR

CONTRACTS ACTIVE DURING FY 88

ENVIRONMENTAL CANCER

Institution/Principal Investigator/Title Contract Number	
Environmental Protection Agency (EPA) W. Farland and F. Ulvedal YO1-CP-80205	Performance of Collaborative Studies in the Area of Environmental Cancer
Technical Resources, Inc. Anthony Lee NO1-CP-71114	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Tracor Jitco, Inc. Stephen S. Olin NO1-CP-41003	Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to Provide Chemical Process, Production and Economic Information as Support to the International Agency for Research on Cancer
Centers for Disease Control Rebecca Schilling YO2-CO-70529	Studies on the Human Health Consequences of Polybrominated Biphenyls (PBB's) Contamination of Farms in Michigan
Gulf Coast Research Laboratory Robin Overstreet NO1-CP-61070	Biochemical, Pharmacological and Tumorigenic Studies on a Composite of Drinking Water Carcinogens and Mutagens Utilizing Aquatic Animals as a Bioassay Animal
National Oceanic and Atmospheric Administration (NOAA) Usha Varanasi YO1-CP-40507	Etiology of Tumors in Bottom Dwelling Marine Fish
Microbiological Associates Inc. John Harbelle Steve Haworth NO1-CP-71084	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay and Salmonella Typhimurium Assay

National Institute for Occupational Safety and Health (NIOSH) Roy M. Fleming Y01-CP-60505	Conduct of Research on Occupational Carcinogenesis
Smithsonian Institution John Harshbarger N01-CP-51031	Operation of a Registry of Tumors in Lower Animals
Hazleton Laboratories America, Inc. Dan W. Dalgard N01-CP-51013	Induction, Biological Markers and Therapy of Tumors in Primates

GRANTS ACTIVE DURING FY 88

International Agency for Research on Cancer Antero Aitio 5-U01-33193-05	IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

REPORT ON INTERNATIONAL AGREEMENTS AND INFORMATION EXCHANGE ACTIVITIES

(Fiscal Year 1988)

The Division of Cancer Etiology (DCE) participates in several of the major international agreements for cooperation in cancer research: U.S.-Peoples Republic of China (1980); U.S.-Germany (1979); U.S.-Italy (1979); U.S.-Japan (1974); and U.S.-U.S.S.R (1972). Collaborative efforts include studies in cancer epidemiology and chemical, physical and biological carcinogenesis, with emphasis on factors related to the etiology and prevention of cancer. Basic and applied research is also conducted in foreign institutions under grants, contracts and cooperative agreements administered through the Division's extramural programs.

U.S.-People's Republic of China. Cancer epidemiology continues to be given high priority under the U.S.-China program, but cooperation extends to areas of molecular biology and other disciplines in cancer etiology. Dispelling the notion that Chinese cigarettes are not harmful, a case-control study in Shanghai showed that smoking was the dominant risk factor for lung cancer. Another study in Shanghai showed the risk of lung cancer among nonsmoking women tended to rise with increasing years lived with a smoking husband, providing further evidence of the health hazard of environmental tobacco smoke (i.e., passive or involuntary smoking). Nutritional hypotheses related to cancer risks are being tested in case-control and intervention studies in China. In Shandong, an area at high stomach cancer risk, elevated risks were associated with intake of salted foods and sour pancakes, while protective effects were seen with a number of vegetables, including those containing carotene, vitamin C and the Allium class (e.g., garlic). A nutritional intervention study in Linxian, an endemic area for esophageal cancer, is midway through its 5-year trial to determine effects of specific micronutrients and trace minerals on risk reduction. Several large cohort studies are underway in China to evaluate cancer risks associated with certain occupational groups. These include studies of workers exposed to benzene; workers with potential silica exposure, including many with silicosis; and tin miners, many of whom were highly exposed to radon and inorganic arsenic from very young ages. These cohorts will enable more precise assessments of carcinogenic risks and dose-response estimations. A study is underway to investigate the possible role of human papillomaviruses in the etiology of penile cancer. In southern Guangdong province, women residing in areas with high natural background radiation from radioactive monazite sands were not found to have a higher prevalence of thyroid nodular disease than women in neighboring areas, suggesting that protracted exposures to very low levels of ionizing radiation throughout life are not associated with thyroid disease. Medical x-ray workers in China were found to have a 50% higher risk of developing cancer than other specialists, with excesses of leukemia, breast, and thyroid cancers being observed. To investigate the role of environmental arsenical air pollution, a case-control study of lung cancer is underway in Shenyang, where China's largest copper smelter is centered in a densely populated residential area. The effect of indoor air pollution on the risk of lung cancer is also being investigated in China. In Shenyang, preliminary results suggest a rising lung cancer risk associated with duration of exposure to coal-burning stoves, which

generate high levels of polycyclic hydrocarbons. In a study in Shanghai, exposure to fumes from high-temperature wok cooking seems to be related to the high risk of lung adenocarcinoma among nonsmoking Chinese women, and use of rapeseed and soybean cooking oils has been implicated as possible risk factors. Radon is also being investigated as an indoor pollutant in the study of lung cancer in Shenyang. Collaborative laboratory research continued during the year. Progress was made on establishing culture conditions for human liver and esophagus, studying the metabolism of chemical carcinogens, assessing biochemical and immunochemical markers in persons at high risk of liver, esophagus, stomach, and lung cancers, and investigating in vitro transformation of human epithelial cells by microbial and chemical agents.

SCIENTIST EXCHANGES

China to U.S.:

Applicant (Laboratory)	Recipient (Laboratory)	Duration	Title of Research
Dr. C. Hsia Dr. T. Sun Cancer Institute (Beijing)	Dr. C. Harris National Cancer Inst. (Bethesda, MD)	4 months	Risk Factors for Liver and Esophageal Cancers

U.S.-Federal Republic of Germany. Substantial progress has continued during the past year in reactivating this program. A highly successful bilateral workshop entitled "The Role of DNA Amplification in Tumor Initiation and Promotion" was convened in Heidelberg in October of 1986 and has formed the basis for planned scientific collaborations between German and American workers in this rapidly developing area of research. A number of research areas for collaborative projects have been presented to the Senate Commission for Cancer Research of the DFG and selection of German scientists to participate in exchanges has been completed. In the field of epidemiology, discussions have been held to explore areas of common interest, and a joint workshop on the role of statistics in cancer epidemiology was held in October of 1987.

U.S.-Italy Agreement. Research pertinent to this Division is included in the Cancer Prevention area of the U.S.-Italy Cancer Program. The program involves a variety of activities related to cancer etiology, with recent emphasis on epidemiology. A collaborative case-control study of stomach cancer, the leading cause of cancer death in parts of northern and central Italy, continued. This multicenter study was stimulated as a result of workshops organized under the Agreement, and analyses of the collected data have begun. A workshop on cancer epidemiology was held in Portofino, with discussions centering on the roles of nutrition and pesticides in cancer etiology. Stomach cancer was the focus of the nutrition discussion, which included biological markers and future research ideas. The session on pesticides centered on the review of ongoing and planned studies, including a collaborative multicenter case-control study of lymphatic and hematopoietic cancers that may be related

to herbicide and insecticide exposures. Under the auspices of the Agreement, Italian epidemiologists visited the NCI to conduct collaborative analyses of studies related to lung, bladder, and stomach cancers in the U.S. and Italy.

U.S.-Japan Agreement. This year marks the fourth year of the third 5-year program of this Agreement, which consists of four broad program areas: Etiology, Cancer Biology and Diagnosis, Cancer Treatment, and Interdisciplinary Research. The cooperation between U.S. and Japanese scientists still remains one of the most active and is especially well suited to the study of malignancies that differ markedly in their occurrences between the two countries. Within the Interdisciplinary areas of the Agreement, two workshops were held during the year. One workshop addressed melanoma, which is rare among Japanese, partly because of the low frequency of dysplastic nevus syndrome (DNS) and partly because of skin pigmentation and shielding from sun exposure. To confirm this low frequency, screening for DNS among atomic bomb survivors will soon start as part of a large-scale skin cancer survey. A highlight of the workshop was a presentation by Prince Hitachi, whose research at the Tokyo Cancer Institute concerns melanoma in giant goldfish (carp). The second workshop addressed the familial breast cancer-soft tissue sarcoma (Li-Fraumeni) syndrome. The workshop participants discussed new research ideas related to this syndrome. The All-Japan Childhood Tumor Registry was used to identify families that may have the syndrome, as well as other families with unusual cancer aggregations. This case-series will be further studied in an investigator exchange visit made under the auspices of the U.S.-Japan Cooperative Cancer Research Program. In addition to these workshops, staff served as the foreign co-organizers of the annual international symposium sponsored by the Princess Takamatsu Cancer Research Foundation, and whose subject was "Rare Events as Clues to Cancer Etiology." Within the Etiology Area of the U.S.-Japan Agreement, seminars were held on "Development of New Medium-Term Bioassay for Carcinogens," "Cancer Cell Membranes: Aberrant Glycosylation and Other Critical Molecular Events," and "Biology of Oncogenes."

Four scientists from Japan and one scientist from the United States participated in exchange programs in etiology research, which has as its mission to provide a fundamental basis for understanding cancer causation that, in turn, would identify effective means for preventing or modulating this process.

SCIENTIST EXCHANGES

Japan to U.S.:

Applicant (Laboratory)	Recipient(s) (Laboratory)	Duration	Title of Research
Dr. Nam-ho Huh Univ. of Tokyo	Dr. Anthony E. Pegg Milton S. Hershey Medical Center Pennsylvania State Univ. Hershey, Pennsylvania	2 weeks	Mechanisms of Carcinogenesis
	Dr. Curtis C. Harris Lab. of Human Carcinogenesis National Cancer Institute Bethesda, Maryland	1 week	
	Dr. Eliezer Huberman Argonne National Laboratory Chicago, Illinois	1 week	
Dr. K. Yaginuma Cancer Institute	Dr. Jesse W. Summers Inst. for Cancer Research Fox Chase Cancer Center Philadelphia, Pennsylvania	1 month	Production of Duck Hepatitis B Virus (DHBV) by a Human Hepatoma Cell Line HuH-7 After Transfection with DHBV DNA
Dr. Masao Hirose Nagoya City Univ.	Dr. H. Witschi Lab. of Energy Related Health Research University of California Davis, California	2 weeks	Roles of Antioxidants in Chemical Carcinogenesis
Dr. Y. Ishizaka National Cancer Ctr. Res. Inst.	Dr. Anthony V. Carrano Lawrence Livermore National Laboratory Livermore, California	1 month	Oncogene and Tumor Biology

U.S. to Japan:

Dr. W. Zacharias Univ. of Alabama at Birmingham Birmingham, Alabama	Dr. Hikoya Hayatsu Okayama University	1 month	Characterization of the Behavior of Mutagenic Probes for Site Reactivity at Unusual DNA Conformations, Especially Left Handed Z-DNA

In addition, the following compounds were shipped to Japan from the United States:

5 mg of benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide(anti),

5 mg of benzo(a)pyrene-trans-7,8,-dihydrodiol-9-10-epoxide(syn),

5 mg of benzo(a)pyrene-4,5-dihydroepoxide,

50 mg each of fecapentaene-12 and fecapentaene-14, and

5 gm of nitrilotriacetic acid trisodium salt, monohydrate.

Also, the following compounds were shipped to the United States from Japan:

1.80 g of 2-amino-3,4,8-trimethylimidazo (4,5-f) quinoxalin

1.91 g of 2-amino-3,8-dimethylimidazo (4,5-f) quinoxalin

U.S.-U.S.S.R. Agreement. In the field of epidemiology, discussions and communications continued regarding collaborative studies of cancers of mutual interest, and protocols and questionnaires for ongoing investigations were exchanged. Correspondence centered around possible cooperative and joint studies to evaluate the following neoplasms: stomach cancer (a leading cause of cancer death in the U.S.S.R.), oral cancer (to evaluate the role of nass, a smokeless tobacco used in high-risk areas), melanoma (to clarify the role of sunlight and various host factors), and colonic polyps (to circumvent problems associated with invasive cancer). In the area of cancer etiology, collaborative studies on perinatal carcinogenesis have been proposed. During the past year, protocol development has been actively pursued between American and USSR scientists. Specific experiments will be initiated during the coming fiscal year. The first exchange of scientists under this agreement will take place in the near future. Plans for this visit, as well as other collaborations in this program area were advanced during the visit of DCE scientists to Leningrad in May-June 1988 to attend the International Symposium on Perinatal and Multigeneration Carcinogenesis, organized jointly by the International Agency for Research on Cancer (Lyon,

France), the Petrov Institute, and NCI. Although this meeting was not funded under the bilateral agreement, it served as a useful opportunity to advance plans for cooperative research negotiated under the agreement.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP03509-25 OD

PERIOD COVERED
October 1, 1986 to September 30, 1987

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Carcinogenesis, Chemotherapy and Biological Markers in Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. M. Sieber	Deputy Director	OD, DCE	NCI
Others:	U. Thorgeirsson	Expert	OD, DCE	NCI

COOPERATING UNITS *(if any)*
Department of Pathology, Louisiana State University, New Orleans, LA (P. Correa); Hazleton Laboratories America, Inc., Vienna, VA (D. Dalgard)

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A wide variety of substances, including antitumor and antineoplastic agents; food additives, food components and environmental contaminants; "model" rodent carcinogens; and nitroso- compounds have been or are being evaluated in four species of nonhuman primates for their potential carcinogenicity and other long-term toxic effects. Of the 29 test compounds, 16 have not as yet demonstrated carcinogenic activity, although some have been on test for less than 4 years. Ten of the compounds are carcinogenic in nonhuman primates, producing tumors in 10-100% of the treated animals. 1-Methyl-1-nitrosourea (MNU) induced squamous cell carcinomas of the oropharynx and esophagus, with the esophageal tumors possessing clinical and morphologic similarities to human esophageal carcinoma. Long-term treatment with procarbazine produced malignant neoplasms, one-half of which were acute nonlymphocytic leukemia, and monkeys receiving melphalan developed fibrosarcomas of the endocervix. The effects of seven of the compounds (diethylnitrosamine [DENA], dipropyl-nitrosamine [DPNA], 1-nitrosopiperidine, aflatoxin B-1, MAM-acetate, urethane and sterigmatocystin) were manifested primarily as hepatocarcinogenicity. Single cases of malignant tumors have been diagnosed in animals treated with adriamycin (acute myeloblastic leukemia), butter yellow (bronchioalveolar carcinoma), cyclophosphamide (transitional cell carcinoma of the urinary bladder), 3-methyl-DAB (hepatocellular carcinoma), 2-acetylaminofluorene (mammary adenocarcinoma), and 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (hepatocellular carcinoma).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. M. Sieber	Deputy Director	OD, DCE	NCI
U. Thorgeirsson	Expert	OD, DCE	NCI

Objectives:

One of the objectives of this project is to obtain comparative data on the response of nonhuman primates to known rodent carcinogens as well as to materials suspected of being human carcinogens. Of particular interest in this regard is to evaluate, in monkeys, several chemicals of social or economic importance (e.g., DDT, saccharin, cyclamate, arsenic) and for which rodent data are difficult to interpret or are contradictory. Another objective of the project is to evaluate the long-term toxic effects (including carcinogenicity) of antineoplastic agents which currently are in clinical use for primary and adjuvant cancer therapy and for treatment of an increasingly broad spectrum of nonmalignant diseases. Other objectives include obtaining model tumor systems in primates for use in testing the effectiveness of new antitumor agents, developing models and strategies for chemoprevention, and developing biological markers and diagnostic tests for early detection of tumors. In addition, this project makes available normal and tumor-bearing animals for a variety of pharmacologic, toxicologic, biochemical and immunological studies. The maintenance of a breeding colony ensures that offspring will be available for use.

Methods Employed:

Twenty-nine substances are currently under investigation or have been investigated, including antineoplastic and immunosuppressive agents (procarbazine, adriamycin, MNU, melphalan, azathioprine and cyclophosphamide), food additives, food components and environmental contaminants (aflatoxin B_1, methylazoxymethanolacetate, sterigmatocystin, 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline [IQ], cyclamate, saccharin, butter yellow, 3-methyl-DAB, DDT, arsenic and cigarette smoke condensate), "model" rodent carcinogens (urethane, 3-MC, 2-acetylaminofluorene [2-AAF], 2,7-AAF, copper chelate of N-OH-AAF, dibenzpyrene and dibenzanthracene), and various nitroso- compounds (dimethylnitrosamine [DMNA], DENA, DPNA, 1-nitrosopiperidine and N-methyl-N'-nitro-N-nitrosoguanidine [MNNG]).

The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound was added to the Similac formula at the time of feeding; when the monkeys are 6 months old, carcinogens given orally are incorporated into a vitamin mixture which is

given to monkeys as a vitamin sandwich on a half slice of bread. An alternate way of giving doses orally is to incorporate the compound into baited foods or to administer it by intubation. The dose level chosen is dependent on the chemical under evaluation. Antineoplastic and immunosuppressive agents are administered at doses likely to be encountered in a clinical situation; other substances, such as environmental contaminants, are given at levels 10- to 40-fold higher than the estimated human exposure level. The remainder of the chemicals tested are administered at maximally tolerated doses which, on the basis of weight gain, blood chemistry and hematology findings, and clinical observations, appear to be devoid of acute toxicity.

The present colony, consisting of 488 animals, is comprised of four species: Macaca mulatta (rhesus), Macaca fascicularis (cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Forty-eight of these monkeys are adult breeders which supply juvenile animals for experimental studies. The majority of the animals are housed in an isolated facility which contains only animals committed to this study, and with the exception of the breeding colony and some juvenile animals, animals are housed in individual cages. The administration of test compounds is continued until a tumor is diagnosed or until a predetermined exposure period is completed. A minimum of 30 animals is usually allotted to each treatment group, since in a sample of this size it is possible to detect a tumor incidence of 10% within 95% confidence limits.

A variety of clinical, biochemical and hematological parameters are monitored weekly or monthly, not only to evaluate the general health status of each animal, but also for the early detection of tumors. Surgical procedures are performed under Ketamine or sodium pentobarbital anesthesia. All animals which die or are sacrificed are carefully necropsied and the tissues subjected to histopathologic examination.

Major Findings:

The six test chemicals categorized as antineoplastic and immunosuppressive agents are procarbazine, MNU, adriamycin, melphalan, azathioprine and cyclophosphamide. Both procarbazine and MNU are carcinogenic in monkeys, inducing tumors in approximately one-third of the animals autopsied thus far. One-half of the tumor-bearing animals in the procarbazine study developed acute nonlymphocytic leukemia; the other half developed solid tumors at a variety of sites. MNU was administered orally and induced squamous cell carcinoma of the mouth, pharynx and/or esophagus. During the past year, highly aggressive, poorly differentiated fibrosarcomas have developed in 10% of a group of 20 animals receiving long-term treatment with melphalan. Two of the chemicals in this series have induced neoplasms in only one animal thus far. In a group of 10 animals receiving monthly IV doses of adriamycin (1.0 mg/kg), 9 developed congestive heart failure and died or were sacrificed in moribund condition; the tenth animal was diagnosed with acute myeloblastic leukemia. This study is

being repeated using lower doses of adriamycin to evaluate further its leukemogenic potential. One of a group of 23 animals receiving cyclophosphamide developed transitional cell carcinoma of the urinary bladder. Animals in this study have been receiving cyclophosphamide for approximately 8 years. The other chemical in this series, azathioprine, has been on test for approximately 12 years, but has provided no evidence of carcinogenicity.

The chemicals categorized as food additives, food components and environmental contaminants include two fungal products, aflatoxin B_1 and sterigmatocystin; MAM-acetate, the synthetic aglycone of cycasin; the artificial sweeteners, cyclamate and saccharin; the azo dyes, butter yellow and its 3-methyl derivative; and the pesticides, DDT and arsenic. Both of the fungal products are carcinogenic in monkeys, inducing hepatocellular carcinoma in 59% (aflatoxin B_1) and 20% (sterigmatocystin) of the animals autopsied thus far. MAM-acetate is also a hepatocarcinogen, inducing tumors in 37% of autopsied monkeys. One animal each in both groups receiving the azo dyes developed a tumor. The tumors, a bronchioalveolar carcinoma in a monkey treated with butter yellow and a hepatocellular carcinoma in an animal treated with 3-methyl-DAB, developed in relatively old monkeys and more than 15 years after the last exposure to the test compounds. Thus, these tumors may be spontaneous rather than chemically induced. The remainder of the test chemicals in this series have not provided any evidence of carcinogenicity, despite the fact that most have been on test for relatively prolonged periods. For example, the saccharin and cyclamate studies have been in progress for 17 years, the DDT and arsenic studies for 11 and 18 years, respectively. The most recently initiated study in this series is an evaluation of the carcinogenicity of IQ, a heterocyclic amine isolated from pyrolysates of amino acids and proteins. Thus far, one animal has developed an hepatocellular carcinoma; the tumor was diagnosed 23 months after dosing with IQ was initiated.

The chemicals categorized as "model" rodent carcinogens are urethane, 3-methylcholanthrene, 2-AAF, 2,7-AAF, the copper chelate of N-hydroxy-AAF and benzpyrene. Of these chemicals only urethane has proved to be consistently carcinogenic in monkeys. Tumors have been detected in 20% of the animals necropsied thus far; many of the animals had multiple primary tumors which, in most cases, included liver hemangiosarcomas. The tumors were diagnosed after an unusually lengthy latent period which averaged 14 years. The surviving animals in all the test groups in this series have been under observation for tumor development for at least 22 years.

The nitroso- class of chemicals tested include dimethyl-, diethyl-, and dipropylnitrosamine, N-nitrosopiperidine and MNNG. As a general class, they are active hepatocarcinogens in nonhuman primates. Dimethylnitrosamine did not induce liver tumors; the high level of liver toxicity exerted by this compound and the associated early mortality probably precluded liver tumor development. The other two nitrosamines, DENA and DPNA, were potent hepatocarcinogens capable of inducing primary hepatocellular carcinoma in virtually every animal exposed.

MNNG has not, as yet, provided evidence of having carcinogenic activity in non-human primates; it has been under evaluation for 13-15 years, although only recently has the dose been increased to the range shown to induce tumors in other species such as dogs and rodents.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04548-16 OD

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Registry of Experimental Cancers/WHO Collab. Ctr. for Tumours of Lab Animals

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

P.I.:	Harold L. Stewart	Scientist Emeritus	DCE	NCI
Others:	Bernard Sass	Veterinary Medical Officer	DCE	NCI
	Margaret K. Deringer	Guest Researcher	DCE	NCI
	Carel F. Hollander	Guest Researcher	DCE	NCI
	Annabel G. Liebelt	Expert	DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Office of the Director

SECTION

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.5	2.5	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 7,337 (4,411 since the 1987 report) single or group accessions from investigators outside the NCI and approximately 68,741 records have been coded. Forty-five investigators have come to the Registry for study and consultation on single or multiple visits.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Harold L. Stewart	Scientist Emeritus	DCE	NCI
Bernard Sass	Veterinary Medical Officer	DCE	NCI
Margaret K. Deringer	Guest Researcher	DCE	NCI
Carel F. Hollander	Guest Researcher	DCE	NCI
Annabel G. Liebelt	Expert	DCE	NCI

Objectives:

1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents); 2) the use of such pathologic material and data for research and educational purposes.

Methods Employed:

The methods employed in the work of the Registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed and many of the data have been entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other Governmental agencies, industrial laboratories, and universities here and abroad. A total of 4,137 (792 since the 1987 report) single or group accessions from investigators outside of NIH have been processed. The Registry prepares Study Sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has Study Sets of slides on "Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms," "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice," "Induced Tumors of the Liver in Rats," "Tumors and Nonneoplastic Lesions of the Lungs of Mice," "Mammary Tumors in Mice," "Pulmonary Metastases in Mice," "Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice," "Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis," "Malignant Schwannomas of Rats," "Harderian Gland Tumors of Mice," "Renal Tumors of Rats," "Spontaneous Gastric Adenomatosis, Polyps and Diverticula; Duodenal Plaques of Mice," "Adrenal Tumors of Mice," "Schwannomas of Mice," and "Malignant Tumors of the Soft Tissues and a Few Miscellaneous Tumors." These Study Sets, with descriptive material, are loaned to investigators who request them. Twenty-eight loans have been made this year, six of which were to countries abroad.

Investigators come to the Registry for study and consultation. There have been single or multiple consultations with 45 individuals since the 1987 report.

During the period from January 1980 until April 30, 1988, the Registry has received 3,751 requests for reprints of Histologic Typing of Liver Tumors of the Rat (JNCI 1980;64:177-206). This histologic classification and typing of rat liver tumors is calculated to promote uniformity of diagnoses from one laboratory to another in this country.

The Director General of the World Health Organization (WHO) designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumours of Laboratory Animals on October 26, 1976 and the Pan American Health Organization renewed this collaboration in March 1988. This is the only such registry in the world to be so designated by the WHO. The Registry will expand communications between U.S. scientists and those of other countries, now numbering 153, which are members of WHO.

Major Findings:

The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Publications

Dunn TB and Deringer MK: Reminiscences of meeting for lunch in the pathology laboratory. J Exp Pathol 1987;3:105-14.

Hayes HM Jr and Sass B: Testicular tumors: species and strain variations. In: Kaiser HE (ed.). Progressive stages of malignant neoplastic growth. Boston, Martinus Nijhoff (In Press)

Hayes HM and Sass B: Testis neoplasms: Part 1 - an epidemiological review and compendium of reported tumor cell-types in laboratory animal species. Lab Anim 1987;16:35-45.

Hayes HM and Sass B: Testis neoplasms: Part 2 - a review and compendium of experimentally induced and environmentally influenced tumor cell-types in laboratory animals. Lab Anim 1987;16 9:27-34.

Hoch-Ligeti C and Stewart HL: Cardiac tumors in laboratory rodents-comparative pathology. In: Kaiser HE (ed.). Progressive stages of malignant neoplastic growth. Boston, Martinus Nijhoff (In Press)

Liebelt AG: Malignant neoplasms in organ transplant recipient. In Kaiser, HE (Ed.): Progressive stages of malignant neoplastic growth. Boston, Martinus Nijhoff (In Press)

Liebelt AG, Sass B and Lombard LS: Mouse ovarian tumors - a review including classification and induction of neoplastic lesions and description of several previously unreported types. J Exp Pathol 1987;3:115-45.

Sass B: Bovine lymphoma - epidemiology, diagnosis, transmission, pathology. In Kaiser HE (ed.). Progressive stages of malignant neoplastic growth. Boston, Martinus Nijhoff (In Press)

Sass B and Hayes HM Jr: Chemodectomas of man and animals. In: Kaiser, HE (ed.): Progressive stages of malignant neoplastic growth. Boston, Martinus Nijhoff (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05551-01 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Liposomes as Carriers for Anti-HIV Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Parker	Expert	OD, DCE	NCI
Others:	S. M. Sieber	Deputy Director	OD, DCE	NCI
	Z. Hao	Visiting Fellow	LBP, DCT	NCI
	J. N. Weinstein	Senior Investigator	LMB, DCB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Liposomes are under investigation as carriers of 2'3'-dideoxynucleotides (ddNTs) in an effort to enhance the activity of these compounds against the human immunodeficiency virus (HIV). Although it is the ddNTs which actually block viral replication by inhibition of HIV-reverse transcriptase (HIV-RT), only their precursors, the 2'3'-dideoxynucleosides (ddNSs), can be used clinically since ddNTs are unstable in the presence of serum and are not readily taken up by cells. Dideoxycytidine triphosphate (ddCTP), the active anabolite of dideoxycytidine (ddCyt), was found to increase anti-HIV activity when entrapped in liposomes as compared to the free compound. Furthermore, the degradation of ddCTP by serum components was significantly decreased when the compound was entrapped in liposomes. Unlike other ddNSs tested, dideoxyuridine (ddUrd) was found to be devoid of anti-HIV activity, although its corresponding ddNT, 2,3'-dideoxyuridine triphosphate (ddUTP), was shown to be a potent inhibitor of HIV-RT. Liposome-entrapped ddUTP is currently being tested for anti-HIV activity. These findings suggest that ddNTs, which are inappropriate for clinical usage for the reasons stated above, could be useful when administered as liposome-entrapped agents.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Parker	Expert	OD, DCE	NCI
S. M. Sieber	Deputy Director	OD, DCE	NCI
Z. Hao	Visiting Fellow	LBP, DCT	NCI
J. N. Weinstein	Senior Investigator	LMB, DCB	NCI

Objectives:

The major goal of this project is to study the effects of liposome entrapment of 2'3'-dideoxynucleotides (ddNTs) in an effort to enhance their activity against HIV. Since liposomes are degraded by phagocytic uptake into macrophages, liposomes may provide a means to direct entrapped antiviral agents to macrophages, which are one of the cell types known to harbor HIV. Furthermore, liposome entrapment has been shown to afford entrapped materials a modest degree of protection against degradation by circulating enzymes and other serum components. These characteristics of liposomes may be of particular importance since ddNTs are not readily taken up by cells and are extensively degraded in the presence of serum.

Methods Employed:

1. Identification and quantitation of ddCyt, ddUrd, ddCTP, ddUTP and their metabolites in media and serum was achieved by a combination of reverse-phase and ion exchange high performance liquid chromatography (HPLC) using both UV and radioactivity detectio Samples containing liposome entrapped ddNTs or ddNSs were treated with Triton-X prior to HPLC analysis in order to release the entrapped material.

2. ddCTP, ddUTP, and ddCyt were entrapped in liposomes composed of phosphatidylserine/lecithin/cholesterol (molar ratio 18:37:45) by extrusion under high pressure. Entrapment efficiencies ranged from 8-10%. Leakage of liposome-entrapped compounds was monitored in stirred dialysis cells at 4° or 37°C in the presence or absence of serum.

3. Antiviral activity of free and liposome entrapped ddCyt and ddCTP was monitored in elutriated human monocytes infected with a monocytotropic strain of HIV (Ba-L). Antiviral activity of free ddUrd and ddUTP was tested in HIV-infected ATH8 human T-lymphocytes. Infected cells were placed in tissue culture wells and treated with free or liposome-entrapped compounds on days 1, 3, 5, and 7 following infection. On days 3, 5, 7, 10, and 12 after infection, samples of supernatant from each well were tested for the presence of p24 viral-core protein by enzyme-linked immunosorbant assay (ELISA).

Major Findings:

Liposomes released <10% of their entrapped ddCTP within 4 days in the presence of cells and culture medium at 37°C. ddCyt was released much more rapidly from liposomes than was ddCTP. Free ddCTP at 62.5 nM inhibited p24 expression >90% in human elutriated monocytes infected with a monocytotropic strain of HIV (Ba-L). Liposome entrapped ddCTP inhibited p24 expression with similar potency while empty liposomes had no effect. Microscopic examination using fluorescently labelled liposomes indicated endocytic uptake of the liposomes by monocytes. HPLC analysis revealed progressive dephosphorylation of the free, but not the liposome-entrapped, ddCTP.

Of the ddNSs containing naturally occurring bases - cytidine, thymidine, uridine, guanosine, adenosine, and inosine - only 2'3'-dideoxyuridine (ddURd) was found to be devoid of anti-HIV activity in the ATH8 test system. Suprisingly, dideoxyuridine triphosphate (ddUTP), the active anabolite of ddUdr, was found to be a potent inhibitor of HIV-RT. This finding suggests that the inactivity of ddUrd against HIV may be due to the absence of the appropriate anabolic enzymes that convert ddUdr to ddUTP or that any ddUTP formed intracellularly is rapidly degraded. The latter possibility was confirmed with cellular studies using labelled ddUdr. Investigations on the effect of liposome-entrapped ddUTP on HIV-infected ATH8 cells are currently in progress.

Publications:

Black CDV, Parker RJ. Behavior and fate of liposomes in vivo. In New RC, ed. Liposomes: a practical approach. Oxford: IRL Press, 1988.

Holton OD, Black CDV, Parker RJ, Covell DG, Barbet J, Sieber SM, Talley MJ, Weinstein JN. Biodistribution of monoclonal IgG1, $F(ab')_2$, and Fab' in mice after intravenous injection. A comparison between anti-B cell (anti-Lyb8.2) and irrelevant (MOPC-21) antibodies. J Immunol 1987;139:3041-9.

Weinstein JN, Black CDV, Holton OD, Covell DG, Parker RJ, Mulshine JL, Lotze MT, Carrasquillo J, Eger R, Lewis A, Larson SM, Keenan A. Delivery of monoclonal antibodies to lymph nodes via the lymphatics. In Winkelhake JL, Holcenberg JS, eds. The pharmacology and toxicology of proteins. New York: Alan R Liss Inc., 1987;75-89.

Weinstein JN, Covell DG, Barbet J, Eger RR, Holton OD, Talley MJ, Parker RJ, Black CDV. Local and cellular factors in the pharmacology of monoclonal antibodies. In Bonavida B, Collier RJ, eds. Membrane mediated cytotoxicity. New York: Alan R. Liss Inc., 1987;279-89.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05576-01 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of ras Oncogene and Collagenase in Primary Tumors vs. Metastases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	M. Ballin	Visiting Fellow	OD, DCE	NCI
	C. C. Sinha	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In some human malignancies tumor progression has been associated with amplification or increased expression of either myc or ras oncogenes. We used an N-nitrosomethylurea (NMU)-induced rat mammary carcinoma model to study ras expression in primary vs. metastatic tumors. Evaluation of a primary tumor and ten lung metastases derived from this primary revealed the following findings. There was considerable variability in the ras-specific DNA levels among the individual metastases; some were lower, while others were higher than the parent primary tumor. Similarly, the ras RNA levels were variable, with three of ten metastases showing barely detectable ras expression by slot blot analysis. There was no significant difference in the ras DNA levels between normal breast and the neoplastic tissues, either primary metastatic, nonmetastatic, or metastases. Furthermore, there was much less variability in the ras levels of the NMU-tumors after multiple passages in syngeneic rats than in autochthonous tumors.

Ongoing studies on metalloproteinase expression by the NMU-tumors have revealed that type IV collagenolytic activity was higher in the tumors than in normal mammary glands. However, the collagenolytic activity in metastases did not differ from the primary tumor. To complete the project the metalloproteinases present in tumor lysates and expressed by cell lines derived from primary tumor and metastases will be studied by gelatin gel electrophoresis. Lastly, comparison of ras expression in different parts of primary tumors and metastases will be made by immunoperoxidase staining of the p21 ras product using antibody raised against the p21 protein.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
M. Ballin	Visiting Fellow	OD, DCE	NCI
C. C. Sinha	Biologist	OD, DCE	NCI

Objectives:

Induction of the metastatic phenotype through transfecting activated *ras* oncogenes into either normal cells or nonmetastatic tumor cells has offered a new approach to study genetic control of metastasis. The major objective of the present study is to assess whether *ras* oncogene amplification or increased expression correlates with more aggressive tumor behaviour. We also wished to find out if *ras* expression is linked to the release of basememt membrane degrading metalloproteinases, since our previous observations had demonstrated that activated *ras* oncogenes can turn on simultaneously the metastatic phenotype and the capacity to degrade basement membrane collagen. The two models used in the study were: a) an NMU-induced rat mammary carcinoma known to possess mutationally activated c-Ha-*ras*; and b) v-Ha-*ras* transfected NIH/3T3 (433) cells carrying a glucocorticoid sensitive mouse mammary tumor virus (MMTV) promoter. The autochthonous NMU-induced rat mammary tumors offer an opportunity to quantitate DNA and RNA levels in mammary tissues ranging from normal breast to metastatic tumors. On the other hand, 433 cells transfected with v-Ha-*ras* can, through treatment with the glucocorticoid dexamethasone, induce their *ras* expression up to 20-fold. It will also be important to examine *ras* expression at a cellular level. This will be done by immunoperoxidase staining of histological sections, using p21 antibody.

Methods Employed:

1. A single injection of NMU (30 µg/g body weight) into Sprague Dawley rats at 50 d of age induces mammary tumors in 90% of the animals within 3-6 months. The tumors are frozen in liquid nitrogen immediately after the removal, pulverized in a mortar and homogenized by douncing in a lysate buffer, composed of 0.05 M Tris, pH 7.4, 0.01 M $CaCl_2$ and 0.25% Triton X-100. The homogenates are centrifuged at 10,000 RPM for 10 min and the supernatant dialyzed against 0.05 M Tris, pH 7.4, 0.2 M NaCl, 5 mm $CaCl_2$ overnight at 4°C, and subsequently stored at -20°C, until tested for type IV collagenolytic and gelatinolytic metalloproteinase activity.

2. Samples of tumor lysates or tumor interstitial fluids (5 µg) are analyzed by gelatin or type IV collagen gel electrophoresis. Preparation of the gel and the running buffer is the same as for regular sodium dodecyl sulfate (SDS) gels except for the addition of 0.1% gelatin or 0.04% type IV collagen

to 7.4% acrylamide separating gel. The electrophoresis is performed under nonreducing conditions at a constant current of 9 mA at 4°C. Following electrophoresis the gels are washed three times in 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100 for 30 min with shaking at room temperature. Gels are then rinsed three times in 50 mM Tris-HCl (pH 7.4) for 5 min, and then incubated in a buffer containing 0.05M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3 pH 7.4 at 37°C for 16 h. The gelatin gels are stained and fixed for 1 h shaking at room temperature using 1% amido black in a mixture of acetic acid:methanol:water (1:3:6) and then destained in the same mixture without amido black. Collagen IV gels are stained with 0.1% Coumassie blue in 10% trichloracetic acid and destained in 7% acetic acid. Bands of proteolytic activity are visualized by negative staining.

3. Assay for type IV collagenolytic activity. The secreted type IV collagen degrading activity is measured in serum-free culture supernatants with and without the addition of TPA. The supernatants are concentrated 100-fold with ammonium sulfate (0-60%), followed by dialysis against 0.2M NaCl, 0.05M Tris-HCl, 0.01M $CaCl_2$, pH 7.4.

   The type IV collagen degradation is assayed as previously described with the use of soluble [^{3}H] proline-labeled type IV collagen as a substrate. The enzyme in duplicate samples is activated by the addition of 10 µg/ml trypsin for 10 min at 37°C, and then assayed in the presence of 50 µg/ml soybean trypsin inhibitor for 16 hours at 37°C. Negative controls included trypsin and soybean trypsin inhibitor incubated only with the labeled collagen substrate. Bacterial collagenase (0.1% w/w) is used as a positive control to achieve the maximum collagen degradation. The reaction is terminated by incubating the samples on ice for 30 min in the presence of 0.6% trichloroacetic acid, 0.6% tannic acid and 25 µg/ml bovine serum albumin. The undigested substrate was removed by centrifugation and the radioactivity of the degraded type IV collagen in the supernatant is measured in a beta-scintillation counter.

4. DNA and RNA is extracted from NMU-induced tumors. For detection of Harvey ras-specific sequences, DNA is digested with PVU II, electrophoresed through 0.8% agarose gel and transferred to nitrocellulose filters by the Southern blotting technique. The filters are hybridized with 10^6 cmp/ml of nick-translated ^{32}P-labelled v-Harvey-ras probe for 24 hours at 37°C. The blot hybridization mixture contained 3X SSC (sodium chloride, sodium citrate, pH 7.0), 0.05 M Tris (pH 7.5), 5X Denhardt's solution, 1 mM EDTA, and 50% formamide. The hybridized blots are washed three times in 2X SSC, 0.1% SDS at room temperature and three times in 0.1X SSC, 0.1% SDS at 60°C. The final blots are exposed to Kodak XR-5 film at 70°C for 2 days. Slot blot analysis is performed on c-Ha-ras-specific DNA and RNA sequences of the NMU-induced tumors. Ten micrograms of denatured DNA or RNA are filtered through nitrocellulose filters and hybridized with a ^{32}P-labeled v-Ha-ras probe as

described above for the Southern blot. Quantitation is made with densitometric tracings and expressed as relative density units.

5. Assays of metastatic 433 cells with and without dexamethasone treatment. The 433 cells are treated with dexamethasone (2×10^{-6}M) for 6 days for both *in vitro* and *in vivo* studies. For experimental metastasis assays, 5×10^5 cells with or without the 6 day dexamethasone treatment are injected in a volume of 0.1 ml into the lateral tail vein of 3-4 week old NIH nude mice. The mice are sacrificed 4 weeks later, and the lungs are fixed immediately through intratracheal injection of Bouin's solution. The lungs are then removed and the metastases enumerated under a dissecting microscope after fixing for 24 hours. Assays for spontaneous metastases involve injection of 10^6 cells in 0.2 ml intramuscularly into the thigh. The mice are sacrificed 6-8 weeks later and are examined for pulmonary and extrapulmonary metastases.

Major Findings:

1. Quantitation of *ras*-specific DNA and RNA levels in NMU-induced rat mammary carcinomas revealed variability in both DNA and RNA levels in ten individual metastases examined. Some were lower, others higher than the primary parent tumor. Three of the metastases had barely detectable *ras* expression.

2. There was no significant difference in *ras*-specific DNA levels among the following groups: normal mammary glands, nonmetastatic tumors, metastatic tumors, and metastases.

3. Multiple passages of the metastatic tumors in syngeneic rats resulted in more homogeneous levels of *ras*-specific DNA and reduction in type IV collagenolytic activity.

4. Type IV collagenolytic activity was higher in NMU-induced tumors than in normal rat mammary glands.

5. Type IV collagenolytic activity in lysates of individual metastases did not exceed that of the primary tumor.

6. NIH/3T3 cells transfected with v-Ha-*ras* under the transcriptional control of a glucocorticoid-sensitive MMTV promoter showed up to a twenty-fold increase in *ras* expression when treated with dexamethasone. However, the dexamethasone treated transfectants produced 2-3 times fewer metastases than the untreated control. Type IV collagenolytic activity was also diminished 2-3 fold in the dexamethasone treated cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05577-01 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ras-Oncogene-Mediated Induction of a 92 kDa Metalloproteinase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Ballin	Visiting Fellow	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	C. C. Sinha	Biologist	OD, DCE	NCI

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.0	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Coordinated ras-mediated induction of the metastatic phenotype and type IV collagenolytic activity was demonstrated previously (Thorgeirsson et al., Mol. Cell. Biol. 5: 259-262, 1985). For further characterization of the metalloproteinases involved, gelatin and type IV collagen sodium dodecyl sulfate (SDS) gel electrophoresis was used. The ras-transfected NIH/3T3 cells and malignant human cells expressed a 92 kDa metalloproteinase which was not detected in the untransfected control or normal human cells. Other gelatinolytic metalloproteinases (m.w. 68, 65 and 61kDa) were similarly expressed by the normal and malignant phenotype. HL-60 leukemia cells produced high levels of the 92 kDa proteinase, which was found to degrade both gelatin and type IV collagen when analyzed by SDS gel electrophoresis with substrates copolymerized into the acrylamide.

These results suggest that the 92 kDa gelatin-type IV collagen degrading metalloproteinase plays an important role in tumor cell invasion.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Ballin	Visiting Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
C. C. Sinha	Biologist	OD, DCE	NCI

Objectives:

The major objective is to identify metalloproteinases associated with the capacity of malignant cells to metastasize. To find out which proteinases appear when the cell becomes metastatic we used ras oncogene transfection of NIH/3T3 cells to induce the metastatic phenotype and gelatin zymograms to compare the profiles of metalloproteinases released. The second approach is to use the same method to compare normal to malignant cell lines of human origin. Since type IV collagenolytic activity has been shown to correlate closely with metastatic potential, it would be of great significance to specify which of the gelatinolytic metalloproteinases also have the capability to degrade type IV collagen.

Methods Employed:

1. Cell cultures. Cell lines are grown to subconfluency, then washed three times with PBS and incubated in serum-free medium. The supernatants are collected at 24 and 48 hours, and all debris removed by centrifiguation. The pooled supernatants are concentrated 100-fold with ammonium sulfate (0-60%), dialyzed at 4°C against buffer containing 0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl, 0.01 M $CaCl_2$ and stored at -70°C until they are assayed.

2. Gelatin and type IV collagen SDS gel electrophoresis. Preparation of the gel and the running buffer was the same as for regular SDS gels except for the addition of 0.1% gelatin or 0.04% type IV collagen to 7.4% acrylamide separating gel. The electrophoresis is performed under nonreducing conditions at a constant current of 9 mA at 4°C. Following electrophoresis the gels are washed three times in 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100 for 30 min with shaking at room temperature. Gels are then rinsed three times in 50 mM Tris-HCl (pH 7.4) for 5 min, and then incubated in a buffer containing 0.05M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3 pH 7.4 at 37°C for 16 h. The gelatin gels are stained and fixed for 1 h shaking at room temperature using 1% amido black in a mixture of acetic acid:methanol:water (1:3:6) and then destained in the same mixture without amido black. Collagen IV gels are stained with 0.1% Coumassie blue in 10% trichloracetic acid and destained in 7% acetic acid. Bands of proteolytic activity are visualized by negative staining.

Major Findings:

1. A calcium-dependent neutral metalloproteinase of 92 kDa was induced through *ras* oncogene transfection of NIH/3T3 cells in parallel with the metastatic phenotype.

2. The calcium-dependent 92 kDa proteinase was completely inhibited by 10 mM EDTA, but not by 10 mM N-ethylmaleimide or 2 mM phenylmethylsulfonamide.

3. Apart from the 92 kDa proteinase, other major gelatinolytic metalloproteinases (68, 65, 61 kDa) were similarly expressed by normal and malignant cell lines (NIH/3T3 and human fibroblasts).

4. HL-60 leukemia cells expressed very high levels of the 92 kDa metalloproteinase which degraded both gelatin and type IV collagen.

5. Demonstration of enzyme activity using type IV collagen copolymerized with acrylamide represents a new approach to detect type IV collagenolytic proteinases.

6. The only normal cell types expressing the 92 kDa metalloproteinases were neutrophils and endothelial cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05578-01 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Cloning of Endothelial Cell Collagenases: Tumor-Endothelial Cell Interaction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Ballin	Visiting Fellow	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	J. Hartzler	Physical Science Aid	OD, DCE	NCI
	D. MacArthur	Biological Lab. Worker	OD, DCE	NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.0	0.5

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A human endothelial cDNA library was screened with a 1.7 kbp human fibroblast mammalian collagenase clone under low stringency conditions in an attempt to isolate cDNA clones of different collagenolytic metalloproteinases. From 500,000 plaques used for primary screening, nine positive clones were plaque purified after 3-4 rounds of screening. Three clones of 1.7, 1.5 and 0.7 kbp were subcloned into pGem 3Z. All three endothelial clones sequenced so far turned out to be identical to the published sequence of the fibroblast mammalian collagenase.

The study on metalloproteinase activity during tumor-endothelial cell interaction has just started. The effect of humoral factors from endothelial cells on tumor cells or vice versa were studied by using serum-free conditioned medium from each cell type. Conditioned medium from human endothelial cells inhibited metalloproteinase activity of A2058 cells in a dose-responsive manner. On the other hand, A2058 melanoma cell conditioned medium did not affect the proteolytic activity of the endothelial cells using 0.1, 1, 5, 10, 15 and 25% conditioned medium, but the activity was increased severalfold in the presence of 50% or higher conditioned medium. These data suggest that the basement membrane degradation during tumor-endothelial cell interaction may depend on the balance between metalloproteinase and tumor inhibitor of metalloproteinase (TIMP) expression by the two cell types. Future studies will involve in situ hybridization techniques to examine expression of metalloproteinases and TIMP in cocultures of tumor and endothelial cells.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Ballin	Visiting Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
J. Hartzler	Physical Science Aid	OD, DCE	NCI
D. MacArthur	Biological Lab. Worker	OD, DCE	NCI

Objectives:

The major objective of the cloning project is to use mammalian fibroblast collagenase cDNA clones for screening of a human endothelial cDNA library in order to isolate metalloproteinase genes which degrade type IV collagen of basement membranes. Isolation of one or more of these genes, which are important participants in the metastatic cascade, is a prerequisite for understanding regulation of tumor cell proteolysis. The capacity of tumor cells to pass through the vascular walls is the most important measure of metastatic behavior. Therefore, interaction of tumor cells with endothelial cells within the primary tumor and in the circulation may be a critical step in the metastatic process. We will first focus on the effect of tumor-endothelial cell contact on the metalloproteinase activity of the two cell types. Once we have the genes for both TIMP and the major metalloproteinases involved in basement membrane degradation, the balance between their expression can be studied at a transcriptional level and in individual cells using the in situ hybridization technique. If these studies reveal that tumor cells and endothelial cells can modulate each others proteolytic mechanism, new projects to isolate the surface proteins involved will be undertaken.

Methods Employed:

1. Cloning of endothelial collagenase. The human endothelial cell λgt 11 library is screened with a human fibroblast mammalian collagenase cDNA probe of 1.7 kbp. For the primary screening, 500,000 plaques were used and hybridized with the ^{32}P-labeled collagenase probe, followed by washing at room temperature. Plaque purified clones are isolated after three or four rounds of screening. The stringency after the primary screening was 0.1x SSC (sodium chloride, sodium citrate, pH 7.0) and 0.1% SDS (sodium dodecyl sulfate) at 50°C. Subcloning was done in the pGem 3Z, and the Sanger technique was used for DNA sequencing.

2. Tumor-endothelial cell interaction. a) Effect of humoral factors on proteolytic activity. Conditioned medium is collected separately from subconfluent cultures and tumor cells and endothelial cells. Tumor cell cultures are then washed free of serum and exposed to conditioned medium from endothelial cells at concentrations of 0.1, 1, 5, 10, 15, 25, 50, 75

and 100%. Conversely, endothelial cell cultures are exposed to conditioned medium from tumor cells in the same manner. Culture supernatants are collected after 24 hours and measured for type IV collagenolytic activity and examined by gelatin gel electrophoresis. Supernatants are also collected after removing the conditioned medium. After washing the monolayers three times with PBS, fresh serum-free medium is added, and 100 µl samples of supernatants collected at 1, 3, 6, 12 and 24 hours for type IV collagenase assay and gelatin gel electrophoresis. b) Co-cultures of different types of endothelial cells and tumor cells are studied with respect to metalloproteinase activity and compared to the proteinase activity of the individual cell types. Expression of different metalloproteinase genes and TIMP is quantitated at a cellular level using in situ hybridization technique.

Major Findings:

1. The cDNA clone for collagenase isolated from human endothelial cells is identical to the human fibroblast mammalian collagenase gene.

2. Serum-free conditioned medium from human endothelial cells block the metalloproteinase activity of A2058 melanoma cells in a dose-responsive manner.

3. Serum-free conditioned medium (50%) from A2058 melanoma cells stimulates the metalloproteinase activity of human endothelial cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05579-01 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 chereciers or less. Title must fit on one line between the borders.)*
Establishment of Cell Lines from Normal and Neoplastic Monkey Tissues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	S. Sieber	Deputy Director	OD, DCE	NCI
	R. Adamson	Director	OD, DCE	NCI
	J. Hartzler	Physical Science Aid	OD, DCE	NCI

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	.33	0.17

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

This is a new project at a stage where cell lines are being established from monkey tissues obtained at autopsies performed immediately after death. Normal fibroblasts from kidney and periaortic tissues, as well as fibrosarcomas from two L-PAM treated monkeys (1010N and 1012N), grew rapidly in culture and have been successfully passed several times. Typical growth patterns of parallel, uniform, spindle-shaped normal fibroblasts vs. irregular criss-cross pattern of polygonally shaped fibrosarcoma cells were observed in culture. Endothelial cells from aorta and vena cava possessed the typical cobblestone pattern, but need to be cloned in order to be free of contaminating fibroblasts. Two hepatocarcinomas which were placed in culture have not done as well. The tumor cells which survived and attached to the tissue culture flask seemed to replicate very slowly or not at all. Use of F12 medium with a specially treated fetal calf serum or monkey serum did not have any effect on stimulating growth.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
S. Sieber	Deputy Director	OD, DCE	NCI
R. Adamson	Director	OD, DCE	NCI
J. Hartzler	Physical Science Aid	OD, DCE	NCI

Objectives:

The main reason for utilizing monkey tissues to establish cell cultures is their similarity to homologous human tissues. Cultures of chemically induced tumors permit a multitude of in vitro studies in the field of chemical carcinogenesis, as well as in tumor biology. Our major objectives are: 1) To establish tumor and endothelial cell lines to study interactions between the two cell types. Microvascular endothelial cell cultures will be established from organs which are commonly seeded by metastatic tumors; and 2) To oncogenically transform fibroblasts or hepatocytes in vitro and either inject them back into the same monkey, or if autopsy material is used, inject them into immunosuppressed monkeys. Tumor growth and progression will be followed by studying biopsies taken periodically.

Methods Employed:

1. Monkey tissues are aseptically obtained from autopsy/biopsy material and placed immediately on ice. Within two to three hours the tissues are minced in a sterile petri dish into 2-3 mm fragments which are placed in 25 cm^2 tissue culture flasks (5-10 in each flask). It is important that the fragments adhere well to the growth surface of the flask. One and a half ml of the appropriate culture medium containing 20% serum is carefully added to the flasks without dislodging the tissue. The flasks are incubated overnight at 37°C in 5% CO_2 and then 5 ml of the same medium added. When cells start to grow out from the periphery of the tissue fragments the medium is changed again. During the first subculturing the tissue fragments are discarded and thereafter the cells passed in a regular manner.

2. Harvesting of endothelial cells. The unopened blood vessels (aorta and vena cava) are clamped and the inside rinsed three times with Hank's basic salt solution (HBSS). Collagenase type I solution (0.1%) made fresh in HBSS is injected into the vessels and incubated at 37°C for 15 min. The collagenase step is repeated once, followed by mild scraping of the endothelial surface to remove loosely attached cells. The cells are centrifuged and washed once with HBSS, then grown in M199 medium containing 10% heat-inactivated fetal bovine serum, 10% Nu serum, 1% Ultroser, 2% endothelial cell mitogen, 80 µg/ml heparin, 1.5% glutamine, 100 µg/ml penicillin-streptomycin.

3. Cloning of endothelial cells by limiting dilution in micro test plates. Actively growing cells are harvested to produce a single cell suspension. Cells are counted and diluted to 3-5 cells/ml in the medium described above. One hundred µl of cells-medium are added to each well and the cell suspension is agitated. One hundred or more µl of medium is added to each well. Incubations are carried out for 24 h and wells containing a single cell are identified. Wells are incubated until they develop a partial monolayer. After trypsinization, cells are added to T25 culture flasks. Identification of endothelial cells is made by using Factor III fluorescent antiserum, which stains the endothelial cells strongly.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP09127-04 OD

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Proteinase Response in Normal and Neoplastic Cells to TPA and Growth Factors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Ballin	Visiting Fellow	OD, DCE	NCI
Others:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
	J. Hartzler	Physical Science Aid	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
0.75	0.5	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Metalloproteinase activity of cells exposed to the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and growth factors was assessed using gelatin sodium dodecyl sulfate (SDS) gel electrophoresis and measurements of type IV collagen degradation. Fibroblasts at different stages of malignant transformation responded variably to TPA. Normal human diploid lung fibroblasts did not respond to TPA at all, but NIH/3T3 cells and transformed fibroblasts expressed up to four times higher type IV collagenolytic activity. Similarly, expression of a 92 kDa gelatinase which is closely associated with the malignant phenotype was increased in the presence of TPA. Conversely, TPA inhibited another major gelatinase of 65 kDa while the 92 kDa gelatinase was induced. The lack of response of normal fibroblasts was not dependent on the stage of confluency in cell culture. Experiments assessing the effect of epidermal growth factor (EFG), transforming growth factor-beta (TGF-beta) and fibroblast growth factor (FGF) on gelatinolytic and type IV collagenolytic activity have been initiated. Preliminary data showed that EGF had no effect on normal and transformed cells, but TGF-beta induced the 92 kDa gelatinase activity of transformed cells in a dose-responsive manner, while the 65 kDa gelatinase activity was only mildly increased.

The results obtained so far suggest that a certain degree of cellular atypia, possibly related to the initiation step of the carcinogenic process is necessary for induction of metalloproteinase activity mediated by the tumor promoter TPA, and possibly also by TGF-beta. TPA has opposite effects on the two major gelatinolytic metalloproteinases expressed by malignant cells, i.e., it stimulates the 92 kDa and inhibits the 65 kDa gelatinase.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Ballin	Visiting Fellow	OD, DCE	NCI
U. P. Thorgeirsson	Expert	OD, DCE	NCI
J. Hartzler	Physical Science Aid	OD, DCE	NCI

Objectives:

1. We are interested in finding out if type IV collagenolytic activity, which is possibly genetically linked to the malignant phenotype, can be induced in fibroblasts by TPA without prior initiation of the neoplastic process.

2. Increasing numbers of reports have shown growth factor-mediated induction of genes for proteolytic enzymes. We wish to find out if growth factors such as EGF, FGF and TGF-beta, separately or combined, affect expression of metalloproteinases responsible for degrading basement membrane collagen IV.

Methods Employed:

1. Cell cultures. All the cultures are grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). For TPA experiments cells are placed in 6-well tissue culture dishes and grown to subconfluency, then washed three times with PBS and placed in the same medium without serum and different concentrations of TPA ranging from 10^{-6}M to 10^{-9}M. Samples of 100 µl from two representative transformed lines are collected at 1,3,6,12, 24 and 48 hours for a time-course study on the expression of the 92 kDa gelatinase in the culture supernatants. Supernatants containing different concentrations of TPA are collected after 48 hours. For growth factor experiments the cells are grown in 24-well tissue culture dishes, and supernatants collected after 48 hours.

2. Assay for type IV collagenolytic activity. The secreted type IV collagen degrading activity is measured in serum-free culture supernatants with and without the addition of TPA. The supernatants are concentrated 100-fold with ammonium sulfate (0-60%), followed by dialysis against 0.2M NaCl, 0.05M Tris-HCl, 0.01M $CaCl_2$, pH 7.4.

   The type IV collagen degradation is assayed as previously described with the use of soluble [^{3}H] proline-labeled type IV collagen as a substrate. The enzyme in duplicate samples is activated by the addition of 10 µg/ml trypsin for 10 min at 37°C, and then assayed in the presence of 50 µg/ml soybean trypsin inhibitor for 16 hours at 37°C. Negative controls included trypsin and soybean trypsin inhibitor without the samples incubated with the

labeled collagen substrate. Bacterial collagenase (0.1% w/w) is used as a positive control to achieve the maximum collagen degradation. The reaction is terminated by incubating the samples on ice for 30 min in the presence of 0.6% trichloroacetic acid, 0.6% tannic acid and 25 µg/ml bovine serum albumin. The undigested substrate is removed by centrifugation and the radioactivity of the degraded type IV collagen in the supernatant measured in a beta-scintillation counter.

3. Gelatin and type IV collagen SDS gel electrophoresis. Preparation of the gel and the running buffer is the same as for regular SDS gels except for the addition of 0.1% gelatin or 0.04% type IV collagen to 7.4% acrylamide separating gel. The electrophoresis is performed under nonreducing conditions at a constant current of 9 mA at 4°C. Following electrophoresis the gels are washed three times in 50 mM Tris-HCl (pH 7.4) containing 2% Triton X-100 for 30 min with shaking at room temperature. Gels are then rinsed three times in 50 mM Tris-HCl (pH 7.4) for 5 min, and then incubated in a buffer containing 0.05M Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100, 0.02% NaN_3 pH 7.4 at 37°C for 16 h. The gelatin gels are stained and fixed for 1 h shaking at room temperature using 1% amido black in a mixture of acetic acid:methanol:water (1:3:6) and then destained in the same mixture without amido black. Collagen IV gels are stained with 0.1% Coumassie blue in 10% trichloracetic acid and destained in 7% acetic acid. Bands of proteolytic activity are visualized by negative staining.

Major Findings:

1. Normal human diploid lung fibroblasts were unresponsive to TPA-mediated gelatinolytic and type IV collagenolytic stimulation.

2. NIH/3T3 fibroblasts and malignant fibroblasts were stimulated by TPA in their capacity to degrade both gelatin and type IV collagen.

3. TPA had the opposite effect on the two major gelatinolytic enzymes expressed by malignant cells, i.e., stimulated the 92 kDa gelatinase and inhibited the 65 kDa gelatinase.

4. The type IV collagenolytic activity was not dependent on the stage of confluency of the normal fibroblasts.

5. EGF had no effect on the gelatinolytic and type IV collagenolytic activity in two malignant cell lines, but TGF-beta stimulated the 92 kDa gelatinase in a dose-responsive manner, while the 65 kDa enzyme was only mildly induced.

6. Type IV collagenolytic activity of the normal fibroblasts without TPA treatment was comparable with that of the NIH/3T3 cells, and 3-4 fold lower than in the malignant fibroblasts.

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 to September 30, 1988

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancer and to elucidate mechanisms of transformation by carcinogenic agents. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man.

A primary emphasis of many ongoing investigations within the laboratory concerns RNA tumor viruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species. Certain retroviruses, the so-called "replication-defective" transforming viruses, have arisen by a recombination with cellular genes or proto-oncogenes. As such, these viruses have offered an unparalleled opportunity to elucidate the processes by which such genes cause malignancies. Investigations within the Laboratory have provided strong evidence that proto-oncogenes are frequent targets of genetic alterations that convert them to oncogenes and lead human cells along the pathways to malignancy. Today, much of our research is aimed at identifying genes that can be implicated as oncogenes in human cancer, their molecular mechanisms of activation, as well as the mode of action of their translational products.

The lentiviruses, a subfamily of the retroviruses, have been implicated as the causative agents of nonneoplastic diseases of certain animal species and of acquired immunodeficiency syndrome (AIDS) in man. Investigations in our laboratory utilize animal lentiviruses as models for treatment and prevention of AIDS.

During the past year, our laboratory has made progress in several areas, as specified below:

sis oncogene/platelet-derived growth factor (PDGF)-2. The v-*sis* oncogene encodes a platelet-derived growth factor (PDGF)-related product whose transforming activity is mediated by its functional interaction with the PDGF receptor. PDGF, as well as processed forms of the v-*sis* gene product, is a disulfide-linked dimer with eight conserved cysteine residues in the minimum region necessary for biologic activity. Site-directed mutagenesis of the v-*sis* gene revealed that each conserved cysteine residue was required directly or indirectly for disulfide-linked dimer formation. However, substitution of serine for cysteine codons at any of four positions had no detrimental effect

on transforming activity of the encoded v-*sis* protein. Results establish that interchain disulfide bonds are not essential in order for this protein to act as a functional ligand for the PDGF receptor. The remaining four substitutions of serine for cysteine each inactivated transforming function of the molecule. In each case this was associated with loss of a conformation shown to involve intramolecular disulfide bonds. These studies provide insight into the role of individual cysteine residues in determining the structure of the *sis*/PDGF molecule critical for biological activity.

The PDGF is a potent mitogen for connective tissue cells. PDGF contains two related but distinct polypeptides, one of which, PDGF-2 has been shown to be homologous to the transforming product of the retroviral v-*sis* oncogene (c-*sis*), a prototype growth factor with transforming potential. The c-*sis*/PDGF-2 transcript was demonstrated to contain a long 5' untranslated sequence (UTS) that is highly GC rich. To examine the influence of this sequence on *sis*/PDGF-2 expression, the c-*sis*/PDGF-2 promoter was localized and this promoter or the simian virus 40 (SV40) early promoter was used to drive expression of the bacterial chloramphenicol acetyltransferase (CAT) or *sis*/PDGF-2 gene. The 5' UTS of c-*sis*/PDGF-2 mRNA had no effect on RNA expression but was shown to exert a potent inhibitory effect on translation. By deletion analysis, the 5' UTS was found to inhibit protein expression by as much as 40-fold. The inhibitory effect was independent of reporter gene, cell type or promoter used. A highly G.C-rich 140-base-pair sequence immediately preceding the c-*sis*/PDGF-2 initiation codon was shown to be nearly as effective as the entire 5' UTS in translational inhibition. Transfection analysis demonstrated that the 5' UTS significantly reduced the transforming efficiency of the *sis*/PDGF-2 gene as well. Findings raised the possibility that changes in regulation at the level of *sis*/PDGF-2 translation may play a role in development of the neoplastic phenotype.

The major mRNA start sites of *sis*/PDGF-2 transcripts derived from normal placenta and from a human tumor cell line, A2780, known not to express the c-*sis*/PDGF-2 transcript, were found to be identical, excluding the possibility that *sis*/PDGF-2 expression in the human tumor cell line transcript reflects the altered transcriptional initiation of the *sis*/PDGF-2 gene. Further investigation of the role of flanking sequences that may affect *sis*/PDGF-2 gene expression in tumor cells expressing the *sis*/PDGF-2 transcript should help to elucidate both the normal transcriptional control of this gene as well as mechanisms that may activate it as an oncogene in certain tumor cells.

Genes for other growth factors as oncogenes. A peptide secreted by some tumor cells in vitro, transforming growth factor alpha (TGFα), imparts anchorage-independent growth to normal rat kidney (NRK) cells. To directly investigate the transforming properties of this factor, the human sequence coding for TGFα was placed under the control of either a metallothionein promoter or a retroviral long terminal repeat (LTR). These constructs failed to induce morphological transformation upon transfection of NIH/3T3 cells, whereas viral oncogenes encoding a truncated form of its cognate receptor, the epidermal growth factor (EGF) receptor, or another growth factor, *sis*/PDGF-2, efficiently induced transformed foci. When NIH/3T3 clonal sublines were

selected by transfection of TGFα expression vectors in the presence of a dominant selectable marker, they were shown to secrete large amounts of TGFα into the medium, to have down-regulated EGF receptors, and to be growth-inhibited by TGFα monoclonal antibody. The results indicated that secreted TGFα interacts with its receptor at a cell surface location. Single cell-derived TGFα-expressing sublines grew to high saturation density in culture. However, when plated as single cells on contact-inhibited monolayers of NIH/3T3 cells, they failed to form colonies, while v-*sis*- and v-*erb*B-transfected cells formed transformed colonies under the same conditions. Moreover, TGFα-expressing sublines were not tumorigenic in nude mice. Results imply that TGFα exerts a growth-promoting effect on the entire NIH/3T3 cell population after secretion into the medium, but little, if any, effect on the individual cell synthesizing this factor. It was thus concluded that the normal coding sequence for TGFα is not a direct-acting oncogene.

Oncogenes encoding growth factor receptors. The epidermal growth factor receptor (EGFR) gene is frequently amplified and/or overexpressed in human malignancies. To investigate the biological effects of its overexpression, a eukaryotic vector containing human EGFR cDNA was constructed. Introduction of this construct led to constitution of functional EGF receptors in NR6 mutant cells, which are normally devoid of this receptor. Transfection of NIH/3T3 resulted in no significant alterations in growth properties. However, the addition of EGF led to the formation of densely growing transformed foci in liquid culture and colonies in semisolid medium. NIH/3T3-EGFR clonal lines, which expressed the EGF at 500- to 1000-fold levels over control NIH/3T3 cells, demonstrated a marked increase in DNA synthesis in response to EGF. Thus, EGF receptor overexpression appears to amplify normal EGF signal transduction. Finally, high levels of EGFR expression, which conferred a transformed phenotype to NIH/3T3 cells in the presence of ligand, were demonstrated in representative human tumor cell lines that contained amplified copies of the EGFR gene.

An expression vector for EGFR was introduced into the 32D myeloid cell line, which is devoid of EGF receptors and absolutely dependent on interleukin-3 (IL-3) for its proliferation and survival. Expression of the EGFR conferred the ability to utilize EGF for transduction of a mitogenic signal. When the transfected cells were propagated in EGF, they exhibited a more mature myeloid phenotype than was observed under conditions of IL-3-directed growth. Moreover, exposure to EGF led to a rapid stimulation of phosphoinositide metabolism, while IL-3 had no detectable effect on phosphoinositide turnover either in control or EGF receptor-transfected 32D cells. Although the transfected cells exhibited high levels of functional EGF receptors, they remained nontumorigenic. In contrast, transfection of v-*erb*B, an amino-terminal truncated form of the EGF receptor with constitutive tyrosine kinase activity, not only abrogated the IL-3 growth factor requirement of 32D cells, but caused them to become tumorigenic in nude mice. The results showed that a naive hematopoietic cell expresses all of the intracellular components of the EGF-signaling pathway necessary to evoke a mitogenic response and sustain continuous proliferation.

Activation of normal human erbB-2 as an oncogene. Studies demonstrated that the human *erb*B-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The level of the *erb*B-2 product was shown to be critical in determining its transforming ability. This in vitro observation is paralleled by in vivo findings that a wide variety of human tumors contain an amplified or overexpressed *erb*B-2 gene, which encodes a growth factor receptor-like protein. When *erb*B-2 complementary DNA was expressed in NIH/3T3 cells under the control of the SV40 promoter, the gene lacked transforming activity despite expression of detectable levels of the *erb*B-2 protein. A further five- to tenfold increase in its expression under influence of the long terminal repeat of Moloney murine leukemia virus was associated with activation of *erb*B-2 as a potent oncogene. The high levels of the *erb*B-2 product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumor cells that overexpressed this gene. These findings demonstrate a new mechanism for acquisition of oncogenic properties by genes encoding growth factor receptor-like proteins and provide a functional basis for the role of their overexpression in the development of human malignancies.

Amplification of the *erb*B/EGF receptor and a structurally related gene, designated *erb*B-2, have been detected in a variety of human tumors. In a series of human mammary tumor cell lines, analysis of transcripts of these genes revealed elevated levels of one or the other in more than 60% of tumors analyzed. Eight cell lines demonstrated *erb*B-2 mRNA levels ranging from four- to 128-fold above those of normal controls. *Erb*B-2 expression was evaluated in comparison to the expression level of actin observed in these cell lines. There was no evidence of aberrantly sized *erb*B-2 transcripts in any of these lines. Immunoblot analysis indicated elevation in levels of the 185-kd product of the *erb*B-2 gene expressed by these cells. In four lines *erb*B-2 gene amplification in the absence of an apparent gene rearrangement was demonstrated. In a representative cell line of this type, SK-BR-3, the amplified *erb*B-2 gene copies were located in an aberrant chromosomal location. Four additional cell lines, which demonstrated four- to eightfold overexpression of *erb*B-2 mRNA, did not exhibit gene amplification. In a representative cell line of this type, ZR-75-1, an apparently normal chromosomal location was found for the *erb*B-2 gene. Findings indicate that overexpression of the *erb*B-2 gene in mammary tumor cell lines is frequent and associated with different genetic abnormalities.

Interactions of growth factors and oncogenes. BALB/MK epidermal keratinocytes require EGF for growth in serum-containing medium and terminally differentiate in response to high Ca^{++} concentration. Several oncogenic retroviruses have been shown to relieve the EGF requirement and to block calcium-induced terminal differentiation. A chemically defined medium was developed to investigate the minimum growth factor requirements for BALB/MK cells, as well as how such requirements might be altered by retroviral oncogenes. In this medium, insulin, apparently acting as IGF-1, and EGF supported cell growth in a manner comparable to serum and EGF. Acidic as well as basic fibroblast growth factors (FGF) substituted for EGF but not insulin in supporting BALB/MK proliferation. Infection with retroviruses containing v-*ras* oncogenes (v-H-

ras, v-Ki-*ras*), oncogenes derived from growth factor receptors (v-*erb*B, v-*fms*) or the v-*mos* oncogene permitted growth in defined medium containing insulin but lacking EGF. The v-*fgr* oncogene, a member of the *src* subfamily, was unique in conferring independence from both insulin and EGF. Findings established the applicability of this system for biologic assay of epithelial cell growth factors as well as identification of specific growth factor requirements that can be altered or complemented by the actions of specific oncogenes.

In other studies, EGF was demonstrated to be an extremely potent mitogen, causing BALB/MK cultures to enter the cell cycle in a synchronous manner associated with a greater than 100-fold increase in DNA synthesis. Analysis of the expression of proto-oncogenes, which have been reported to be activated during the cascade of events following growth factor stimulation of fibroblasts or lymphoid cells, revealed a very rapid but transient 100-fold increase in c-*fos* RNA but little or no effect on the other proto-oncogenes analyzed. Exposure of EGF-synchronized BALB/MK cells to high levels of calcium was associated with a striking decrease in the early burst of c-*fos* RNA as well as the subsequent peak of cell DNA synthesis. Since the inhibitory effect of high calcium on c-*fos* RNA expression was measurable within 30 minutes, studies imply that the EGF proliferative and calcium differentiation signals must interact very early in the pathway of EGF-induced proliferation. The results also establish that c-*fos* RNA modulation is an important early marker of cell proliferation in epithelial as well as mesenchymal cells.

The role of physiologic concentrations of EGF on phosphoinositide metabolism in BALB/MK mouse keratinocytes was investigated. Results indicated that EGF rapidly activates phospholipase C-mediated phosphoinositide metabolism, resulting in the generation of the second messenger inositol (1,4,5)-triphosphate and diacylglycerol. These metabolites control intracellular Ca^{++} levels and activate protein kinase C, respectively. Protein kinase C activation in response to EGF was evidenced by the phosphorylation of the acidic 80-kd endogenous protein substrate (p80) specific for this kinase. In contrast, insulin had no effect on phosphoinositide metabolism nor led to any additional stimulation when added in combination with EGF. Taken together, results showed that phosphoinositide metabolism and subsequent protein kinase C activation are involved in the normal mitogenic response of epithelial cells to EGF.

<u>*Ras* oncogenes and proto-oncogenes</u>. The availability of molecular clones of the normal and activated alleles of human *ras* proto-oncogenes made it possible to determine the molecular mechanisms responsible for the malignant conversion of these genes. Findings during the past year include the following:

Quiescent mouse NIH/3T3 cells responded to microinjection of activated *ras* p21 with a rapid and sustained rise in intracellular pH (~0.17 pH units). The p21-induced pH change was inhibited by amiloride treatment of growth of cells in medium low in sodium, suggesting a role for the Na^+/H^+ antiporter. Amiloride was found to suppress p21-induced mitosis also.

Glycosphingolipid alterations upon viral transformation are well documented. Transformation of mouse 3T3 cells with murine sarcoma viruses results in marked decreases in the levels of gangliosides GM1 and GD1a and an increase in gangliotriasylceramide. The transforming oncogenes of these viruses have been identified as members of the *ras* gene family. NIH/3T3 cells transfected with human H-, K- and N-*ras* oncogenes were analyzed for their glycolipid composition and expression of cell surface gangliosides. Using conventional thin-layer chromatographic analysis, the level of GM3 was found to be increased and that of GD1a slightly decreased or unchanged, and GM1 was present but not in quantifiable levels. Cell surface levels of GM1 were determined by cholera toxin binding to cells treated with sialidase prior to toxin binding. All *ras*-transfected cells had decreased levels of surface GM1 and GD1a as compared to logarithmically growing normal NIH/3T3 cells. Levels of GM1 and, to a lesser extent, GD1a increased as the latter cells became confluent. Using a monoclonal antibody assay, we found that gangliotriasylceramide was present in all *ras*-transfected cells studied but not in logarithmically growing untransfected cells. Interestingly, a gangliotriasylceramide appeared when the latter cells became confluent. These results indicated that *ras* oncogenes derived from human tumors are capable of inducing alterations in glycolipid composition.

The p21 products of *ras* proto-oncogenes are thought to be important components in pathways regulating normal cell proliferation and differentiation. These proteins acquire transforming properties as a result of activating lesions that convert *ras* genes to oncogenes in a wide array of malignancies. In *Xenopus laevis* oocytes, microinjection of transforming *ras* p21 is a potent inducer of maturation, whereas microinjection of a monoclonal antibody to *ras* p21 inhibits normal maturation induced by hormones. The phosphoinositide pathway is a ubiquitous system that appears to play a key role in diverse cellular functions. By use of the *Xenopus* oocyte system, it was possible to quantitate the effects of *ras* p21 microinjection on individual components of the phosphoinositide pathway. Within 20 minutes of microinjection, levels of phosphatidylinositol, 4,5-bisphosphate, inositol 1-phosphate, and inositol bisphosphate increased one and one-half to twofold. The most striking effects were on diacylglycerol, which increased fivefold under the same conditions. In contrast, the normal *ras* p21 protein induced no detectable alteration in any of the metabolites analyzed. The earliest effects of the transforming p21 on phosphoinositol turnover were observable within two mintues, implying a very rapid effect of *ras* p21 on the enzymes involved in phospholipid metabolism.

Genes involved in the transduction of signals required for normal cell proliferation commonly appear to be subverted in the neoplastic process. One such group is the highly conserved family of *ras* genes, which are present in a wide variety of naturally occurring tumors. By analogy with other known G proteins, the p21 proteins encoded by *ras* genes may act as regulatory proteins in the transduction of signals that lead to DNA synthesis. A major pathway involved in the DNA synthesis induced by growth factors is mediated by phosphatidylinositol turnover: cleavage of phosphoinositides by phospholipase

C produces 1,2-diacylglycerol and inositol phosphates. The former acts as an essential cofactor for protein kinase C, and inositol-(1,4,5)-triphosphate mobilizes Ca^{++} from nonmitochondrial intracellular stores. A reproducible increase in 1,2-diacylglycerol, in the absence of a detectable increase in inositol phosphates, in transformed cells containing H-*ras* oncogenes and with different membrane targeting signals for the *ras* p21 protein was demonstrated. These findings suggest that a source other than phosphoinositides exists for the generation of 1,2-diacylglycerol and that the H-*ras* oncogene specifically activates this novel pathway for 1,2-diacylglycerol production.

Microinjection of purified protein kinase C (PKC) into Swiss/3T3 fibroblasts pretreated with the phorbol ester, phorbol-12,13-dibutyrate, had previously been demonstrated to restore the mitogenic response of the cells to phorbol-12,13-dibutyrate. Present studies demonstrate that the mitogenic activity of the H-*ras* oncogene in H-*ras* p21-microinjected quiescent cells is markedly reduced under conditions in which PKC is down-regulated by chronic phorbol ester treatment. The ability to reconstitute the mitogenic response upon microinjection of both H-*ras* p21 and PKC implies involvement of functional PKC in the mitogenic activity of the H-*ras* oncogene product.

DNA prepared from the 3-methylcholanthrene (3MC)-transformed human 3I2H cell line induced foci on NIH/3T3 cells, whereas DNAs prepared from 7,12-dimethylbenz[a]-anthracene-transformed and the dimethylsulfoxide control 3I2H cell lines failed to induce foci. The transformed gene from the 3MC-transformed 3I2H cells was identified as an activated form of the human cellular transforming H-*ras* oncogene. Analysis of the *ras* oncogene p21 product in this transformant by immunoprecipitation and gel electrophoresis suggested that this gene was activated by mutation in the 61st codon. These findings demonstrate that activation of a member of the *ras* gene family can occur in a chemically transformed human cell line.

Activation of the cellular oncogene c-N-*ras* has been frequently observed in DNA from leukemic cells in acute myeloid leukemia (AML). *Ras* gene activation sufficient to mediate in vitro transformation and rodent tumorigenesis usually results from point mutations and amino acid substitutions in the 12th or 61st codons. In AML and the related myelodysplastic syndromes, amino acid substitution at the 13th codon has been observed. An activated c-N-*ras* gene from a 45-year-old patient with AML was isolated by transfection analysis and subjected to molecular cloning and sequence analysis. A point mutation of the 12th codon (GGT to GAT) resulting in aspartic acid substitution for glycine was observed. In other neoplasms, such as colon cancer, specific *ras* mutations occur predominantly (e.g., K-*ras*, codon 12). This predominance has been of demonstrable value in analyzing large cohorts for *ras* activation with techniques that are rapid and economical, such as oligonucleotide hybridization. It had previously been thought that such a predominance for activation of c-N-*ras* at codon 13 existed in AML; however, this study, in concert with others, underscores the importance of 12th codon c-N-*ras* mutations, along with 13th and 61st codon mutations in the molecular pathogenesis of AML. Guanylate to adenylate transition mutations are commonly observed in AML and may provide insight into potential environmental

leukemogens. Addressing all commonly prevalent *ras* activating mutations bears impact in the future design of molecular surveys of the role of *ras* activation in leukemogenesis.

The frequency of active *ras* oncogenes in human bladder cancers associated with schistosomiasis, the cause of which is suspected to be a chemical carcinogen(s) in urine, was examined. Of nine squamous cell carcinomas of the bladder surgically obtained in Egypt, none scored as positive in the regular DNA transfection assay using NIH/3T3 cells as recipients. The restriction fragment length polymorphism assay at codon 12 of the H-*ras* gene confirmed the absence of an activating mutation at this site in all of them. Western blotting analysis of electrophoretic mobilities of the *ras* p21 proteins, a method which can detect at least some of the point mutations within codons 12 and 61 of *ras* genes, suggested a point mutation within codon 61 in one out of the seven tumors analyzed. In contrast to the low frequency of detection of mutationally activated *ras* oncogenes, enhanced expression of the *ras* p21 proteins was demonstrated in four of them by this analysis. The carcinogenic process involved in the endemic bilharzial bladder cancers is thus not associated with detectable point mutations within *ras* genes at a higher frequency than those in nonbilharzial bladder cancers in Japan or the USA.

In a further survey of human urinary tract tumors, including 16 primary kidney tumors, one transitional cell carcinoma and 15 renal cell carcinomas, two H-*ras* oncogenes were detected by the NIH/3T3 transfection assay. Analysis of *ras* protein p21 suggested single point mutations within codons 12 and 61 in each case. Results indicated that the genetic lesions affecting *ras* genes do occur in human renal cell carcinomas and probably represent one step in the multistep carcinogenic process.

Human *fgr* proto-oncogene. The c-*fgr* proto-oncogene, the human analogue of v-*fgr*, the transforming gene of the Gardner-Rasheed feline sarcoma virus (GR-FeSV), is detected in cell lines derived from lymphoproliferative disorders, including B-cell lines derived from African and American undifferentiated lymphomas of the Burkitt's and non-Burkitt's types. In studies to characterize the primary structure of the *fgr* proto-oncogene product, normal human c-*fgr* cDNA clones were constructed by using normal peripheral blood mononuclear cell mRNA as a template. Nucleotide sequence analysis of two such clones revealed a 1,587-base-pair-long open reading frame which predicted the primary amino acid sequence of the c-*fgr* translational product. Homology of this protein with the v-*fgr* translational product stretched from codons 128 to 516, where 32 differences among 388 codons were observed. Sequence similarity with human c-*src*, c-*yes* and *fyn* translational products began at amino acid position 76 of the predicted c-*fgr* protein and extended nearly to its C-terminus. In contrast, the stretch of 75 amino acids at the N-terminus demonstrated a greatly reduced degree of relatedness to these same proteins. To verify the deduced amino acid sequence, antibodies were prepared against peptides representing amino- and carboxy-terminal regions of the predicted c-*fgr* translational product. Both antibodies specifically recognized a 55-kilodalton protein expressed in COS-1 cells transfected with a c-*fgr* cDNA expression plasmid. Moreover, the same protein was immunoprecipitated from an

Epstein-Barr virus (EBV)-infected Burkitt's lymphoma cell line which expressed c-*fgr* mRNA but not in its uninfected *fgr* mRNA-negative counterpart. These findings identified the 55-kilodalton protein, $p55^{c\text{-}fgr}$, as the product of the human *fgr* proto-oncogene.

The dbl oncogene. The *dbl* transforming gene was originally identified by transfection of NIH/3T3 cells with DNA from a human B-cell lymphoma. This gene was found to have arisen as a result of recombination of the 3' portion of the *dbl* proto-oncogene coding sequences with an unrelated segment of human DNA. It encodes a cytoplasmic protein that is equally distributed between cytosol and crude membrane fractions. To further characterize this transforming gene, a biologically active cDNA clone of the *dbl* transforming gene mRNA was isolated. Analysis of the sequence of the *dbl* oncogene cDNA revealed a long open reading frame that encodes a hybrid protein whose first 50 amino acids (at least) derive from a complete exon of a different locus. No significant homology with known oncogenes or any known protein sequences was demonstrated. The computer analysis of the predicted *dbl* protein indicated it is highly hydrophilic with no hydrophobic domains characteristic of a membrane-spanning region or signal peptide. Thus, the *dbl* oncoprotein is distinct among known transforming gene products.

In further characterizations, the transcribed sequences of *dbl* were shown to be distributed over a 30-kb span within a molecularly cloned 45-kb segment of human DNA which contained the transforming gene. By restriction mapping, its transcribed region corresponded to that of its normal allele except at the 5' end where a rearrangement involved transcribed *dbl* oncogene sequences from another locus. An independent isolate of a *dbl*-related transforming gene was obtained following transfection of NIH/3T3 cells with DNA of a human nodular poorly differentiated lymphoma (NPDL). Physical mapping indicated that this transforming gene, designated NPDL-*dbl*, shared considerable homology with the *dbl* oncogene, but differed at both 5' and 3' termini. Its point of divergence from the normal allele at the 5' end was at least 10 kb upstream from that of the *dbl* oncogene. The oncogenes each expressed truncated transcripts compared to the 5.3-kb normal transcript. The *dbl* and NPDL-*dbl* oncogene translational products of 66 and 76 kDa, respectively, were consistent with their corresponding major 2.8- and 3.5-kb transcripts. It was not possible to detect evidence of the 5' structural rearrangements associated with these oncogenes in either of the original tumors. Thus, if these rearrangements were critical to their activation, they occurred in the process of gene transfer or in vivo in only a minority of tumor cells.

Chromosomal localization of the human *fyn* gene. A novel human gene, termed *fyn*, was isolated recently and the primary structure of its protein product was determined. This gene had been cloned from human cDNA libraries and, to date, no naturally occurring transforming counterpart has been found. However, comparison of *fyn* and *src* coding sequences has revealed that their protein products share a number of structural features and are 74% identical in amino acid sequence within their kinase domains. The differences between *src* and *fyn* gene products lie at their amino termini where they share only five of 82 amino acid residues. Although little is presently known regarding

expression of this gene, *fyn* mRNA has been detected in a wide variety of cell types. One approach towards assessing the role of proto-oncogenes in the neoplastic process has relied upon determining their location within the human genome. Such information makes it possible to focus on human cancers possessing specific chromosomal aberrations for alterations affecting the structure or expression of nearby proto-oncogenes. In collaboration with the Laboratory of Biology, NCI (Dr. Popescu), the *fyn* gene was localized to human chromosome 6 at band p21 (6q21) which places the *fyn* gene within the same region as *ros* and *myb*, raising the possibility that these genes may be closely linked at the molecular level. Attention is now being focused on neoplasms commonly exhibiting aberrations at 6q21 for evidence of alterations affecting the structure or expression of the *fyn* proto-oncogene.

In vitro assay for quantitating invasive potential of tumor cells. A matrix of basement membranes were reconstituted onto a filter in a Boyden chamber and the ability of various malignant and nonmalignant cells to penetrate through the coated filter assessed. Cells from all the malignant cell lines tested were able to cross the matrix in five to six hours, whereas human fibroblasts as well as mouse 3T3 and 10T-1/2 cell lines, which are not tumorigenic, were not invasive. In addition, normal primary prostate epithelial cells and benign prostatic hyperplasia cells were not invasive when tested in this assay, whereas malignant prostate carcinoma cells were highly invasive. Parallel experiments with these prostatic cells, using the intrasplenic assay for metastasis detection in the nude mouse, confirmed the benign behavior of the former cells and the metastatic phenotype of the latter ones. These results suggest that this in vitro test allows the rapid and quantitative assessment of invasiveness and a means to screen for drugs which alter the invasive phenotype of tumor cells.

Activation of oncogenes in B6C3F1 mouse liver as measure of risk assessment. The validity of mouse liver tumor end points in assessing the potential hazards of chemical exposure to humans is a controversial but important issue, since liver neoplasia in mice is the most frequent tumor target tissue end point in two-year carcinogenicity studies. The ability to distinguish between promotion of background tumors versus a genotoxic mechanism of tumor initiation by chemical treatment would aid in the interpretation of rodent carcinogenesis data. Activated oncogenes in chemically induced and spontaneously occurring mouse liver tumors were examined and compared as one approach to determine the mechanism by which chemical treatment caused an increased incidence of mouse liver tumors. Data suggest that furan and furfural caused an increased incidence in mouse liver tumors, at least in part, by induction of novel weakly activating point mutations in *ras* genes even though neither chemical induced mutations in *Salmonella* assays. In addition to *ras* oncogenes, two activated *raf* genes and four non-*ras* transforming genes were detected. The B6C3F1 mouse liver may thus provide a sensitive assay system to detect various classes of proto-oncogenes that are susceptible to activation by carcinogenic insult. As illustrated with mouse liver tumors, analysis of activated oncogenes in spontaneously occurring and chemically induced rodent tumors will provide information at a molecular level to aid in the use of rodent carcinogenesis data for risk assessment.

Correlation of cellular radiosensitivity with malignant transformation. The radiobiology of human tumors suggests that multiple factors are involved in clinical radioresponsiveness. In the absence of direct experimental evidence to correlate intrinsic cellular radiosensitivity with the steps of malignant transformation, an in vitro multistage model of epithelial neoplasia was developed in collaboration with scientists at Georgetown University School of Medicine using human epidermal keratinocytes to examine the effects of malignant transformation on radiation response. Cells were first immortalized as a result of infection with a hybrid virus (Ad12-SV40) and subsequently transformed either by infection with a second virus (Ki-MSV) or by treatment with a chemical carcinogen (N-methyl-N'-nitro-N-nitrosoguanidine or 4-nitroquinoline-1-oxide). Primary human epidermal keratinocytes were demonstrated to be radiation resistant as compared with human fibroblasts. This resistance was retained in the immortalized as well as the transformed cell lines. These findings provide direct experimental evidence that radiation sensitivity of malignant human keratinocytes is an intrinsic property of the precursor cell that appears to be conserved through the stages of neoplastic transformation.

In vitro model system for studying interaction of retroviral oncogenes in human epithelial cells. The development of tissue culture systems for propagation of human epithelial cells has aided the investigation of events that lead epithelial cells to become neoplastic. Nontumorigenic human epidermal keratinocytes immortalized by Ad12-SV40 virus or pSV_3-neo were transformed by a variety of retroviruses containing *bas*, H-*ras*, *fes*, *fms*, *erb*B and *src* oncogenes. Transformants showed morphological alterations and induced carcinomas when transplanted into nude mice. Findings demonstrate malignant transformation of human primary epithelial cells in culture by the combined action of Ad12-SV40 virus and retroviral oncogenes and support a multistep process for neoplastic conversion.

Enhanced G_2 chromosomal radiosensitivity, deficient DNA repair, and susceptibility to cancer. In collaborative studies with Howard University, skin fibroblasts or peripheral blood lymphocytes from individuals with genetic disorders predisposing to cancer or with familial cancer were demonstrated to show a higher than normal incidence of chromatid breaks and gaps when irradiated during G_2 phase of the cell cycle. The incidence is also higher in human tumor cells and cells transformed in culture than in normal controls. This enhanced G_2 chromatid radiosensitivity is thus associated with both genetic susceptibility to cancer and neoplastic transformation. It is observed only in cells harvested at least 1.5 hours after irradiation and appears to result from a deficiency(ies) in DNA repair during G_2 phase. This deficiency has a genetic basis, behaving as a recessive trait. Furthermore, G_2 chromatid radiosensitivity provides a means for identifying individuals with genetic susceptibility to cancer.

Skin fibroblasts from 25 members of nine kindreds with familial dysplastic naevus syndrome (DNS), 12 apparently normal spouses, and 11 additional

unrelated normal individuals were tested for G_2 cell-cycle phase sensitivity to ionizing radiation. The cells from individuals with DNS or hereditary cutaneous malignant melanoma with DNS (HCMM/DNS) had significantly more chromatid breaks and gaps when entering metaphase 01.5-1.5 hours after G_2 phase x-irradiation than those from unaffected controls. In two cases, the test results positively identified individuals before the clinical diagnosis of DNS. A clinically normal obligate carrier of the HCMM/DNS gene showed the enhanced G_2 radiosensitivity. Moreover, in a test on one proband, the sensitivity was apparent in peripheral blood lymphoblasts. Enhanced G_2 chromatid radiosensitivity may be a marker of genetic susceptibility to HCMM/DNS.

Possible role of genes in efficiency of chromatin repair in murine model. In collaborative studies with the Laboratory of Genetics and Biostatistics Branch (NCI) and Howard University, evidence was developed in genetically defined, inbred mouse strains suggesting there may be multiple genes in the mouse that affect efficiency of DNA repair and susceptibility to pristane-induced plasmacytoma formation. Tests are underway to determine the correlation, if any, of genes associated with susceptibility and efficiency of DNA repair. In complementary studies early-passage skin fibroblasts from different inbred and congenic strains of mice were x-irradiated and the number of chromatid breaks determined at two hours after irradiation. The cells from DBA/2N, C3H/HeN, STS/A, C57BL/6N, BALB/cJ and AKR/N mice had 25 to 42 chromatid breaks per 100 metaphase cells (efficient repair phenotype). NZB/N/J had <78, and B ALB/cAn had 87 to 100 chromatid breaks per 100 cells (inefficient repair phenotype). Differences between BALB/cAn and BALB/c.DBA/2 congenic strains, which carry less than 1% of the DBA/2 genome, indicate that two genes, one on chromosome 1 linked to *Pep*-3-*bc1*-2 and the other on chromosome 4, closely linked to *Fv-1*, affect the efficiency with which the cells repair radiation-induced chromatin damage.

Lentivirus studies. Certain dideoxynucleosides have been shown to markedly inhibit the infectivity of human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). Collaborative studies between this laboratory and Dr. Samuel Broder's group (Division of Cancer Treatment, NCI) demonstrated that these drugs are broad spectrum antiretroviral agents capable of inhibiting the infectivity of evolutionarily divergent mammalian type C viruses and animal lentiviruses. Under some conditions, virus infectivity could be inhibited by more than six orders of magnitude. However, the potency of these agents was shown to be greatly influenced by cell-specified determinants. Drug exposure during the initial 24 hours was almost as effective as prolonged treatment on the inhibition of a single cycle of virus infection and expression. Moreover, virus infection was shown to be directly inhibited at the level of proviral DNA synthesis. Thus, the time during which reverse transcription and provirus integration occur is the critical period required for drug action. These findings have implications concerning strategies to be considered in attempts to utilize 2',3'-dideoxynucleosides in control and treatment of retrovirus-induced diseases of animals and humans.

Human T-cell leukemia virus (HTLV-III) was isolated from tears, conjunctival epithelium and corneoscleral tissue of AIDS patients. This raises a note of caution regarding possible transmission of virus via corneal transplants and during ophthalmological examinations by way of examiner's hands, through instruments and during contact lens fittings. It also indicates the need for testing various eye disinfectants for HTLV-III inactivation and/or inhibition.

The nucleotide sequence of the integrated form of the genome of the equine infectious anemia virus (EIAV) was determined. By comparison with LTR sequences of other retroviruses, signals for the control of viral gene transcription and translation could be identified in the EIAV LTR. Open reading frames for *gag* and *pol* genes were identified and their sequences matched very closely to those determined previously by others. However, in the present study, the *pol* gene reading frame was open throughout its entire length. The open reading frame for the *env* gene product was constructed from the sequences of two independent EIAV clones. Thus, a noninfectious genomic-length clone was shown to contain a frameshift mutation approximately in the middle of the presumed *env* gene coding sequence, whereas the sequence of another clone was open in this region. The deduced amino acid sequences of the EIAV *gag* and *pol* products showed closer evolutionary relationships to those of known lentiviruses than to other retroviruses. There was also partial sequence homology between predicted *env* gene products of EIAV, visna virus and HIV. Sequences analogous to the *sor* region of other lentiviruses could not be identified in our EIAV clone. A short open reading frame at the 3' end of the genome that overlapped *env* but not the 3' LTR was present but lacked significant sequence similarity to the 3' open reading frames of other lentiviruses. Thus, the sequence and general structure of EIAV most closely resemble those of known lentiviruses.

The *Escherichia coli* chloramphenicol acetyltransferase gene (CAT) was used to study sequences that influence expression of the EIAV genome. The EIAV LTR-directed CAT activity in a canine cell line, but at levels much lower than those achieved with other eucaryotic viral promoters. In the same cells infected with EIAV or cotransfected with molecularly cloned EIAV genomic DNA, LTR-directed activity was markedly enhanced. Comparison of CAT mRNA and protein levels in these cells indicated that this *trans*-activating effect could be accounted for by a bimodal mechanism in which both transcriptional and posttranscriptional events are enhanced. *Trans*-activation but not promoter activity was abolished by deletion of the R-U5 region of the EIAV LTR. EIAV sequences responsible for the *trans*-activating function could be localized to a region encompassing the 3' and 5' termini of the *pol* and *env* genes, respectively (nucleotides 4474 to 5775). Interestingly, this stretch harbors a short open reading frame with some amino acid sequence similarity to the HIV-1 *tat* gene product.

Studies of human B-lymphotropic virus (HBLV). Human B-lymphotropic virus (HBLV), also known as human herpesvirus-6 (HHV-6), was isolated in 1986 from AIDS patients and patients with other lymphoproliferative disorders. HBLV is distinct from known human herpesviruses, biologically, immunologically and by

molecular analysis. It can infect and replicate in fresh and established hematopoietic cells and cells of neural origin, suggesting wide tropism. Of 299 sera tested from patients with AIDS-related complex (ARC), persistent generalized lymphopathy (PGL) and AIDS, 66% were HBLV antibody-positive with an average titer of 1:160. Among 25 sera from asymptomatic homosexuals, 64% were HBLV antibody-positive, with an average titer of 1:40. By comparison, 47% of the sera from 17 HIV-1 antibody-negative homosexual men were HBLV antibody-positive, with an average titer of 1:20, and only 24% of sera from healthy donors contained HBLV antibody, with an average titer of 1:20 or greater. These data indicated an increased association of HBLV with HIV-1-infected individuals and also with a population at high risk of developing AIDS. Since HBLV can infect both fresh and established B and T cells, it is possible that infection by HBLV may contribute to the rapidity of the development of symptoms or to the severity of the immune consequences in HIV-infected individuals.

HBLV was also detected by Southern blot analysis and *in situ* hybridization in Burkitt's lymphoma (BL) cells obtained from an African child. Preliminary results also demonstrated HBLV DNA in seven out of ten other African BLs by *in situ* hybridization. All of the BL cells contained EBV DNA as well, which was always in excess of HBLV DNA. HBLV and EBV antibody titers to virus capsid antigen were elevated in all patients. These data provide impetus for assessing the possible interaction of EBV and HBLV.

In addition to their intramural research efforts, investigators within the LCMB serve on the editorial boards of major journals in their fields, serve as members of various review bodies, and participate in a large number of collaborative efforts with scientists in laboratories within and outside of the United States. The ultimate goal of these multidisciplinary studies of virus/carcinogen-induced and spontaneously occurring cancers is to apply the basic information derived to its most important application, the prevention of cancer in man.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04930-17 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Biology of Natural and Induced Neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	P. Arnstein	Veterinary Director	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. S. Rhim	Microbiologist	LCMB	NCI
	K. C. Robbins	Chief, Mol. Genetics Section	LCMB	NCI
	J. S. Pierce	Microbiologist	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	D. Ron	Visiting Associate	LCMB	NCI
	M. Kraus	Visiting Scientist	LCMB	NCI

COOPERATING UNITS (if any)
CA Dept. Health Services, Berkeley, CA (J. Riggs and R. Emmons); University CA, Berkeley (H. Rubin and M. Stampfer); Children's Hospital Med. Ctr., Oakland, CA (K. Walen)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Collaborative studies on oncogenes integrated into cellular and viral genomes are pursued by parallel in vivo and in vitro studies. Newborn mice inoculated with viral vectors containing the oncogene mac produced unique vascular tumors, whereas similar recombinants containing the genes dbl, transforming growth factor (TGF)-alpha and platelet-derived growth factor (PDGF) have been negative in similar experiments. Fibroblast cultures transfected with the oncogenes dbl, PDGF, basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF) were consistently sarcomagenic, whereas those containing TGF-alpha or epidermal growth factor receptor (EGFR) proved benign. In contrast, combined transfection with TGF-alpha and EGFR proved highly malignant in several cultures.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. Arnstein	Veterinary Director	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rhim	Microbiologist	LCMB	NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
J. H. Pierce	Microbiologist	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
M. H. Kraus	Visiting Scientist	LCMB	NCI
D. Ron	Visiting Associate	LCMB	NCI
S. Blam	Guest Researcher	LCMB	NCI

Objectives:

1. Characterize selected oncogenes of particular interest to LCMB staff, with special emphasis on in vivo manifestation of neoplasia and parallel effects in vitro. The ultimate goal is to determine the full potential of pathogenic expression (or lack of pathogenicity) for each putative oncogene.

2. Characterize neoplastic transformation in selected tissue culture systems and correlate morphologic transformation with neoplastic transplantability in nude athymic mice.

3. Test spontaneous mammalian neoplasms, especially human, for xenograft malignancy and explore correlations with oncogene expression in the original tissue.

Methods Employed:

Specified oncogenes selected by other LCMB investigators and incorporated into viral vectors are tested for pathogenicity (particularly carcinogenicity) by inoculation into normal (euthymic) and immunodeficient (athymic nude) siblings by several routes (intraperitoneal, subcutaneous, intramuscularly, intracerebrally), usually at birth. The same oncogenes integrated into cellular genomes are tested by transplantation into athymic nude adult hosts. The hosts chosen include three levels of immunodeficiency: (a) T-cell deficiency, (b) T + B cell deficiency, (c) T + B + LAK cell deficiency.

Special studies on oncogenic transformation of primate cells and neoplastic behavior of spontaneously expressed oncogenes in human malignancies (in collaboration with Drs. Aaronson, Project No. Z01CP04940-21 LCMB; Rhim, Project NO. Z01CP05060-10 LCMB; and Kraus, Project No. Z01CP05366-05 LCMB) utilize similar techniques.

Major Findings:

Additional studies on the *mac* oncogene, in collaboration with Dr. Pierce (Project No. Z01CP05164-08 LCMB), have confirmed its predilection for endothelial and cardiac cells (see 1986-87 report) and its more prompt expression in euthymic (vs. athymic nude) hosts. The cardiac sarcomas, even though fatal to the host, are difficult to transplant and establish in vitro. Several tissue cultures, putatively neoplastic, have been derived from the tumors and are being analyzed.

The oncogene *dbl*, isolated from a human diffuse B cell lymphoma, proved to be an efficient transformant (in studies conducted with Dr. Eva, Project No. Z01CP05062-10 LCMB) for murine cells transfected with its genomic DNA. These cells repeatedly transplanted as rapidly progressive sarcomas. A similar (or identical) gene found in normal human tissues and propagated and transfected into 3T3 cells by Dr. Ron also gave rise to sarcomas. Both of these genes, however, have to date proven negative for direct in vivo tumor induction when integrated into the usual type C viral vectors.

The oncogene TGFα gave mixed results (approximately 50% malignant, 50% benign) when transfected into 3T3 cells.

The oncogene EGFR did not cause these cells to produce tumors. In two experiments, however, 3T3 cells transfected with both EGFR and TGFα produced rapidly progressive sarcomas. This finding suggests future trials with paired or multiple putative oncogenic sequences as a model for tumor progression where a single gene is ineffective.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04940-21 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
	J. H. Pierce	Microbiologist	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	J. C. Lacal	Visiting Associate	LCMB	NCI
	M. H. Kraus	Visiting Scientist	LCMB	NCI
	P. P. Di Fiore	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*
Dept. Surgery, Duke U. Med. Center (D. Iglehart); California Biotechnology Inc. (J. Abraham, J. Fiddes); Ludwig Institute for Cancer Research, Uppsala, Sweden (C. Heldin, C. Betsholtz); Genetics Institute, Cambridge, MA (J. Knoph)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to determine the cellular alterations responsible for naturally occurring malignancies. Topics of present interest include: (1) transforming genes of retroviruses and cancer cells; (2) the biology of endogenous retroviruses; (3) the molecular biology of retrovirus replication and transformation; and (4) the application of knowledge gained from these studies to the search for the causes and mechanisms involved in human neoplastic transformation.

Some of the highlights of the past year include: (1) The epidermal growth factor receptor (EGFR) gene was found to be frequently amplified and/or overexpressed in human malignancies. High levels of EGFR expression, which conferred a transformed phenotype to NIH/3T3 cells in the presence of ligand, were demonstrated in human tumor cell lines that contained amplified copies of the EGFR gene. (2) The human erbB-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The high levels of the erbB-2 product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumor cells that over-expressed this gene. These findings demonstrate a new mechanism for acquisition of oncogenic properties by genes encoding growth factor receptor-like proteins. (3) The p21 proteins encoded by ras genes may act as regulatory proteins in the transduction of signals that lead to DNA synthesis. (4) The dbl transforming gene product, isolated from a human B-cell lymphoma, was demonstrated to be distinct among known transforming gene products. (4) The mechanism of action of the dideoxynucleosides, which markedly inhibit lentivirus infectivity, was further characterized.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
J. C. Lacal	Visiting Associate	LCMB	NCI
M. H. Kraus	Visiting Scientist	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI
G. Kruh	Medical Staff Fellow	LCMB	NCI
M. Pech	Visiting Scientist	LCMB	NCI

Objectives:

1. To study the mechanisms of action of RNA tumor viruses and transforming genes.

2. To apply knowledge gained from experimental systems to the search for etiologic agents and mechanisms involved in neoplastic transformation of human cells.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology and molecular biology.

Major Findings:

Sis oncogene/platelet-derived growth factor (PDGF)-2. The v-*sis* oncogene encodes a platelet-derived growth factor (PDGF)-related product whose transforming activity is mediated by its functional interaction with the PDGF receptor. PDGF, as well as processed forms of the v-*sis* gene product, is a disulfide-linked dimer with eight conserved cysteine residues in the minimum region necessary for biologic activity. Site-directed mutagenesis of the v-*sis* gene revealed that each conserved cysteine residue was required directly or indirectly for disulfide-linked dimer formation. However, substitution of serine for cysteine codons at any of four positions had no detrimental effect on transforming activity of the encoded v-*sis* protein. Results establish that interchain disulfide bonds are not essential in order for this protein to act as a functional ligand for the PDGF receptor. The remaining four substitutions of serine for cysteine each inactivated transforming function of the molecule. In each case this was associated with loss of a conformation

shown to involve intramolecular disulfide bonds. These studies provide insight into the role of individual cysteine residues in determining the structure of the *sis*/PDGF molecule critical for biological activity.

The human platelet-derived growth factor (PDGF) is a potent mitogen for connective tissue cells. PDGF contains two related but distinct polypeptides, one of which, PDGF-2, has been shown to be homologous to the transforming product of the retroviral v-*sis* oncogene (c-*sis*), a prototype growth factor with transforming potential. The c-*sis*/PDGF-2 transcript was demonstrated to contain a long 5' untranslated sequence (UTS) that is highly GC rich. To examine the influence of this sequence on *sis*/PDGF-2 expression, the c-*sis*/PDGF-2 promoter was localized and this promoter or the simian virus 40 (SV40) early promoter was used to drive expression of the bacterial chloramphenicol acetyltransferase (CAT) or *sis*/PDGF-2 gene. The 5' UTS of c-*sis*/PDGF-2 mRNA had no effect on RNA expression but was shown to exert a potent inhibitory effect on translation. By deletion analysis, the 5' UTS was found to inhibit protein expression by as much as 40-fold. The inhibitory effect was independent of reporter gene, cell type or promoter used. A highly GC-rich 140-base-pair sequence immediately preceding the c-*sis*/PDGF-2 initiation codon was shown to be nearly as effective as the entire 5' UTS in translational inhibition. Transfection analysis demonstrated that the 5' UTS significantly reduced the transforming efficiency of the *sis*/PDGF-2 gene as well. Findings raised the possibility that changes in regulation at the level of *sis*/PDGF-2 translation may play a role in development of the neoplastic phenotype.

The major mRNA start sites of *sis*/PDGF-2 transcripts derived from normal placenta and from a human tumor cell line, A2780, known not to express the c-*sis*/PDGF-2 transcript, were found to be identical, excluding the possibility that *sis*/PDGF-2 expression in the human tumor cell line transcript reflects the altered transcriptional initiation of the *sis*/PDGF-2 gene. Further investigation of the role of flanking sequences that may affect *sis*/PDGF-2 gene expression in tumor cells expressing the *sis*/PDGF-2 transcript should help to elucidate both the normal transcriptional control of this gene as well as mechanisms that may activate it as an oncogene in certain tumor cells.

Genes for other growth factors as oncogenes. A peptide sescreted by some tumor cells in vitro, transforming growth factor alpha (TGFα), imparts anchorage-independent growth to normal rat kidney (NRK) cells. To directly investigate the transforming properties of this factor, the human sequence coding for TGFα was placed under the control of either a metallothionein promoter or a retroviral long terminal repeat (LTR). These constructs failed to induce morphological transformation upon transfection of NIH/3T3 cells, whereas viral oncogenes encoding a truncated form of its cognate receptor, the epidermal growth factor (EGF) receptor, or another growth factor, *sis*/PDGF-2, efficiently induced transformed foci. When NIH/3T3 clonal sublines were selected by transfection of TGFα expression vectors in the presence of a

dominant selectable marker, they were shown to secrete large amounts of TGFα into the medium, to have down-regulated EGF receptors, and to be growth-inhibited by TGFα monoclonal antibody. The results indicated that secreted TGFα interacts with its receptor at a cell surface location. Single cell-derived TGFα-expressing sublines grew to high saturation density in culture. However, when plated as single cells on contact-inhibited monolayers of NIH/3T3 cells, they failed to form colonies, while v-*sis*- and v-*erb*B-transfected cells formed transformed colonies under the same conditions. Moreover, TGFα-expressing sublines were not tumorigenic in nude mice. Results imply that TGFα exerts a growth-promoting effect on the entire NIH/3T3 cell population after secretion into the medium, but little, if any, effect on the individual cell synthesizing this factor. It was thus concluded that the normal coding sequence for TGFα is not a direct-acting oncogene.

Oncogenes encoding growth factor receptors. The epidermal growth factor receptor (EGFR) gene is frequently amplified and/or overexpressed in human malignancies. To investigate the biological effects of its overexpression, a eukaryotic vector containing human EGFR cDNA was constructed. Introduction of this construct led to constitution of functional EGF receptors in NR6 mutant cells, which are normally devoid of this receptor. Transfection of NIH/3T3 resulted in no significant alterations in growth properties. However, the addition of EGF led to the formation of densely growing transformed foci in liquid culture and colonies in semisolid medium. NIH/3T3 EGFR clonal lines, which expressed the EGF at 500- to 1000-fold levels over control NIH/3T3 cells, demonstrated a marked increase in DNA synthesis in response to EGF. Thus, EGF receptor overexpression appears to amplify normal EGF signal transduction. Finally, high levels of EGFR expression, which conferred a transformed phenotype to NIH/3T3 cells in the presence of ligand, were demonstrated in representative human tumor cell lines that contained amplified copies of the EGFR gene.

An expression vector for EGFR was introduced into the 32D myeloid cell line, which is devoid of EGF receptors and absolutely dependent on interleukin-3 (IL-3) for its proliferation and survival. Expression of the EGFR conferred the ability to utilize EGF for transduction of a mitogenic signal. When the transfected cells were propagated in EGF, they exhibited a more mature myeloid phenotype than was observed under conditions of IL-3-directed growth. Moreover, exposure to EGF led to a rapid stimulation of phosphoinositide metabolism, while IL-3 had no detectable effect on phosphoinositide turnover either in control or EGF receptor-transfected 32D cells. Although the transfected cells exhibited high levels of functional EGF receptors, they remained nontumorigenic. In contrast, transfection of v-*erb*B, an amino-terminal truncated form of the EGF receptor with constitutive tyrosine kinase activity, not only abrogated the IL-3 growth factor requirement of 32D cells, but caused them to become tumorigenic in nude mice. The results showed that a naive hematopoietic cell expresses all of the intracellular components of the

EGF-signaling pathway necessary to evoke a mitogenic response and sustain continuous proliferation.

Activation of normal human *erb*B-2 as an oncogene. Studies demonstrated that the human *erb*B-2 gene can be activated as an oncogene by its overexpression in NIH/3T3 cells. The level of the *erb*B-2 product was shown to be critical in determining its transforming ability. This in vitro observation is paralleled by in vivo findings that a wide variety of human tumors contain an amplified or overexpressed *erb*B-2 gene, which encodes a growth factor receptor-like protein. When *erb*B-2 complementary DNA was expressed in NIH/3T3 cells under the control of the SV40 promoter, the gene lacked transforming activity despite expression of detectable levels of the *erb*B-2 protein. A further five- to 10-fold increase in its expression under influence of the long terminal repeat of Moloney murine leukemia virus was associated with activation of *erb*B-2 as a potent oncogene. The high levels of the *erb*B-2 product associated with malignant transformation of NIH/3T3 cells were observed in human mammary tumor cells that overexpressed this gene. These findings demonstrate a new mechanism for acquisition of oncogenic properties by genes encoding growth factor receptor-like proteins and provide a functional basis for the role of their overexpression in the development of human malignancies.

Interactions of growth factors and oncogenes. BALB/MK epidermal keratinocytes require EGF for growth in serum-containing medium and terminally differentiate in response to high Ca^{++} concentration. Several oncogenic retroviruses have been shown to relieve the EGF requirement and to block calcium-induced terminal differentiation. A chemically defined medium was developed to investigate the minimum growth factor requirements for BALB/MK cells, as well as how such requirements might be altered by retroviral oncogenes. In this medium, insulin, apparently acting as IGF-1, and EGF supported cell growth in a manner comparable to serum and EGF. Acidic as well as basic fibroblast growth factors (FGF) substituted for EGF but not insulin in supporting BALB/MK proliferation. Infection with retroviruses containing v-*ras* oncogenes (v-H-*ras*, v-Ki-*ras*), oncogenes derived from growth factor receptors (v-*erb*B, v-*fms*) or the v-*mos* oncogene permitted growth in defined medium containing insulin but lacking EGF. The v-*fgr* oncogene, a member of the *src* subfamily, was unique in conferring independence from both insulin and EGF. Findings established the applicability of this system for biologic assay of epithelial cell growth factors as well as identification of specific growth factor requirements that can be altered or complemented by the actions of specific oncogenes.

In other studies, EGF was demonstrated to be an extremely potent mitogen, causing BALB/MK cultures to enter the cell cycle in a synchronous manner associated with a greater than 100-fold increase in DNA synthesis. Analysis of the expression of proto-oncogenes which have been reported to be activated

during the cascade of events following growth factor stimulation of fibroblasts or lymphoid cells revealed a very rapid but transient 100-fold increase in c-*fos* RNA but little or no effect on the other proto-oncogenes analyzed. Exposure of EGF-synchronized BALB/MK cells to high levels of calcium was associated with a striking decrease in the early burst of c-*fos* RNA as well as the subsequent peak of cell DNA synthesis. Since the inhibitory effect of high calcium on c-*fos* RNA expression was measurable within 30 minutes, studies imply that the EGF proliferative and calcium differentiation signals must interact very early in the pathway of EGF-induced proliferation. The results also establish that c-*fos* RNA modulation is an important early marker of cell proliferation in epithelial as well as mesenchymal cells.

The role of physiologic concentrations of EGF on phosphoinositide metabolism in BALB/MK mouse keratinocytes was investigated. Results indicated that EGF rapidly activates phospholipase C-mediated phosphoinositide metabolism resulting in the generation of the second messengers inositol (1,4,5) triphosphate and diacylglycerol. These metabolites control intracellular Ca^{++} levels and activate protein kinase C, respectively. Protein kinase C activation in response to EGF was evidenced by the phosphorylation of the acidic 80-kd endogenous protein substrate (p80) specific for this kinase. In contrast, insulin had no effect on phosphoinositide metabolism nor led to any additional stimulation when added in combination with EGF. Taken together, results showed that phosphoinositide metabolism and subsequent protein kinase C activation are involved in the normal mitogenic response of epithelial cells to EGF.

Ras oncogenes and proto-oncogenes. The availability of molecular clones of the normal and activated alleles of human *ras* proto-oncogenes made it possible to determine the molecular mechanisms responsible for the malignant conversion of these genes. Findings during the past year include the following:

Quiescent mouse NIH/3T3 cells responded to microinjection of activated *ras* p21 with a rapid and sustained rise in intracellular pH (~0.17 pH units). The p21-induced pH change was inhibited by amiloride treatment of growth of cells in medium low in sodium, suggesting a role for the Na^+/H^+ antiporter. Amiloride was found to suppress p21-induced mitosis also.

Glycosphingolipid alterations upon viral transformation are well documented. Transformation of mouse 3T3 cells with murine sarcoma viruses results in marked decreases in the levels of gangliosides GM1 and GD1a and an increase in gangliotriasylceramide. The transforming oncogenes of these viruses have been identified as members of the *ras* gene family. NIH/3T3 cells transfected with human H-, K- and N-*ras* oncogenes were analyzed for their glycolipid composition and expression of cell surface gangliosides. Using conventional thin-layer chromatographic analysis, the level of GM3 was found to be

increased and that of GD1a slightly decreased or unchanged, and GM1 was present but not in quantifiable levels. Cell surface levels of GM1 were determined by cholera toxin binding to cells treated with sialidase prior to toxin binding. All *ras*-transfected cells had decreased levels of surface GM1 and GD1a as compared to logarithmically growing normal NIH/3T3 cells. Levels of GM1 and, to a lesser extent, GD1a increased as the latter cells became confluent. Using a monoclonal antibody assay, we found that gangliotriasylceramide was present in all *ras*-transfected cells studied but not in logarithmically growing untransfected cells. Interestingly, a gangliotriasylceramide appeared when the latter cells became confluent. These results indicated that *ras* oncogenes derived from human tumors are capable of inducing alterations in glycolipid composition.

The p21 products of *ras* proto-oncogenes are thought to be important components in pathways regulating normal cell proliferation and differentiation. These proteins acquire transforming properties as a result of activating lesions that convert *ras* genes to oncogenes in a wide array of malignancies. In *Xenopus laevis* oocytes, microinjection of transforming *ras* p21 is a potent inducer of maturation, whereas microinjection of a monoclonal antibody to *ras* p21 inhibits normal maturation induced by hormones. The phosphoinositide pathway is a ubiquitous system that appears to play a key role in diverse cellular functions. By use of the *Xenopus* oocyte system, it was possible to quantitate the effects of *ras* p21 microinjection on individual components of the phosphoinositide pathway. Within 20 minutes of microinjection, levels of phosphatidylinositol, 4,5-bisphosphate, inositol 1-phosphate, and inositol bisphosphate increased one and one-half to twofold. The most striking effects were on diacylglycerol, which increased fivefold under the same conditions. In contrast, the normal *ras* p21 protein induced no detectable alteration in any of the metabolites analyzed. The earliest efects of the transforming p21 on phosphoinositol turnover were observable within two mintues, implying a very rapid effect of *ras* p21 on the enzymes involved in phospholipid metabolism.

Genes involved in the transduction of signals required for normal cell proliferation commonly appear to be subverted in the neoplastic process. One such group is the highly conserved family of *ras* genes, which are present in a wide variety of naturally occurring tumors. By analogy with other known G proteins, the p21 proteins encoded by *ras* genes may act as regulatory proteins in the transduction of signals that lead to DNA synthesis. A major pathway involved in the DNA synthesis induced by growth factors is mediated by phosphatidylinositol turnover: cleavage of phosphoinositides by phospholipase C produces 1,2-diacylglycerol and inositol phosphates. The former acts as an essential cofactor for protein kinase C, and inositol-(1,4,5)-triphosphate mobilizes Ca^{++} from nonmitochondrial intracellular stores. A reproducible increase in 1,2-diacylglycerol, in the absence of a detectable increase in inositol phosphates, in transformed cells containing Ha-*ras* oncogenes and with different membrane targeting signals for the *ras* p21 protein was demonstrated.

These findings suggest that a source other than phosphoinositides exists for the generation of 1,2-diacylglycerol and that the Ha-*ras* oncogene specifically activates this novel pathway for 1,2-diacylglycerol production.

Microinjection of purified protein kinase C (PKC) into Swiss/3T3 fibroblasts pretreated with the phorbol ester phorbol-12,13-dibutyrate had previously been demonstrated to restore the mitogenic response of the cells to phorbol-12,13-dibutyrate. Present studies demonstrate that the mitogenic activity of the H-*ras* oncogene in H-*ras* p21-microinjected quiescent cells is markedly reduced under conditions in which PKC is downregulated by chronic phorbol ester treatment. The ability to reconstitute the mitogenic response upon microinjection of both H-*ras* p21 and PKC implies involvement of functional PKC in the mitogenic activity of the H-*ras* oncogene product.

The *dbl* oncogene. The *dbl* transforming gene was originally identified by transfection of NIH/3T3 cells with DNA from a human B-cell lymphoma. This gene was found to have arisen as a result of recombination of the 3' portion of the *dbl* proto-oncogene coding sequences with an unrelated segment of human DNA. It encodes a cytoplasmic protein that is equally distributed between cytosol and crude membrane fractions. To further characterize this transforming gene, a biologically active cDNA clone of the *dbl* transforming gene mRNA was isolated. Analysis of the sequence of the *dbl* oncogene cDNA revealed a long open reading frame that encodes a hybrid protein whose first 50 amino acids (at least) derive from a complete exon of a different locus. No significant homology with known oncogenes or any known protein sequences was demonstrated. The computer analysis of the predicted *dbl* protein indicated it is highly hydrophilic with no hydrophobic domains characteristic of a membrane-spanning region or signal peptide. Thus, the *dbl* oncoprotein is distinct among known transforming gene products.

In further characterizations, the transcribed sequences of *dbl* were shown to be distributed over a 30-kb span within a molecularly cloned 45-kb segment of huuman DNA which contained the transforming gene. By restriction mapping, its transcribed region corresponded to that of its normal allele except at the 5' end where a rearrangement involved transcribed *dbl* oncogene sequences from another locus. An independent isolate of a *dbl*-related transforming gene was obtained following transfection of NIH/3T3 cells with DNA of a human nodular poorly differentiated lymphoma (NPDL). Physical mapping indicated that this transforming gene, designated NPDL-*dbl*, shared considerable homology with the *dbl* oncogene, but differed at both 5' and 3' termini. Its point of divergence from the normal allele at the 5' end was at least 10 kb upstream from that of the *dbl* oncogene. The oncogenes each expressed truncated transcripts compared to the 5.3-kb normal transcript. The *dbl* and NPDL-*dbl* oncogene translational products of 66 and 76 kDa, respectively, were consistent with their corresponding major 2.8- and 3.5-kb transcripts. It was not possible to detect evidence of the 5' structural rearrangements associated with these oncogenes in either of the original tumors. Thus, if these rearrangements

were critical to their activation, they occurred in the process of gene transfer or in vivo in only a minority of tumor cells.

Characterization of equine infectious anemia virus (EIAV), a lentivirus related to human immunodeficiency virus (HIV). The nucleotide sequence of the integrated form of the genome of the equine infectious anemia virus was determined. By comparison with LTR sequences of other retroviruses, signals for the control of viral gene transcription and translation could be identified in the EIAV LTR. Open reading frames for *gag* and *pol* genes were identified and their sequences matched very closely to those determined previously by others. However, in the present study, the *pol* gene reading frame was open throughout its entire length. The open reading frame for the *env* gene product was constructed from the sequences of two independent EIAV clones. Thus, a noninfectious genomic-length clone was shown to contain a frameshift mutation approximately in the middle of the presumed *env* gene coding sequence, whereas the sequence of another clone was open in this region. The deduced amino acid sequences of the EIAV *gag* and *pol* products showed closer evolutionary relationships to those of known lentiviruses than to other retroviruses. There was also partial sequence homology between predicted *env* gene products of EIAV, visna virus and HIV. Sequences analogous to the *sor* region of other lentiviruses could not be identified in our EIAV clone. A short open reading frame at the 3' end of the genome that overlapped *env* but not the 3' LTR was present but lacked significant sequence similarity to the 3' open reading frames of other lentiviruses. Thus, the sequence and general structure of EIAV most closely resemble those of known lentiviruses.

Broad-spectrum antiretroviral activity of 2',3'-dideoxynucleosides. Certain dideoxynucleosides have been shown to markedly inhibit the infectivity of HIV, the causative agent of acquired immunodeficiency syndrome (AIDS). Collaborative studies between this laboratory and Dr. Samuel Broder's group (Division of Cancer Treatment, NCI) demonstrated that these drugs are broad spectrum antiretroviral agents capable of inhibiting the infectivity of evolutionarily divergent mammalian type C and animal lentiviruses. Under some conditions, virus infectivity could be inhibited by more than six orders of magnitude. However, the potency of these agents was shown to be greatly influenced by cell-specified determinants. Drug exposure during the initial 24 hours was almost as effective as prolonged treatment on the inhibition of a single cycle of virus infection and expression. Moreover, virus infection was shown to be directly inhibited at the level of proviral DNA synthesis. Thus, the time during which reverse transcription and provirus integration occur is the critical period required for drug action. These findings have implications concerning strategies to be considered in attempts to utilize 2',3'-dideoxynucleosides in control and treatment of retrovirus-induced diseases of animals and humans.

In vitro assay for quantitating invasive potential of tumor cells. A matrix of basement membranes was reconstituted onto a filter in a Boyden chamber and the ability of various malignant and nonmalignant cells to penetrate through the coated filter assessed. Cells from all the malignant cell lines tested were able to cross the matrix in five to six hours, whereas human fibroblasts as well as mouse 3T3 and 10T-1/2 cell lines, which are not tumorigenic, were not invasive. In addition, normal primary prostate epithelial cells and benign prostatic hyperplasia cells were not invasive when tested in this assay, whereas malignant prostate carcinoma cells were highly invasive. Parallel experiments with these prostatic cells, using the intrasplenic assay for metastasis detection in the nude mouse, confirmed the benign behavior of the former cells and the metastatic phenotype of the latter ones. These results suggest that this in vitro test allows the rapid and quantitative assessment of invasiveness and a means to screen for drugs which alter the invasive phenotype of tumor cells.

Activation of oncogenes in B6C3F1 mouse liver as measure of risk assessment. The validity of mouse liver tumor end points in assessing the potential hazards of chemical exposure to humans is a controversial but important issue, since liver neoplasia in mice is the most frequent tumor target tissue end point in two-year carcinogenicity studies. The ability to distinguish between promotion of background tumors versus a genotoxic mechanism of tumor initiation by chemical treatment would aid in the interpretation of rodent carcinogenesis data. Activated oncogenes in chemically induced and spontaneously occurring mouse liver tumors were examined and compared as one approach to determine the mechanism by which chemical treatment caused an increased incidence of mouse liver tumors. Data suggest that furan and furfural caused an increased incidence in mouse liver tumors at least in part by induction of novel weakly activating point mutations in *ras* genes even though neither chemical induced mutations in *Salmonella* assays. In addition to *ras* oncogenes, two activated *raf* genes and four non-*ras* transforming genes were detected. The B6C3F1 mouse liver may thus provide a sensitive assay system to detect various classes of proto-oncogenes that are susceptible to activation by carcinogenic insult. As illustrated with mouse liver tumors, analysis of activated oncogenes in spontaneously occurring and chemically induced rodent tumors will provide information at a molecular level to aid in the use of rodent carcinogenesis data for risk assessment.

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Patents:

Kruh GD, Aaronson SA. US Patent (Pending): Definition of a Human Gene Related to but Distinct from the Abelson Proto-oncogene.

King CR, Kraus MH, Aaronson SA. US Patent (Pending): A Human Gene Related to but Distinct from EGF Receptor Gene.

Lacal JC, Aaronson SA. US Patent (Pending): Deletion Mutants and Monoclonal Antibodies Against *ras* Proteins.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04941-16 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biochemical Characterization of Retroviruses and onc Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. R. Tronick	Chief, Gene Structure Section	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI
	A. Eva	Visiting Scientist	LCMB NCI
	D. Ron	Visiting Associate	LCMB NCI
	D. Archambault	Guest Researcher	LCMB NCI
	N. Popescu	Microbiologist	LB NCI
	O. W. McBride	Chief, Mol. Regulatory Section	LB NCI

COOPERATING UNITS *(if any)*
Sackler Sch. Med., Tel Aviv, Israel (A. Yaniv, A. Gazit); Pan-Data, Inc., Rockville, MD (J. Dahlberg); North Carolina State U., Raleigh, NC (L. Coggins, F. Fuller); Nat. Inst. Allergy & Infectious Dis. (C. Flexner, B. Moss)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies on animal lentiviruses have led to the development of potential vaccines for equine infectious anemia. Equine infectious anemia virus (EIAV) Pr55gag expressed in bacterial and vaccinia virus systems are undergoing tests for efficacy. The transcriptional patterns of EIAV and caprine arthritis encephalitis virus (CAEV) have been studied and reveal mRNAs specific for potential viral regulatory genes. The isolation of an infectious molecular clone of EIAV will facilitate understanding of determinants of lentivirus pathogenicity. A number of molecular clones of defective forms of the CAEV genome were isolated from unintegrated DNA and their effects on the course of CAEV infections are being determined.

The nucleotide sequences of the human dbl oncogene and proto-oncogene have been determined and analyzed. The results show that dbl represents a new class of transforming gene and that the proto-dbl translational product shares features with proteins that constitute structural elements of cells. The chromosomal localization of dbl oncogene sequences has been determined. These studies show that dbl was generated by recombination between three sequences originating from two different human chromosomes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
D. Ron	Visiting Associate	LCMB	NCI
D. Archambault	Guest Researcher	LCMB	NCI

Objectives:

The purposes of this project are the following: to biochemically characterize animal lentiviruses in order to understand the mechanisms by which these viruses cause disease; to study proto-oncogenes and their activated counterparts in order to determine their role in the causation of human cancers and how these genes may participate in normal physiological processes such as growth and differentiation.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing, and electron microscopy; immunological techniques (RIA, Western, and ELISA).

Major Findings:

Coding sequences for the precursor polypeptide of the EIAV *gag* gene were cloned in a plasmid vector (pRC23) and expressed in *E. coli*. The protein was purified by either high salt extraction of cell lysates or by gel electrophoresis. The protein derived by either procedure was antigenically indistinguishable from the authentic viral protein. In collaboration with Dr. Coggins, this material is being tested in horses for its immunogenicity. Pr55^{gag} sequences were also introduced into the pSC11 plasmid which contains the vaccinia virus TK gene. This plasmid was transfected into cells infected with vaccinia virus and recombinant vaccinia viruses were isolated that express EIAV Pr55^{gag}. Horses inoculated with the recombinant virus will be tested for their immune responses to EIAV *gag*. If succesfully immunized, the animals will be challenged with a pathogenic field strain of EIAV. These studies are being performed in collaboration with C. Flexner and L. Coggins.

The molecular cloning and nucleotide sequence of the integrated form of the 888πEIAV DNA was previously reported. This clone was not infectious, however. In subsequent attempts, using similar methodology, an infectious clone

(designated C2) has been obtained. Restriction enzyme analysis of the two clones revealed no obvious differences. The *in vivo* pathogenicity of virus derived from transfection of dog cells with clone C2 is now being tested in collaboration with Coggins and Fuller. Nucleotide sequence analysis of clone C2 is in progress. The pattern of EIAV gene transcription in infected cells was analyzed. Messenger RNAs representing genomic, and *env* sequences were detected (8 and 4.4 Kb) in addition to two smaller messages that represent the *trans*-activator and 3' open reading frame (2.2 and 2.0 Kb).

The nucleotide sequence of CAEV proviral DNA has been nearly completed. Comparative analyses demonstrated closer relatedness between CAEV and visna virus than to any other lentiviruses. Open reading frames encoding putative SOR, *tat* and 3'-open reading frame products were present. However, sequences analogous to human lentiviral *trs/art*, X or R open reading frames could not be discerned. Point mutations in *gag*, *pol* and *env* open reading frames explained the lack of biological activity of this clone. Further attempts to clone infectious CAEV genomes has thus far resulted in the isolation of a number of defective forms from super-coiled circular unintegrated DNA. Clones derived from high molecular weight DNA are now being analyzed.

Nucleotide sequence analysis of cDNAs of the *dbl* oncogene and the *dbl* proto-oncogene defined the changes that took place during the conversion of the latter to a transforming gene. Thus, the amino terminal 497 amino acids of proto-*dbl* were lost due to recombination with a discontiguous human locus. This rearrangement resulted in substitution of 50 new amino acids at the N-terminus of *dbl*. Computer analyses revealed that normal and oncogenic *dbl* proteins are hydrophilic and do not possess a membrane binding domain characteristic of integral membrane proteins. There were no significant similarities between *dbl* sequences to those of any other oncogenes. However, the amino terminal regions of proto-*dbl* showed a low but statistically significant degree of similarity to the coiled-coil domain of the intermediate filament vimentin. Furthermore, there was a characteristic heptad repeat motif in this proto-*dbl* sequence. Chromosome mapping using probes derived from the *dbl* genomic cosmid clone revealed that the sequences encoding the amino terminal 50 amino acids were derived from chromosome 3. The other transcribed sequences were localized to the long arm of chromosome X and non-transcribed sequences present at the 3' end of the clone (and not required for transforming activity) were mapped to chromosome 16.

Publications:

Aaronson SA, Tronick SA. Oncogenes, growth regulation and cancer. In: Advances in cyclic nucleotide and protein phosphorylation research. Proceedings of the VIth international cnference on cyclic nucleotides, calcium and protein phosphorylation (In Press)

Dahlberg JE, Chiu I-M, Yaniv A, Gazit A, Tronick SR. Molecular cloning of equine infectious anemia virus and detection of genetic relatedness to lentiviruses and HTVL III/LAV. In: Proceedings of conference on equine infectious anemia, prevention and control. (In Press)

Eva A, Srivastava S, Vecchio G, Ron D, Tronick S, Aaronson SA. *dbl*: A new transforming gene isolated from a human diffuse B-cell lymphoma. In: Cimino F, Birkmayer GD, Klavins JV, Pimentel E, Salvatore F, eds. Human tumor markers. Berlin/New York: Walter De Gruyter & Co., 1987;83-92.

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Katamine S, Notario V, Rao CD, Miki T, Cheah MSC, Tronick SR, Robbins KC. Primary structure of the human *fgr* proto-oncogene product, $p55^{c\text{-}fgr}$. Mol Cell Biol 1988;8:259-66.

Kawakami T, Sherman L, Dahlberg J, Gazit A, Yaniv A, Tronick SR, Aaronson SA. Nucleotide sequence analysis of equine infectious anemia virus proviral DNA. Virology 1987;158:300-12.

Lacal JC, Tronick SR. The *ras* oncogenes. In: Reddy EP, Curran T, Skalka A, eds. The oncogene handbook. New York: Elsevier (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: ZP1CP04976-11 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Carcinogenesis of Mammalian Cells in Culture

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
Others:	S. Takai	Visiting Fellow	LCMB	NCI
	J. S. Rhim	Microbiologist	LCMB	NCI
	M. Potter	Chief	LG	NCI
	K. H. Kraemer	Research Scientist	LMC	NCI
	R. E. Tarone	Mathematical Statistician	BB, EBP	NCI
	R. Gantt	Chemist	REB, CPCP	NCI

COOPERATING UNITS *(if any)*
Howard U. College Med. (R. Parshad); Children's Hospital of Los Angeles (W. E. Benedict); Tel Aviv U. (Y. Shiloh); Walter Reed Department of Medicine (R. Knight)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cultures of skin fibroblasts, peripheral blood lymphocytes and lymphoblastoid cell lines from normal and cancer-prone individuals, as well as neoplastic cells transformed in culture or in vivo, are utilized in evaluating the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, genetic instability, cancer susceptibility and neoplastic transformation. An increased incidence of chromatid damage after x-irradiation, specifically during G-2 phase of the cell cycle, is associated with both a predisposition to cancer and neoplastic transformation and can provide the basis of a test for genetic susceptibility to cancer. Efforts are currently directed toward developing such a cytogenetic assay using skin fibroblasts, peripheral blood lymphocytes or lymphoblastoid cell lines. A genetic basis for this radiosensitivity with localization of genes to specific chromosomes is indicated from studies with somatic cell hybrids, inbred strains of mice and congenic mouse strains; studies are in progress to localize and map such gene loci. The chromosomal radiosensitivity appears to result from deficient DNA repair during G-2. Another aspect of this project is to develop a reproducible transformation system with human epidermal keratinocytes as an in vitro model for following the progression of biologic, cytomorphologic and biochemical changes leading to neoplastic transformation, with particular emphasis on the acquisition of DNA repair deficiencies and genetic instability.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
S. Takai	Visiting Fellow	LCMB	NCI
J. S. Rhim	Microbiologist	LCMB	NCI
M. Potter	Chief	LG	NCI
K. H. Kraemer	Scientist	LMC	NCI
R. E. Tarone	Mathematical Statistician	BB, EBP	NCI
M. A. Tucker	Oncologist	EEB	NCI
R. Gantt	Chemist	REB, CPCP	NCI

Objectives:

The objective of this project is to elucidate, through cell culture studies, mechanisms of neoplastic transformation in human cells. Current emphasis is on the relationship betwen radiation-induced chromosomal DNA damage, deficient DNA repair, genetic instability, cancer susceptibility and malignant transformation. Efforts are also directed toward developing a transformation system with human epidermal keratinocytes as an in vitro model of human cell carcinogenesis with particular emphasis on the acquisition of DNA repair deficiencies and genetic instability. Specific goals are: (1) to develop a cytogenetic assay for genetic predisposition to cancer using skin fibroblasts, lymphoblastoid cell lines or peripheral blood lymphocytes; (2) to determine, at the molecular and cytogenetic level, consequences of radiation-induced chromatin damage and repair during G_2 phase; (3) to determine the relationship between G_2 chromatin repair deficiency, genetic instability and neoplastic transformation in vitro and in vivo; and (4) to identify genes for chromatin repair in mouse and human cells.

Methods Employed:

Chromatid breaks and gaps persisting after G_2 phase x-irradiation (100 R) or exposure to fluorescent light (effective wavelength 405 nm, two hours, $8W/m^2$) are quantified in skin fibroblasts, stimulated peripheral blood lymphocytes or lymphoblastoid cell lines. DNA repair inhibitors, caffeine and cytosine arabinoside, are used following irradiation to analyze mechanisms. Since repair of lesions is influenced by the stage of the cell cycle and since the chromatid damage is scored in metaphase cells only, the stage of the cell cycle at the time of irradiation can be experimentally manipulated by varying the interval from treatment to fixation of cells.

Major Findings:

1. In a blind study skin fibroblasts from 25 members of nine kindreds with familial dysplastic naevus syndrome (DNS), 12 apparently normal spouses, and 11 additional unrelated normal individuals were tested for G_2 cell-cycle phase sensitivity to ionizing radiation. The cells from individuals with DNS or hereditary cutaneous malignant melanoma with DNS (HCMM/DNS) had significantly more chromatid breaks and gaps when entering metaphase 0.5 - 1.5 hours after G_2 phase x-irradiation (1 Gy) than those from unaffected controls. In two cases, the test results positively identified individuals before the clinical diagnosis of DNS. A clinically normal obligate carrier of the HCMM/DNS gene showed the enhanced G_2 radiosensitivity. Moreover, in a test on one proband, the sensitivity was apparent in stimulated peripheral blood lymphocytes. Enhanced G_2 chromatid radiosensitivity may be a marker of genetic susceptibility to HCMM/DNS.

2. The frequencies of chromatid breaks and gaps in metaphase cells two hours after G_2 phase x-irradiation (1 Gy) were in almost all cases at least two- to threefold higher in skin fibroblasts from individuals with genetic or familial conditions predisposing to cancer (ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, familial polyposis, hereditary malignant melanoma and DNS, Gardner's syndrome, retinoblastoma, Wilms' tumor, xeroderma pigmentosum and cancer family members) than in comparable cells from clinically normal controls. We have summarized our data concerning these frequencies which were determined in a series of studies on coded samples during the past six years and identified factors that affect assay results. These factors include pH, temperature, cell density, culture medium or serum, microbial contamination and visible light exposure (effective wavelength 405 nm). Because of experimental variability, known normal controls should be used in each group of assays. With adequate control of the above factors, this method could provide the basis of a test for detecting individuals genetically predisposed to cancer.

3. Xeroderma pigmentosum (XP) is a rare autosomal-recessive, progressive, degenerative disease associated with sun sensitivity, cutaneous neoplasia, abnormal DNA repair and sometimes neurologic degeneration. Obligate carriers of the XP gene(s) are clinically normal individuals who cannot be recognized by any test known to date. However, their identification is important for genetic counseling, epidemiolgic studies and cancer control. With coded samples of either skin fibroblasts or peripheral blood lymphocytes, we were able to detect obligate carriers utilizing a cytogenetic assay shown previously to detect individuals with cancer-prone genetic disorders (Lancet 2:1111, 1987). Metaphase cells of stimulated (phytohemagglutinin) T-lymphocytes from eight individuals heterozygous for XP were compared with those from nine normal controls at 140 and

200 minutes after x-irradiation (1 Gy) during the G_2 phase of the cell cycle. Cells from the XP heterozygotes had twofold higher frequencies of chromatid breaks or chromatid gaps than normal ($P < 10^{-5}$). Cells from six homozygotes had aberration frequencies threefold higher than normal. Skin fibroblasts from the father of an XP patient had a twofold higher frequency of chromatid breaks and a four-fold higher frequency of gaps than a normal control and approximately half the frequencies observed in the patient when examined 115 minutes after G_2 phase x-irradiation (1 Gy). These findings may provide the basis of a test for identifying carriers of the XP gene(s).

Publications:

Parshad R, Sanford KK, Gantt R. G_2 chromatid radiosensitivity in relation to DNA repair and cancer susceptibility. In: Sobti RC, Natarajan AT, eds. The eukaryotic chromosome: structural and functional aspects. (In Press)

Potter M, Sanford KK, Parshad R, Huppi K, Mock B. Susceptibility and resistance to plasmacytogenesis: possible role of genes that modify efficiency of chromatin repair. In: Mock B, Potter M, eds. Current Topics in Microbiology and Immunology 137 (In Press)

Potter M, Sanford KK, Parshad R, Tarone RE, Price FM, Mock B, Huppi K. Genes on chromosomes 1 and 4 in the mouse are associated with repair of radiation-induced chromatin damage. Genomics (In Press)

Sanford KK, Parshad R, Greene MH, Tarone RE, Tucker MA, Jones GM. Hypersensitivity to G_2 chromatid radiation damage in familial dysplastic naevus syndrome. Lancet 1987;2:1111-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05060-10 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Mechanisms of Carcinogenesis In Vitro: Oncogenic Transformation of Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. S. Rhim	Microbiologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. Arnstein	Veterinary Director	LCMB	NCI
	K. K. Sanford	Chief, In Vitro Carcino-genesis Section	LCMB	NCI
	C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*: Institut de Recherches Scientifiques sur le Cancer, France (R. Cassingena)

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Office of the Chief

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Objectives of this project are (1) to establish and define a cell culture transformation system for identification of carcinogenic agents and humans at high risk for cancer; (2) to develop human cell transformation systems, with particular emphasis on epithelial cells, in order to study host factors regulating cell transformation and the mechanisms of carcinogenesis by chemicals, viruses, hormones and x-irradiation; and (3) to isolate and characterize oncogenes from human tumors.

In line with these objectives, we have (1) established nontumorigenic human bronchial epithelial cell lines infected with simian virus-40 (SV40) or adenovirus-12 (Ad 12)-SV40 hybrid virus, or transfected via strontium phosphate coprecipita-tion with plasmid containing the SV40 early region genes; (2) demon-strated accelerated malignant conversion of human HBL-100 cells by the v-K-ras oncogene; (3) demonstrated the susceptibility of a human epidermal keratinocyte line to human herpesviruses; (4) demonstrated activation of the met oncogene in an early passage of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-transformed human osteosarcoma cells; (5) determined the frequency of active ras oncogenes in human bladder cancers associated with schistosomiasis; (6) demonstrated mutated H-ras oncogenes in human kidney tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. S. Rhim	Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. Arnstein	Veterinary Director	LCMB	NCI
K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI

Objectives:

1. To develop sensitive in vitro transformation assays to identify carcinogenic agents and humans at high risk for cancer.

2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation to elucidate mechanisms of cell transformation by carcinogenic agents and viruses.

3. To search for human oncogenes in human cancers.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients to detect activated cellular transforming genes in malignant tumors. A number of biochemical and molecular biological techniques, including gel electrophoresis, Southern blotting, immunoprecipitation and restriction endonuclease analysis are used to characterize the activated oncogenes. Gene cloning into phage and plasmid is used for characterization of oncogenes.

Major Findings:

Establishment of nontumorigenic human bronchial epithelial cell lines for studies of multistage bronchial carcinogenesis. Normal human bronchial epithelial cells were infected with SV40 virus or an Ad12-SV40 hybrid virus, or transfected via strontium phosphate coprecipitation with plasmids containing the SV40 early region genes. Colonies of morphologically altered cells were isolated and cultured; these cells had extended culture life spans compared to normal human bronchial epithelial cells. All cultures eventually underwent senescence, with the exception of one which appears to have unlimited proliferative potential. Colonies arising after viral infection were screened for virus production by cocultivation with Vero cells; only viral nonproducer cultures were analyzed further. The cells retained electron microscopic features of epithelial cells, and keratin and SV40 T antigen were

detected by indirect immunofluorescence. All of the cultures were aneuploid with karyotypic abnormalities characteristic of SV40-transformed cells. No tumors formed after subcutaneous injection of cells in nude mice. These cells should be useful for studies of multistage bronchial epithelial carcinogenesis.

Accelerated malignant conversion of human HBL-100 cells by the v-Ki-*ras* oncogene. The human epithelial HBL-100 cell line harbors SV40 genetic information and has an unlimited growth potential. Despite displaying properties characteristic of transformation since its early in vitro passages, it is capable of producing progressively growing tumors in nude mice only after long-term culture. This is a reproducible phenomenon and apparently not the consequence of a selection of preexisting malignant cells. Superinfection of early passage nontumorigenic HBL-100 cells with Kirsten murine sarcoma virus, which contains a Ki-*ras* oncogene having undergone multiple activating events, induces morphologic alterations and rapidly converts the cells to neoplastic cells, further supporting the hypothesis of multistep carcinogenesis. The HBL-100 cell line might be useful in defining the oncogenes representative of different families which are capable of complementing SV40 in this system.

Susceptibility of a human epidermal keratinocyte line to herpesviruses (HSV). Since human epidermal keratinocyte lines are sensitive to infection and transformation by retroviruses, we have examined their susceptibility to HSV. The human epidermal keratinocyte line (RHEK-1) was susceptible to HSV infection. However, the infectivity titers of HSV obtained in RHEK-1 cells were one to two logs lower than those obtained in Vero cells.

Activation of the *met* oncogene in an early passage of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-transformed human osteosarcoma (HOS) cells. A new cellular transforming gene, *met*, has been isolated by the NIH/3T3 transfection of DNA from a late passage (>150) MNNG-transformed cell line. The *met* gene is activated by gene rearrangement. We were interested in learning whether earlier passages of MNNG/HOS cells contained a similar cellular transforming gene. We detected and identified the met oncogene in an earlier passage (P-98) of MNNG/HOS cells. However, so far DNA from malignantly transformed 7,12-methylbenz[a]anthracene (DMBA)/HOS cells has been negative in our transfection assay, as reported by others.

Frequency of active *ras* oncogenes in human bladder cancers associated with schistosomiasis. The frequency of active *ras* oncogenes in human bladder cancers associated with schistosomiasis, the cause of which is suspected to be a chemical carcinogen(s) in urine, was examined. Of nine squamous cell carcinomas of the bladder, surgically obtained in Egypt, none scored as positive in the regular DNA transfection assay using NIH/3T3 cells as recipients. The restriction fragment length polymorphism assay at codon 12 of the H-*ras* gene confirmed the absence of an activating mutation at this site in all of them. Western blotting analysis of electrophoretic mobilities of the

ras p21 proteins, a method which can detect at least some of the point mutations within codons 12 and 61 of *ras* genes, suggested a point mutation within codon 61 in one out of the seven tumors analyzed. In contrast to the low frequency of detection of mutationally activated *ras* oncogenes, enhanced expression of the *ras* p21 proteins was demonstrated in four of them by this analysis. The carcinogenic process involved in the endemic bilharzial bladder cancers is thus not associated with detectable point mutations within *ras* genes at a higher frequency than those in nonbilharzial bladder cancers in Japan or the United States.

Activated H-ras oncogenes in human kidney tumors. Two H-*ras* oncogenes were detected by NIH/3T3 transfection assay out of 16 primary kidney tumors, 15 renal cell carcinomas (RCC) and one transitional cell carcinoma in 16 patients. Analysis of *ras* protein p21 suggested single point mutations within codon 12 and 61 in each case. The restriction endonuclease analysis of H-*ras* gene at codon 12 confirmed this in one of them, and the remaining 15 tumors did not have a mutation at this site. DNAs from the noncancerous portions of the kidney with codon 12-mutated tumor, but not leukocytes from the same patient, showed an abnormal resistance to the endonucleases MspI and HpaII, suggesting a presence of codon 12-mutated H-*ras* gene in the noncancerous cells. No amplification of *ras* genes was detected in the 16 tumors analyzed. In one of eight tumors from patients heterozygous for H-*ras*-related BamHI restriction fragments, one allele was lost in the tumor but not in the noncancerous portion of the same kidney. Although cytogenetic studies have previously suggested nonrandom involvement of c-*raf*-1 gene in RCC, no abnormality in the size nor amount of *raf* transcript was detected in the 15 RCCs. Our results thus indicated that the genetic lesions affecting *ras* genes do occur in human RCC, and probably serve as one of multisteps in the carcinogenic process.

Publications:

Dahlberg JE, Ablashi DV, Rhim JS, Fladger A, Salahuddin SZ. Analysis of the replication of a transforming primate herpesvirus, HVS, in human cells. Intervirology (In Press)

Fujita J, Kraus MH, Inoue H, Srivastava SK, Ebi Y, Kitamura T, Rhim JS. Activated H-*ras* oncogenes in human kidney tumors. Cancer Res (In Press)

Fujita J, Nakayama H, Inoue H, Rhim JS, El-Bolkainy MN, El-Aaser AA, Kitamura Y. Frequency of active *ras* oncogenes in human bladder cancers associated with schistosomiasis. Jap J Cancer Res 1987;78:915-20.

Kasid UN, Dritschilo A, Rhim JS. Human epidermal keratinocytes retain radiation resistance following *in vitro* immortalization and malignant transformation. Radiat Res 1987;111:565-71.

Reddel RR, Ke Y, Gerwin BI, McMenamin M, Lechner JF, Su RT, Brash DE, Park JB, Rhim JS, Harris CC. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus 12-SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 1988;48:1904-9.

Rhim JS. Viruses, oncogenes and cancer. Cancer Detec Prev 1988;11:139-149.

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Rhim JS, Park JB, Kawakami T. Techniques for establishing human epithelial cell cultures: sensitivity of cell lines for propagation of herpesviruses. J Virol Methods (In Press)

Saint-Ruf C, Nardeux P, Estrade S, Brouty-Boye D, Rhim JS, Cassingena R. Accelerated malignant conversion of human HBL-100 cells by the v-Ki-*ras* oncogene. Exp Cell Res (In Press)

Patents:

Reddel RR, Yang K, Rhim JS, Brash D, Su RT, Lechner JF, Gerwin BI, Harris CC. US Patent 7-114,508: Immortalized Human Cell Lines, March 24, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05062-10 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transforming Genes of Naturally Occurring and Chemically-induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Eva	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	D. Ron	Visiting Associate	LCMB	NCI
	G. Graziani	Visiting Fellow	LCMB	NCI
	J. H. Pierce	Microbiologist	LCMB	NCI
	L. Varesio	Visiting Scientist	LMI	NCI
	J. Ward	Chief, Tumor Path. Pathogen. Sec.	LCC	NCI
	W. McBride	Chief, Cellular Regulation Section	LB	NCI

COOPERATING UNITS *(if any)*
NIEHS, Res. Triangle Park, NC (M. Anderson); Dana-Farber Cancer Inst., Boston, MA (G. M. Cooper); Baylor College Med., Houston, TX (P. Overbeek); Georgetown U. Medical Sch., Washington, DC (S. Srivastava)

LAB/BRANCH
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SECTION
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INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cDNA clone of the dbl transforming gene mRNA was isolated and sequenced. Computer analysis of the predicted dbl protein indicated it to be highly hydrophilic with no hydrophobic domain characteristic of a membrane-spanning region or signal peptide. cDNA clones representing the human dbl proto-oncogene were also isolated. Nucleotide sequence analysis indicated that a stretch of 300 amino acids within the N-terminal half of proto-dbl showed structural similarity to the intermediate filament vimentin. Under the influence of the same strong promoter, both genes would readily transform NIH/3T3 cells, but the dbl oncogene cDNA exhibited higher transforming capability. The expression of the dbl oncogene in human normal tissues and childhood tumors of neuroectodermal origin was investigated. Proto-dbl RNA species were detected only in fetal brain and adrenal fluids as well as in adult testes and ovaries. Moreover, the dbl oncogene was found to be transcribed in Ewing's sarcoma as a single 5.3-kbπ mRNA species, while it was not present in two related categories of tumors, neuroblastoma and neuroepithelioma. Using peptide antisera raised against specific dbl peptides, the proto-oncogene product was identified as a 115-kDa protein, phosphorylated in serine and threonine, and localized in the crude membrane and soluble fraction of the cell.

DNAs from 14 of 33 mouse liver tumors induced by a single injection of N-nitrosodiethylamine (DEN) at 12-15 days of age were able to induce morphological transforma-tion of NIH/3T3 cells. Analysis of the transformants obtained revealed the presence of activating mutations in the H-ras gene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Eva	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
D. Ron	Visiting Associate	LCMB	NCI
G. Graziani	Visiting Fellow	LCMB	NCI
J. H. Pierce	Microbiologist	LCMB	NCI
L. Varesio	Visiting Scientist	LMI	NCI
J. Ward	Chief, Tumor Pathology & Pathogenesis Section	LCC	NCI
W. McBride	Chief, Cellular Regulation Section	LB	NCI

Objectives:

Studies are directed to identify transforming genes associated with specific human hematopoietic malignancies. Isolation and characterization of these genes are pursued in order to determine their mechanisms of activation and their specific involvement in the human malginant process.

Methods Employed:

Standard and developmental techniques in cell biology, biochemistry and recombinant DNA are used in these studies.

Major Findings:

The *dbl* oncogene cDNA was isolated and completely sequenced. Computer analysis of the sequence revealed a long open reading frame that encodes a hybrid protein from which at least the first 50 amino acids derive from a complete exon of a different locus. The predicted *dbl* product was found to be markedly hydrophilic in its overall characteristics. Further analysis revealed no hydrophobic domains indicative of a membrane-spanning region nor was there a presecretory signal peptide. Two putative sites for serine phosphorylation by a cyclic AMP-dependent protein kinase were identified, in agreement with previous findings that the *dbl* p66 product is a phosphoprotein with phosphorylation occurring specifically at serine residues.

cDNA clones representing the human *dbl* proto-oncogene transcript were isolated and sequenced. Comparison with the *dbl* oncogene cDNA revealed that the oncogene rearrangement occurred in proto-*dbl* codon 497 and that all proto-*dbl* sequences upstream of this site were deleted from the *dbl* oncogene. Proto-*dbl* and *dbl* oncogene cDNAs were cloned in a eukaryotic expression vector which utilizes transcriptional regulatory sequences of the Moloney murine leukemia virus long terminal repeats. Foci were induced by both DNA constructs but the focus-forming activity of the oncogene was found to be 20-50 times higher than

its normal counterpart. Thus, while overexpression is sufficient to activate proto-*dbl* as a transforming gene, gene structural alterations affecting its 5' coding sequence can significantly enhance its transforming potential.

We surveyed a wide variety of human normal tissues for evidence of *dbl* expression. Proto-*dbl* mRNA was detected as a 5-kb transcript only in fetal brain, fetal adrenal glands and adult testes and ovaries. In the course of a survey study attempting to establish the pattern of expression of the *dbl* oncogene in several human tumors, we observed that Ewing's sarcoma consistently expresses a *dbl*-related transcript whose size was comparable to that of the *dbl* transcript present in normal tissues. Attempts to detect expression of the *dbl* gene in two other types of tumors of neuroectodermal origin (neuroepithelioma and neuroblastoma) have been unsuccessful, revealing a preferential expression of these sequences in Ewing's sarcomas.

Peptide antisera were prepared against specific peptides from the predicted proto-*dbl* protein. The proto-*dbl* product was identified with these peptide antisera in COS-1 and NIH/3T3 cells transfected with the biologically active proto-*dbl* cDNA construct as a 115-kDa protein. The p115 protein was found to be a cytoplasmic protein which is distributed between both the cytosolic and the membrane fractions. As previously observed for the oncogene product p66, the p115 is also primarily phosphorylated on serine residues. Unlike p66, a low degree of threonine phosphorylation was detected on p115. The p115 protein is associated with the triton-insoluble matrix and, further, is not an integral membrane protein, in agreement with the analysis of the predicted amino acid sequence. Examination of the stability of both proteins revealed that p115 had a significantly shorter half-life. This suggests that the truncation/rearrangement that generated the *dbl* oncogene may account for the increased stability of its translational product and possibly for its higher transforming potential.

DNA from N-nitrosodiethylamine (DEN)-induced liver tumors from $B6C3F_1$ mice and Fischer 344 rat liver tumors were examined for the presence of activated proto-oncogenes. Activated H-*ras* proto-oncogenes were detected, by transfection of NIH/3T3 cells, in DNAs from 14 of 33 of the mouse liver tumors induced by a single dose of DEN given prior to weaning. DNA from only one of the 28 DEN-induced rat liver tumors could transform NIH/3T3 cells. Characterization of the activating mutations in the activated H-*ras* gene of DEN-induced $B6C3F_1$ mouse liver tumors showed that they each reside in the first or second base of the 61st codon of the H-*ras* gene.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05063-10 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on HBLV, EBV and HIV

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. V Ablashi	Microbiologist	LCMB	NCI
Others:	S. Z. Salahuddin	Expert	LTCB	NCI
	R. C. Gallo	Chief	LTCB	NCI
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COOPERATING UNITS *(if any)*
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human B-lymphotropic (human herpesvirus-6) virus (HBLV), isolated from acquired immunodeficiency syndrome (AIDS) patients and patients with other malignancies and lymphopro-liferative disorders, known to possess B-cell surface markers, was also found to infect T-cells obtained from peripheral blood, bone marrow, spleen, thymus and tonsils. HBLV could also replicate and produce large quantities of virus or viral antigens in established B-, T- and megakaryocyte cell lines. Coincident infection by human immunodeficiency virus (HIV)-1 and HBLV of T-cells possessing T4 receptors showed enhanced cytopathic effects and enhanced reverse transcriptase activity of HIV.. Both viruses could be found in the same T4 cell, suggesting that interaction between HIV-1 and HBLV could enhance impairment of immune function by accelerated destruction of target cells. These findings were supported by higher HBLV antibody in HIV-1-positive asymptomatic individuals (AIDS-related complex [ARC], persistent generalized lymphopathy [PGL] and AIDS) and the presence of HIV-1 DNA in about 80% of peripheral lymphocytes from AIDS patients. We also found that HBLV could only infect Epstein-Barr virus (EBV)-genome-positive B-cell lines or B-cell lines converted to EBV positivity, suggesting that EBV induces a receptor common for HIV and HBLV. HBLV and EBV antibody titers in African Burkitt's lymphoma (BL) sera were elevated; seven of ten BL tumors contained EBV and HBLV DNA. This suggests that HBLV has a role as a cofactor in some B-cell tumors. HBLV DNA was also detected in two Sjogren's B-cell tumors and a large B-cell follicular lymphoma. These findings are compatible with the implication of HBLV in some malignant, lymphoproliferative and immunosuppressive disorders.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. V. Ablashi	Microbiologist	LCMB	NCI
S. Z. Salahuddin	Expert	LTCB	NCI
R. C. Gallo	Chief	LTCB	NCI
S. Joseph	Chemist	LTCB	NCI
F. Wong-Staal	Chief, Molecular Genetics of Hematopoietic Cells Section	LTCB	NCI

Objectives:

1. To determine the role of HBLV (isolated by us in 1986) in lymphoproliferative disorders and malignant diseases.

2. To further characterize HBLV biologically, immunologically and by molecular analysis in order to develop diagnostic and prognostic parameters.

3. To study the in vitro interaction of HBLV, EBV and HIV in order to understand their roles in malignant disease and immunosuppression.

Methods Employed:

Primary cell cultures were established from peripheral blood lymphocytes and tumor tissues from AIDS and other patients with tumors were used for virus isolation and infection with HIV-1, HBLV and EBV. Continuous B- and T-cell human lines were also used for propagation of EBV, HIV, and other viruses. Human cord blood mononuclear cells were used for infecting viral stocks and for use in immunologic assays. Monoclonal and polyclonal antibodies to HIV and human herpesviruses were used in immunologic assays. Biochemical and molecular studies were performed for characterization of virus isolates and detection of viral genomes.

Major Findings:

1. HBLV-infected cells from fresh lymphocytes of peripheral blood, thymus, spleen and bone marrow expressed receptors for CD_7, CD_5, CD_2 and CD_4, and CD_8 to a lesser extent, as detected by monoclonal antibody and radioimmunoprecipitation. Moreover, HBLV could also infect B-, T-, megakaryocyte and glioblastoma cell lines, suggesting an expanded tropism. Earlier difficulties in producing large amounts of HBLV for biologic, molecular and immunologic investigations were recently overcome, since large amounts of virus antigens could be obtained by propagating the virus in an immature T-cell line (HSB_2), a mature T-cell line (JM2.7) and

an EBV-genome-positive, nonproducer B-cell line (ET-62). HSB_2, JM2.7 and other T-cell lines did not contain EBV, cytomegalovirus (CMV), *Herpesvirus saimiri* (HSV), varicella zoster virus (VZV) or other human retroviruses. Thus they can be used for testing antibody to HBLV and for other molecular studies.

2. Since HBLV can infect CD_4 cells (>60%), we examined the effect of HIV and HBLV on CD_4 cells. Coincident infection of a CD_4-positive T-cell line (6D5) by HIV-1 and HBLV showed enhanced cell degeneration and an increase in reverse transcriptase (RT) to HIV-1 expression of HIV-1P19 and P24 antigens. Moreover, peripheral blood mononuclear cells from three AIDS patients expressing low levels of HIV-1 RT activity and antigens after infection with HBLV showed a two- to fivefold increase in expression of HIV-1. In addition, 66% of HIV antibody-positive symptomatic AIDS patients contained higher IgG antibody titers to HBLV virus capsid antigen (VCA) as detected by the indirect immuno-fluorescence assay (IFA). Approximately 80% of peripheral lymphocytes from AIDS patients contained HBLV DNA. These data seem to indicate that HBLV, in conjunction with HIV-1, may further impair immune function by killing target cells in HIV-1-infected people. The effect of both HBLV and HIV-1 infections in the same patient merits extensive analysis.

3. HBLV failed to infect EBV-genome-negative cell lines (Bjab, Louks, Ramos). However, upon conversion of such cell lines to EBV positivity, these cell lines became infectable with HBLV. Our previous findings also showed that such cell lines could only be infected with HIV-1 when they became EBV-genome-positive. This suggests that a receptor is being induced by infection with EBV which is common to both HBLV and HIV-1 infection. The nature of this receptor is still under investigation. A high prevalence of HBLV antibody (87%) was found in 15 African B-cell lymphomas (Burkitt's lymphoma) by IFA. These sera also contained elevated antibody titers to both EBV-VCA and HBLV-VCA ($\geq$1:640). Ten BL biopsies, upon examination by in situ technique using EBV-Bam W fragment and clone ZVH 14 of HBLV (9.0-kb fragment), detected EBV and HBLV DNA in seven of the biopsies. However, the EBV DNA was always in excess of HBLV DNA. Thus it is apparent that HBLV could be a possible cofactor in BL. This observation requires further investigation.

4. Elevated antibody to HBLV VCA was detected in sera from Hodgkin's disease (>1:1280), acute lymphocytic leukemia (>1:320) and other non-Hodgkin's lymphomas (>1:640). HBLV antibody was also prevalent in sera obtained from Sjogren's syndrome patients (55%). Tissues derived from two Sjogren's B-cell lymphomas contained HBLV DNA as detected by Southern blot hybridization. No EBV DNA was detected. One out of four follicular large B-cell lymphomas was found to contain HBLV DNA and no EBV or CMV DNA was detected in this tumor. Ten AIDS-associated Kaposi's sarcoma DNAs lacked HBLV DNA.

Although the role of HBLV in these tumors is unclear, infection of some cells with HBLV induces a blast-like appearance in the infected cells and these cells appear to have a selective growth advantage in culture prior to the release of virus during the lytic phase of infection. If the lytic phase is prevented, a direct growth-promoting effect could occur, thereby initiating transformation to the malignant phenotype. The high seroprevalence and antibody titers noted in certain malignant, lymphoproliferative and immunosuppressive disorders does not indicate whether they are due to recent infection, latent infection with reactivation, reexposure to HBLV or a heightened immunologic response associated with underlying disease. The precise role of HBLV in these diseases requires additional laboratory and clinical investigation, including prospective epidemiological studies. Moreover, with the availability of large quantities of HBLV, more improved and specific reagents will be developed for further characterization of HBLV and for identification of proteins which are disease associated.

These findings further suggest that HBLV has a wide cell tropism and thus may be associated with other malignancies or disorders in addition to a possibly important role in AIDS, certain B-cell malignancies and autoimmune diseases.

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Patents:

Salahuddin Z, Ablashi DV, Saxinger C, Gallo RC. US Patent (Pending): HBLV Testing.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05164-08 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Oncogenes, Growth Factor Pathways and Hematopoietic Cell Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. H. Pierce	Microbiologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. P. Di Fiore	Visiting Scientist	LCMB	NCI
	M. Ruggiero	Visiting Scientist	LCMB	NCI
	O. Segatto	Visting Fellow	LCMB	NCI
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	T. Fleming	Guest Researcher	LCMB	NCI
	W. Davidson	Visiting Associate	LG	NCI

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SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

An epidermal growth factor receptor (EGFR) expression vector was introduced into an interleukin-3 (IL-3)-dependent myeloid cell line, 32D, which is devoid of EGF receptors. EGFR expression allowed these cells to utilize EGF for signal transduction and continued proliferation. While 32D-EGFR cells remained nontumorigenic and factor-dependent on either IL-3 or EGF, transfection of v-erbB abrogated IL-3 dependence and induced tumorigenicity. These results establish that components of the EGF-signaling pathway necessary for transmitting intracellular signals must be available in these cells. The normal human EGFR expression vector was also transfected into NIH/3T3 cells to determine whether this growth factor receptor could induce transformation when overexpressed in NIH/3T3 cells. EGFR overexpression was shown to induce transformed foci or colonies in soft agar only after the addition of exogenous EGF. These findings indicate that overexpression of EGFR in human malignancies would confer a strong selective growth advantage to these cells in the presence of the ligand (EGF or transforming growth factor [TGF]-alpha).

Cell lines transformed by H-ras or myc/raf-containing retroviruses were found to coexpress antigens usually restricted to the B-cell or myeloid pathways of differentiation. Detailed analyses of these lines suggested that the initial transforming event giving rise to these lines occurred in a precursor common to the B-cell and myeloid lineages. The availability of cell lines with both myeloid and B-cell differentiation potentials provides a unique opportunity to explore the molecular and biochemical events that define irrevocable commitment to these distinct hematopoietic lineages.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. H. Pierce	Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI
M. Ruggiero	Visiting Scientist	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
M. Kraus	Visiting Scientist	LCMB	NCI
T. Fleming	Guest Researcher	LCMB	NCI
W. Davidson	Visiting Associate	LG	NCI

Objectives:

(a) To determine mechanisms by which oncogenes abrogate growth factor dependence and induce transformation of factor-dependent hematopoietic cells; (b) to determine the role of growth factors and growth factor receptor expression on the signal transduction pathway controlling either growth or differentiation in cells of connective tissue or hematopoietic origin; and (c) investigate the diversity of target cells for neoplastic transformation by various oncogene-containing retroviruses to provide insights into pathways of transformation and, in particular, the relationship of the differentiated state of the cell to its susceptibility to oncogene action.

Methods Employed:

Standard hematopoietic culture techniques, including an in vitro hematopoietic colony-forming assay developed to detect transformation of hematopoietic cells by retroviruses, use of feeder layers to establish continuous cell lines, and cloning of established cell lines in soft agar. Other procedures included generation of growth factors and retrovirus infection of factor-dependent cell lines.

Identification of hematopoietic phenotype of retrovirus-transformed hematopoietic cells was performed utilizing histochemical staining, immunofluorescence techniques, radioimmunoassays and enzymatic assays.

DNA transfection by the calcium phosphate and electroporation methods were utilized to identify growth factor receptor-related oncogenes. Analysis of transformants was performed by Southern and Northern hybridization analysis, radioimmunoprecipitation and growth factor-binding assays.

Major Findings:

We sought to determine the effects of introduction of the epidermal growth factor receptor (EGFR) or v-*erb*B genes into a nontumorigenic hematopoietic cell line, designated 32D, which lacks EGF receptors and is normally dependent

on interleukin-3 (IL-3) for proliferation and survival. Expression vectors containing either the complete human EGF receptor (LTR/EGFR) or the avian v-*erb*B (LTR-v-*erb*B) were introduced into 32D cells by electroporation. The parental 32D line was unresponsive to the addition of fetal calf serum alone or in the presence of EGF but showed a marked increase in [^{3}H]-thymidine incorporation in response to IL-3. In contrast, the 32D-EGFR line responded dramatically to addition of EGF. Dose-response experiments revealed a half-maximal stimulation by EGF at approximately 25 pg/ml. The presence of functional EGF receptors did not impair the responsiveness of the 32D-EGFR cells to IL-3, as evidenced by their ability to incorporate [^{3}H]-thymidine at levels equivalent to those of the parental 32D line after treatment with IL-3. In contrast, the 32D-v-*erb*B transfectant required only serum to elicit a maximal proliferative response. The addition of exogenous IL-3 or EGF did not significantly enhance its growth. Therefore, it appeared that expression of v-*erb*B, the oncogenic counterpart of the avian EGF receptor, was able to abrogate the IL-3 requirement of these cells.

We next investigated the effects of EGF receptor or v-*erb*B expression by 32D cells on the ability of the cells to proliferate indefinitely. The parental 32D line remained viable only in the presence of IL-3 and serum. When 32D-EGFR cells were transferred to medium supplemented with EGF and serum, cell growth remained slow during the first few weeks after switching from IL-3 to EGF supplementation. After this adaptation period, 32D-EGFR cells could be cultured continuously in EGF-containing medium but remained EGF-dependent. In contrast, when the 32D-v-*erb*B transfectant was transferred to medium containing only serum, the cells continued to proliferate indefinitely. These results, as well as the ability of 32D-v-*erb*B cells to readily form colonies in soft agar in the absence of IL-3, confirmed that transfection of v-*erb*B into 32D cells completely abrogated their IL-3 dependence. To analyze the malignant potential of 32D and transfected lines, each was tested in vivo. Neither the parental 32D or 32D-EGFR lines yielded any detectable tumors. In contrast, 32D-v-*erb*B cells produced large tumors. These results confirmed that a bypass of the pathway of IL-3-induced proliferation by v-*erb*B was associated with conversion of the cells to the malignant phenotype.

IL-3-dependent 32D cells can be induced to terminal granulocytic and monocytic differentiation when IL-3 is removed and granulocyte colony-stimulating factor (G-CSF) is added to the culture. When 32D-EGFR cells were propagated in EGF, there was an increase in the presence of histochemical markers for both monocytes and granulocytes. These results suggest that while IL-3 induced a proliferative signal in these cells and G-CSF primarily triggers a differentiation program, EGF appeared to modulate proliferation coupled with a shift in the differentiation state of the cells toward more mature myeloid phenotypes.

One signaling pathway implicated in growth control involves the hydrolysis of inositol lipids. In an attempt to determine whether IL-3 and EGF induced similar or different intracellular responses, we measured phosphoinositide turnover in 32D-EGFR cells after treatment with EGF or IL-3. The production

of [^{32}P]-phosphatidic acid in response to IL-3 was not increased over control unstimulated values. In contrast, EGF induced phosphatidic acid formation to a level threefold that of the control. Thus, the effects of IL-3 and EGF on phosphoinositide turnover could be readily distinguished in 32D-EGFR cells. In summary, these results show that a naive hematopoietic cell expresses all of the intracellular components of the EGF signaling pathway necessary to evoke a mitogenic response and continued proliferation.

The epidermal growth factor receptor (EGFR) gene is frequently amplified and/or overexpressed in human malignancies. Introduction of the eukaryotic vector containing human EGFR cDNA led to reconstitution of functional EGF receptors in NR6 mutant cells, which are normally devoid of this receptor. Transfection of NIH/3T3 cells resulted in no significant alterations in growth properties. However, EGF addition led to the formation of densely growing transformed foci in liquid culture and colonies in semisolid medium. NIH/3T3-EGFR clonal lines, which expressed the EGFR at 500- to 1000-fold levels over control NIH/3T3 cells, demonstrated a marked increase in DNA synthesis in response to EGF. Thus EGF receptor overexpression appears to amplify normal EGF signal transduction. High levels of EGFR expression, which conferred a transformed phenotype to NIH/3T3 cells in the presence of ligand, were demonstrated in representative human tumor cell lines that contained amplified copies of the EGFR gene. Our studies provide a mechanistic basis for EGFR amplification in human malignancies. Levels of EGFR overexpression that conferred a strong selective growth advantage to NIH/3T3 cells in the presence of the ligand were demonstrated in representative human tumor cell lines that contained amplified copies of an apparently normal EGFR gene. By analogy with the NIH/3T3 model system, in vivo conditions in which such tumor cells might be exposed to EGF or TGFα, which also binds the EGF receptor, should significantly enhance their growth. TGFα is known to be expressed by some tumor cells. While expression of high levels of TGFα does not confer the malignant phenotype to NIH/3T3 cells, preliminary evidence reveals that the expression of TGFα in NIH/3T3 cells overexpressing EGFR leads to a malignant phenotype.

Although the stages of differentiation within the B-cell lineage are increasingly well understood at both the phenotypic and molecular levels, little is known about the timing and mechanisms signaling irrevocable commitment to this lineage or those controlling the orderly progression of cells from one stage of differentiation to the next. The study of regulatory genes in normal B cell differentiation is limited by difficulties in identifying, isolating and propagating early precursors. As an alternative approach to these questions, we have chosen to study spontaneously or virally transformed cell lines. HAFTL-1, a cell line with the hallmarks of an early B-cell progenitor, can be induced by treatment with lipopolysaccharide (LPS) to differentiate with high frequency into Ly-1^{+}, Ia^{+}, ThB$^{+/-}$ pre-B-cell-like cells or, with low frequency, into Ly-1^{+} monocytes and macrophages. The mature morphology, phenotype and functional characteristics of the HAFTL-1

macrophages, as well as the presence of intermediate monocytic cells, suggests that well-coordinated, lineage-specific programs of differentiation are activated in the progenitor cells. Both HAFTL-1 and the previously described HAFTL-3 progenitor and macrophage lines, therefore, offer a unique opportunity to study genes that regulate myeloid differentiation in well-defined clonal systems.

In a separate study, murine bone marrow cells infected with replication-defective retroviruses containing v-*raf* alone or v-*myc* alone yielded pre-B-cell lines at low frequencies, whereas a retroviral construct containing v-*raf* and v-*myc* transformed cells with more than 10-fold higher efficiency. The *raf*/*myc* transformants included clonally related populations of mature B-cells and mature macrophages, suggesting a common lineage progenitor as the target for transformation. The geneology of these transformants demonstrated that mature myeloid cells were derived from cells with apparent B lineage commitment including functional Ig gene rearrangements. This system should facilitate studies of developmental relationships in hematopoietic differentiation and analyses of *onc* gene interactions in lineage determination. In addition, the results of the studies and a previous report allow us to predict the existence of a normal bipotential progenitor cell capable of giving rise to both Ly-1$^+$ B cells and Ly-1$^+$ macrophages.

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King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: frequent overexpression in human mammary adenocarcinomas and induction of transformation *in vitro*. In: Proceedings of UCLA Symposium (In Press)

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Malone DG, Pierce JH, Falco JP, Metcalfe DD. Production of granulocyte-macrophage colony-stimulating factor by primary cultures of unstimulated rat microvascular endothelial cells. Blood 1988;71:684-9.

Pierce JH, Ruggiero M, Fleming TP, Di Fiore PP, Greenberger JS, Schlessinger J, Rovera G, Aaronson SA. Signal transduction through the EGF receptor transfected into interleukin-3-dependent hematopoietic cells. Science 1988;239:628-31.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05167-08 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Transformation Induced by the sis Gene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	T. Miki	Visiting Scientist	LCMB	NCI
	N. Giese	IRTA Fellow	LCMB	NCI
	T. Fleming	Guest Researcher	LCMB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our previous studies have demonstrated the importance of human sis/platelet-derived growth factor (PDGF)-2 gene deregulation in its activation as an oncogene in cells responsive to PDGF stimulation. Current studies have focused on the structure, regulation and function of this gene. We have shown that human tumor cells arising from PDGF-responsive cell types express sis/PDGF-2 mRNA and mitogenically active sis/PDGF-2 homodimers. Utilizing cDNA cloning, S1 nuclease mapping, and primer extension techniques, the normal human sis/PDGF-2 transcriptional unit has been defined. These studies also suggested the presence of transcriptional and translational regulatory signals within the sis/PDGF-2 locus, and have provided an approach for elucidating mechanisms by which this gene is controlled.

Knowledge that the v-sis oncogene encodes a PDGF-related product whose transforming activity requires functional interaction with the PDGF receptor has suggested the importance of identifying the active site of the v-sis translational product. Site-directed mutagenesis of v-sis has localized an 89-codon stretch as its minimum transforming region and has shown a requirement for each of eight cysteine codons within the region for proper folding of the v-sis gene product. These studies have also predicted three testable models for the active conformation of this protein and represent an important step in identifying the receptor binding domain of this oncogenic growth factor.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. C. Robbins	Chief, Molecular Gsenetics Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI
N. Giese	IRTA Fellow	LCMB	NCI
T. Fleming	Guest Researcher	LCMB	NCI

Objectives:

1. To define the platelet-derived growth factor (PDGF) receptor binding domain of the simian sarcoma virus (SSV)-transforming protein, $p28^{sis}$.

2. To assess the role of the *sis*-proto-oncogene in human neoplasia.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of gene products; in vitro synthesis and immunoprecipitation; peptide synthesis; antibody production; fractionation of cellular components and protein purification to analyze gene products; molecular hybridization techniques to analyze genes; recombinant DNA techniques for the purification and amplification of genes; analysis of genetic structure using restriction endonuclease mapping, nucleotide sequencing, S1 nuclease mapping, primer extension, cDNA cloning, site-directed mutagenesis and electron microscopy techniques; DNA transfection and construction of virus mutants for analysis of transforming activity.

Major Findings:

The v-*sis* oncogene encodes a protein structurally and functionally related to human PDGF. We have shown that the primary translational product of the human *sis* proto-oncogene is a 26-kd protein, $p26^{c\text{-}sis}$. This product is processed to yield a disulfide-linked homodimer, $p52^{c\text{-}sis}$, which is further processed to a 35,000-dalton dimer, $p35^{c\text{-}sis}$. Like the v-*sis* gene product, the PDGF-2 precursor undergoes N-linked glycosylation, implying its processing through the endoplasmic reticulum. The PDGF-2 product was shown to possess functional properties of PDGF. Although lysates of control COS-1 cells lacked mitogenic activity, lysates of COS-1 cells transfected with a c-*sis*/PDGF-2 expression vector specifically stimulated DNA synthesis of quiescent fibroblasts. Moreover, this activity was completely inhibitable by PDGF antibody. Identical forms of the *sis*/PDGF-2 product were identified in human tumor

cells that expressed c-*sis*/PDGF-2 transcripts. These proteins were shown to be specifically associated with the membrane component of the tumor cells and were not detectably secreted into the culture medium. These findings support the concept that expression of the *sis*/PDGF-2 product in human cells responsive to its proliferative actions can be an important step in the processes leading to malignancy.

The v-*sis* oncogene encodes a PDGF-related product whose transforming activity is mediated by its functional interaction with the PDGF receptor. PDGF, as well as processed forms of the v-*sis* gene product, are disulfide-linked dimers with eight conserved cysteine residues in the minimum region necessary for biologic activity. Site-directed mutagenesis of the v-*sis* gene revealed that each conserved cysteine residue was required directly or indirectly for disulfide-linked dimer formation. However, substitutions of serine for cysteine codons at any of four positions had no detrimental effect on transforming activity of the encoded v-*sis* protein. These results establish that interchain disulfide bonds are not essential for this protein to act as a functional ligand for the PDGF receptor. The remaining four substitutions of serine for cysteine each inactivated transforming function of the molecule. In each case, this was associated with loss of a conformation shown to involve intramolecular disulfide bonds. Thus, our findings predict specific models for the role of individual cysteine residues in determining the structure of the *sis*/PDGF-2 molecule critical to its actions as a growth factor with transforming properties.

The structure of the normal human c-*sis*/PDGF-2 transcript was determined by a combination of cDNA cloning, nuclease S1 mapping and primer extension. Nucleotide sequence analysis revealed that the 3373-nucleotide *sis*/PDGF-2 mRNA contained only a 723-base-pair (bp) coding sequence for the PDGF-2 precursor polypeptide. The coding sequence was flanked by long 5' (1022 bp) and 3' (1625 bp) untranslated regions. The 5' noncoding region, as well as upstream flanking genomic sequences, contained clusters of specific short repeat sequences. A consensus transcriptional promoter sequence, TATAAA, was identified 24 bp upstream of the mRNA start site, and an enhancer-like "TG element" was detected about 180 bp downstream from the site of poly-adenylation. These findings identify putative regulatory elements of the *sis*/PDGF-2 gene.

Publications:

Aaronson SA, Igarashi H, Rao CD, Finzi E, Fleming TP, Segatto O, Robbins KC. Role of genes for normal growth factors in human malignancy. In: Aaronson SA, Bishop JM, Sugimura T, Terada M, Toyoshima K, Vogt PI, eds.: Oncogenes and cancer. Tokyo: Japan Scientific Societies Press, 1987;95-108.

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Rao CD, Pech M, Robbins KC, Aaronson SA. The 5' untranslated sequence of the c-*sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. Mol Cell Biol 1988;8:284-92.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05366-05 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Role of Proto-oncogenes Encoding Growth Factor Receptors in Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Matthias Kraus	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	W. Issing	Guest Researcher	LCMB	NCI
	L. Marazzi	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*
Department of Surgery, Duke University, Durham, NC (D. Iglehart)

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A second member, erbB-2, in the erbB proto-oncogene family, had been identified based on gene amplification in a mammary carcinoma and close structural homology to the epidermal growth factor (EGF) receptor gene. Subsequently, overexpression in the presence or absence of gene amplification has been determined as the mechanism activating erbB-2 in human mammary tumor cell lines. These observations were paralleled by the finding that in vitro overexpression of the human erbB-2 coding sequence at protein levels similar to those observed in human mammary tumor cells was capable of conferring the transformed phenotype onto NIH/3T3 cells. Analysis of erbB-2 and EGF receptor protein levels in mammary tumor tissues revealed overexpression of erbB-2 in 45% (24/53) and EGF receptor of erbB-2 in 9% (4/47) of the patients analyzed. Gene amplification of erbB-2 and EGF receptor was associated with high protein expression levels in 19% and 4%, respectively, of the patients analyzed. Concordance of increased receptor gene expression in primary and metastatic lesions, combined with the observation that such alterations are detectable in mammary tumors as early as stages I and II, indicate that proto-oncogene activation resulting in the overexpression of growth factor receptor molecules can occur at a relatively early stage in the development of human mammary neoplasia. ErbB-3, a novel member of the erbB proto-oncogene family, was identified in normal genomic human DNA using v-erbB as a probe under reduced stringency hybridization conditions. Characterization of genomic exon sequences revealed structural homology to members of the tyrosine kinase family. The predicted amino acid sequence of these genomic exons indicated the closer homology of this region to the tyrosine kinase domains of the EGF receptor and erbB-2 protein than to any other known tyrosine kinase protein. Northern blot analysis using an exon-containing erbB-3 probe identified a 6.5-kb gene-specific mRNA in normal and neoplastic cells of various tissue origin.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. H. Kraus	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
W. Issing	Guest Researcher	LCMB	NCI
L. Marazzi	Visiting Fellow	LCMB	NCI

Objectives:

1. Identification and characterization of novel proto-oncogenes encoding growth factor receptors based on nucleotide sequence and structural homology to known growth factor receptor genes.

2. Identification of the normal protein and its functions in normal cell growth and differentiation.

3. Investigation of mechanisms activating such genes in transformed cells and role of activated versions of growth factor receptor encoding proto-oncogenes in human neoplasia.

Methods Employed:

Southern blot, Northern blot, and dot blot analyses; recombinant DNA technology including genomic and cDNA cloning; nucleotide sequence analysis; generation of polyclonal peptide antisera and gene product analysis by immunoblotting and immunoprecipitation.

Major Findings:

1. *Erb*B-2 or EGF receptor protein is overexpressed at high frequency in human mammary tumor tissues. The highest expression levels are associated with gene amplification. Intermediate receptor protein overexpression occurs in the absence of gene amplification.

2. Detection of such alterations in early stages of human mammary cancer and concordance of receptor gene overexpression in primary and metastatic lesions derived from the same patient indicate that such alterations affecting receptor-like molecules of the *erb*B proto-oncogene family are likely being selected for during tumor growth rather than representing an incidental consequence of tumorigenesis.

3. Using v-*erb*B as a probe under reduced hybridization stringency conditions, a novel member of the *erb*B proto-oncogene family, *erb*B-3, was identified and partially isolated from normal genomic human DNA.

The predicted amino acid sequence of three contiguous exons revealed the most extensive sequence homology to the EGF receptor and *erb*B-2 protein when aligned with tyrosine kinase family members. Identification of a 6.5-kb gene-specific mRNA in a variety of human tissues indicated that *erb*B-3 represents a novel functional gene in the *erb*B proto-oncogene family.

Publications:

King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: Frequent overexpression in human mammary adenocarcinomas and induction of transformation in vitro. In: Proceedings of UCLA Symposium (In Press)

Kraus MH, Di Fiore PP, Pierce JH, Aaronson SA. Different mechanisms are responsible for oncogene activation in human mammary neoplasia. In: Lippman ME, Dickson B, eds. Breast cancer: cellular and molecular biology. Boston: Martinus Nijhoff Publ (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05457-04 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Growth Factor Receptors in Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. P. Di Fiore	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. H. Pierce	Microbiologist	LCMB	NCI
	O. Segatto	Visiting Fellow	LCMB	NCI
	E. Di Marco	Visiting Fellow	LCMB	NCI
	F. Lonardo	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*
Rorer Biotechnology, Inc., King of Prussia, PA (J. Schlessinger)

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The mechanism of activation of the epidermal growth factor receptor (EGFR) is being studied. A eukaroytic expression vector for the EGFR cDNA (long terminal repeat [LTR]/EGFR) has been engineered to direct the synthesis of this cDNA under Moloney murine leukemia virus (Mo-MLV) LTR transcriptional control. Upon introduction of this vector into NIH/3T3 cells, the overexpressed EGFR was able to confer a conditional (EGF-dependent) transformed phenotype to the recipient cells. The high levels of EGFR necessary to confer this phenotype to NIH/3T3 cells were found in human tumor cell lines displaying amplification and overexpression of the EGFR gene.

The LTR/EGFR vector was introduced into a naive cell line, the hematopoietic progenitor line 32D. In this cell, EGFR was able to confer responsiveness to EGF, which can now substitute as a mitogenic stimulus for interleukin-3 (IL-3), the physiological growth stimulus for 32D. The mitogenic dose-response analysis of 32D EGFR to epidermal growth factor (EGF) and the analysis of pathway activation (phosphatidyl-inositol [PI] turnover) by interleukin (IL-3) and EGF in these cells demonstrate that EGF is not likely acting through the IL-3 pathway but rather on an EGF-specific signaling pathway.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. P. Di Fiore	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. H. Pierce	Microbiologist	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
E. Di Marco	Visiting Fellow	LCMB	NCI
F. Lonardo	Visiting Fellow	LCMB	NCI

Objectives:

1. To study the mechanisms of oncogenic activation of genes which normally encode for growth factor receptors. This will lead to a better understanding of how chronically activated mitogenic signals can convert cells from the normal to malignant state.

2. To study how the above "activated" genes alter the growth properties and the differentiated program of certain cell cytotypes, like hematopoietic and epithelial cells.

Methods Employed:

A eukaryotic expression vector for the EGFR cDNA was engineered using a pSV2 gpt-based vector molecule in which a MLV genome was cloned. This was used to clone the EGFR cDNA under the transcriptional control of murine LTRs. This vector (MLV/LTR) was introduced into NIH/3T3 murine fibroblasts by the transfection method and into 32D by electroporation.

Analysis of the EGFR product has been conducted by either immunoprecipitation of metabolically labeled extracts with a specific antipeptide antibody directed against the EGFR or by ^{125}I-EGF binding to intact cells.

Major Findings:

1. The EGFR is an oncogene when overexpressed in NIH/3T3 cells. It confers a conditional transformed phenotype which is dependent on the addition of EGF to the culture medium. When NIH/3T3 cells overexpressing EGFR were assayed for their mitogenic response to EGF, they showed a 100- 110-fold incorporation over background, compared with the three- to four-fold increase in normal NIH/3T3 cells. This suggests that the mechanism of transformation by EGFR is through the amplification of the mitogenic signal normally delivered by EGF. When the levels of overexpression of the EGFR protein was assayed in human tumor cell lines overexpressing the EGFR, they were found comparable to the levels at which the same protein is able to induce transformation of NIH/3T3. Thus our study

established a mechanistic basis for growth factor-receptor gene amplification as repesenting a causal driving force in the evolution of a tumor, rather than being an incidental consequence of tumorigenesis.

2. The expression of EGFR into a naive cell, the hematopoietic cell line 32D, confers EGFR responsiveness to the cell. The half maximal stimulation of DNA synthesis in 32D EGFR cells was achieved with EGF concentration as low as 25 pg. This result indicates an exquisite sensitivity of the 32D EGFR to EGF and seems to indicate that the EGFR should work in this line through a specific EGF triggering pathway, rather than through unorthodox substrates. EGF is capable of triggering, in 32D EGFR, the turnover of phosphoinositides, a property not shared by IL-3, which is the physiological mitogenic stimulus for 32D cells in culture. Since EGF can substitute for IL-3 in 32D EGFR, these results seem to indicate that EGFR and IL-3 are not triggering the same signaling pathways.

Publications:

Di Fiore PP, Falco J, Borrello I, Weissman B, Aaronson SA. Calcium signal for BALB/MK keratinocyte terminal differentiation counteracts epidermal growth factor (EGF) very early in the EGF-induced proliferative pathway. Mol Cell Biol 1988;8:557-63.

Di Fiore PP, Pierce JH, Fleming TP, Hazan P, Ullrich A, King CR, Schlessinger J, Aaronson SA. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH/3T3 cells. Cell 1987;51:1063-70.

Di Fiore PP, Pierce JH, Kraus M, Segatto O, King CR, Aaronson SA. A human growth factor receptor-like gene (*erb*B-2) is a potent oncogene when overexpressed in NIH/3T3 cells. Science 1987;237:178-82.

Falco JP, Taylor WG, Di Fiore PP, Weissman BE, Aaronson SA. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of BALB/MK keratinocytes. Oncogene (In Press)

Fusco A, Berlingieri MT, Di Fiore PP, Portello G, Grieco M, Vecchio G. One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes. Mol Cell Biol 1987;7:3365-70.

Giancotti V, Pani B, D'Andrea P, Berlingieri MT, Di Fiore PP, Fusco A, Vecchio G, Philip R, Crane-Robinson C, Nicolas RH, Wright CA, Goodwin GH. Elevated levels of a specific class of nuclear phosphoproteins in cells transformed with v-*ras* and v-*mos* oncogenes and by cotransfection with c-*myc* and polyoma middle T genes. EMBO J 1987;7:1981-7.

King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: frequent overexpression in human mammary adenocarcinomas and induction of transformation in vitro. In Proceedings of UCLA Symposium (In Press)

Kraus MH, Di Fiore PP, Pierce JH, Aaronson SA. Different mechanisms are responsible for oncogene activation in human mammary neoplasia. In Lippman ME, Dickson B, eds.: Breast Cancer: Cellular and Molecular Biology. Boston: Martinus Nijhoff Publishing (In Press)

Pierce JH, Ruggiero M, Fleming TP, Di Fiore PP, Greenberger JS, Schlessinger J, Rovera G, Aaronson SA. Signal transduction through the EGF receptor transfected into interleukin-3-dependent hematopoietic cells. Science 1988;239:628-31.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05459-03 LCMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Structural and Functional Characterization of ras p21 Proteins

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principel Investigetor.) (Name, title, leboretory, and institute affiliation)*

PI:	J. C. Lacal	Visiting Associate	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	T. P. Fleming	Guest Researcher	LCMB	NCI
	S. H. Yuspa	Chief	CCTP	NCI
	P. M. Blumberg	Chief, Molecular Mechanisms of Tumor Promotion Section	CCTP	NCI

COOPERATING UNITS *(if any)*

Human Genetics Branch, NICHHD (P. de la Pena); Centro de Biologia Molecular, Madrid, Spain (L. Serrano); Instituto de Investigaciones Biomedicas, Madrid (A. Valencia); Depart. Med. Exper. Hosp. Principal, Madrid (P. Garcia-Barreno)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the space provided.)*

We have found that microinjection of the transforming but not the normal p21 protein into Xenopus laevis oocytes induced the production of 1,2-diacylglycerol (DAG) and inositol trisphosphate (IP-3). While the transforming H-ras p21 was an effective mitogen for normal 3T3 cells, its mitogenic function was substantially reduced (about 80%) in protein kinase C (PKC)-depleted cells. The activity was almost completely recovered by co-microinjection of the ras p21 protein and PKC. These results provide evidence for a functional requirement of PKC for the mitogenic activity of the H-ras protein. In contrast with the results obtained in Xenopus oocytes, 3T3 cells transformed by a variety of ras oncogenes do not show any increase of basal levels of IP-3. However, DAG levels are increased about 40-50% over control, normal cells, suggesting a different source for the production of DAG than the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP-2). We have observed elevated levels of the catabolites resulting from the hydrolysis of other major phospholipids, like phosphatidyl-choline (PC) and phosphatidyl-ethanolamine (PE). Transformation by the sis oncogene, as well as treatment with serum or PDGF, induced production of DAG and IP-3 but not the hydrolysis of PC or PE. These results provide evidence for at least two independent mechanisms for the production of DAG, and that both mechanisms can be activated by individual oncogene products. In a different set of experiments, we have been able to express in E. coli and purify to homogeneity the product of the rho gene from Aplysia californica, a ras-related gene. We have demonstrated that, indeed, the gene product of the rho gene (p21 rho) is a G-protein with a similar GTPase activity to that of the normal ras protein. Finally, we have been able to restore membrane localization and transforming activities of ras p21 mutants devoid of both activities by insertion of a membrane signaling peptide of the p60src product from the amino terminal.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. C. Lacal	Visiting Associate	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
T. P. Fleming	Guest Researcher	LCMB	NCI
S. H. Yuspa	Chief	CCTP	NCI
P. M. Blumberg	Chief, Molecular Mechanisms of Tumor Promotion Section	CCTP	NCI

Objectives:

1. To define the mechanisms of *ras* p21 activation at the biochemical and biological levels.

2. To define the pathways(s) in which *ras* p21 functions in both normal and transformed cells.

Methods Employed:

Expression of *ras* and *rho* p21 proteins in *E. coli*, purification of the products and characterization of their in vitro activities; microinjection of purified proteins into 3T3 cells and *Xenopus laevis* oocytes; analysis of phospholipid metabolism in *Xenopus* oocytes and 3T3 cells; in vitro mutagenesis of *ras* genes.

Major Findings:

1. We have demonstrated that microinjection of *ras* p21 proteins into *Xenopus* oocytes induces the production of diacylglycerol (DAG) and inositol phosphates.

2. We have provided evidence that the mitogenic activity of the *ras* p21 protein requires functional protein kinase C.

3. We have observed that in 3T3 cells transformation by *ras* oncogenes does not increase the basal levels of inositol phosphates, although the levels of DAG are above those of the normal cells.

4. We found elevated levels of the catabolites of phosphatidyl-choline (PC) and phosphatidyl-ethanolamine (PE) hydrolysis. These phospholipids seem to be the source of the elevated levels of DAG found in *ras*-transformed cells.

5. We have demonstrated that localization to the plasma membrane of *ras* p21 proteins by a signal peptide from the $p60^{v\text{-}src}$ protein restores

transforming activity of nonpalmitoylable *ras* mutants, suggesting that no processing other than palmitoylation and membrane location is required for its function.

6. We have provided evidence that the product of the *rho* gene (p21 *rho*), a *ras*-related gene, is a G protein with the ability to bind and hydrolyze GTP, similar to that of *ras* p21.

7. We have found that point mutations at position 12 or 61, but not a Thr^{59} substitution, induce conformational alterations in the *ras* p21 protein, detected by circular dichroism analysis.

Publications:

Anderson PS, Lacal JC. Expression of the *Aplysia californica rho* gene in *Escherichia coli*: purification and characterization of its encoded p21 product. Mol Cell Biol 1987;7:3620-8.

Harper JR, Reynolds SH, Greenhalgh DA, Strickland JC, Lacal JC, Yuspa SH. Analysis of the ras^{H} oncogene and its p21 product in chemically-induced skin tumors and tumor-derived cell lines. Carcinogenesis 1987;8:1821-5.

Jeng AY, Srivastava SK, Lacal JC, Blumberg PM. Phosphorylation of *ras* oncogene product by protein kinase C. Biochem Biophys Res Commun 1987;145:782-8.

Lacal JC, de la Pena P, Moscat J, Garcia-Barreno P, Anderson PS, Aaronson SA. Rapid stimulation of diacylglycerol production in *Xenopus* oocytes by microinjection of H-*ras* p21. Science 1987;238:533-6.

Lacal JC, Fleming TP, Warren BS, Blumberg PM, Aaronson SA. Involvement of functional protein kinase C in the mitogenic response to the H-*ras* oncogene product. Mol Cell Biol 1987;7:4146-9.

Lacal JC, Moscat J, Aaronson SA. Novel source of 1,2-diacylglycerol elevated in cells transformed by Ha-*ras* oncogene. Nature 1987;330:269-72.

Lacal PM, Pennington CY, Lacal JC. Transforming activity of *ras* proteins translocated to the plasma membrane by a myristoylation sequence from the *src* gene product. Oncogene (In Press)

Valencia A, Serrano L, Cabellero R, Anderson PS, Lacal JC. Conformational alterations detected by circular dichroism induced in the normal *ras* p21 protein by activating point mutations at position 12, 59 or 61. Eur J Biochem (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05461-04 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Characterization of Normal Counterpart of dbl Oncogene

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Ron	Visiting Associate	LCMB	NCI
Others:	A. Eva	Visiting Scientist	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Dbl oncogene genesis involved the loss of the first 497 amino acids of proto-dbl and the acquisition of a new N-terminus from an unknown human gene. Both the dbl oncogene and proto-dbl products can transform NIH/3T3 cells when expressed under the influence of retroviral long terminal repeat (LTR). However, the transforming activity of dbl was significantly higher. To assess which of the structural alterations in dbl is responsible for this enhancement, two deletion mutants were constructed, one from proto-dbl and one from dbl in which only the sequences which are shared by both were kept. The mutants were cloned in an LTR-based expression vector and their transforming ability was determined in the transfection assay. Both mutants transformed NIH/3T3 cells at similar efficiency, which was as high as that of the dbl oncogene product. The subcellular localization of the mutant protein was similar to that of the parental gene. In contrast to the parental gene products, none of the mutant protein was phosphorylated.

In order to search for evidence of proto-dbl involvement in human malignancies, the expression of proto-dbl-related transcript was surveyed in human tumors. Five of 11 leiomyosarcomas were positive, while tissues derived from normal smooth muscle were negative.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. Ron	Visiting Associate	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. To explore the relationship between proto-*dbl* structure and its transforming function using in vitro mutagenesis technique.

2. To assess the role of proto-*dbl* in the genesis of human tumors by determining the levels of expression of proto-*dbl* gene products in normal and malignant tissues.

3. To analyze the transforming properties of proto-*dbl* by introducing its cDNA into cultured cells and into animals.

4. To clone at least a portion of the mouse proto-*dbl* coding sequences to identify the specific cell type in which proto-*dbl* is expressed in vivo and to study its expression during developmental regulation.

Methods Employed:

In vitro mutagenesis to construct proto-*dbl* deletion mutants; standard methods of RNA extraction from tissue and cell lines; Northern blotting to screen for *dbl*-related transcripts; cDNA library screening, as well as other molecular cloning techniques, to subclone *dbl*-related cDNAs; dideoxy sequencing technique; Southern blotting; restriction enzyme analysis; construction of eukaryotic expression vectors; transfection; gel electrophoresis; and preparation of high molecular weight DNA.

Major Findings:

1. The amino terminal sequences acquired by the *dbl* oncogene from an unknown human gene as a result of its activation are not contributing to its transforming activity.

2. The N-terminal region of proto-*dbl* exerts a negative effect on its transforming activity.

3. Phosphorylation of proto-*dbl* is not required for transformation.

4. The proto-*dbl*-related transcript is expressed in leiomyosarcomas but not in normal muscles.

Publications:

Eva A, Srivastava S, Vecchio G, Ron D, Tronick S, Aaronson SA. Biochemical characterization of *dbl* oncogene and its product. In: Cornaglia-Ferraris P, Massimo L, Tonini GP, eds. Oncogenes in pediatric tumors. Erice, Italy: Serono Symposia Publ (In Press)

Eva A, Srivastava S, Vecchio G, Ron D, Tronick S, Aaronson SA. *dbl*, a new transforming gene isolated from a human diffuse B-cell lymphoma. In: Cimino F, Birkmayer GD, Pimentel E, Klavin JV, Salvatore F, eds. Human tumor markers. Berlin/New York: Walter De Gruyter, 1987;83-92.

Eva A, Vecchio G, Diamond M, Tronick SR, Ron D, Cooper GM, Aaronson SA. Independently activated *dbl* oncogenes exhibit similar yet distinct structural alterations. Oncogene 1987;1:355-60.

Ron D, Tronick SR, Aaronson SA, Eva A. Molecular cloning and characterization of the human *dbl* proto-oncogene: Evidence that its overexpression is sufficient to transform NIH/3T3 cells. EMBO J (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05463-04 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Participation of Growth Factors and Oncogene Products in Growth Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. G. Taylor	Biologist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. S. Rubin	Biotechnology Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*
Johns Hopkins Oncology Center, Baltimore, MD (J. Falco); Childrens Hospital of Los Angeles, CA (B. Weissman)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long-term objective of this program is to understand the mechanism(s) of cellular changes fundamental to neoplastic transformation. As nonneoplastic mammalian cells have specific hormone and growth factor requirements for initiation of DNA synthesis and mitosis, and retroviral oncogene (v-onc) gene product(s) can subvert normal growth regulatory mechanisms and cause neoplastic transformation in culture, the interaction(s) between v-onc gene products and subversion of signal transduction in the normal mitotic pathways or prototype epithelial cells was assessed in a serum-free bioassay system(s). Infection of BALB/MK keratinocytes with retroviruses containing v-ras oncogenes (v-H-ras, v-Ki-ras) with oncogenes which encode growth factor receptors (v-erbB, v-fms) or with v-mos permitted growth in defined medium containing insulin but lacking EGF. The v-fgr oncogene was unique as it conferred independence from both exogenous insulin and EGF. No evidence of a novel, secreted mitogen was detected in conditioned medium (CM) generated by monolayers of the fgr transformant. To determine if low passage, morphologically normal mesenchymal cells derived from human tumor cells secrete undefined mitogens, CM generated by monolayers from several organs was assayed. Modest mitogenic activity (greater than fivefold background for BALB/MK keratinocytes) was observed with CM from colon and stomach fibroblasts. If the incidence of intermediate activity (two- to fivefold background for BALB/MK cells) is included, CM from lung fibroblasts also contained significant undefined mitogenic activity. To date, the additional epithelial strains derived from tissues functionally or anatomically more similar to the tumor tissue than keratinocytes have not demonstrated a marked tissue specificity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

W. G. Taylor	Biologist	LCMB NCI
S. A. Aaronson	Chief	LCMB NCI
J. S. Rubin	Biotechnology Fellow	LCMB NCI

Objectives:

A fundamental event in carcinogenesis is subversion of normal growth regulation. A current model proposes that certain retroviral (v-*onc*) gene products, which have structural and functional homology with normal cellular proteins, interact with mitogenic signal transduction pathway(s). As a consequence constitutive expression at an inappropriate time in the cell cycle, overexpression or synthesis of an altered gene product may exert a sustained mitotic stimulus on cells secreting the mitogen(s) (autocrine) or on neighboring cells (paracrine). Interaction(s) between extracellular mitogens and membrane-bound receptors with the mitogenic pathways of normal and tumorigenic cells are of both theoretical and practical interest, either as a molecular model of growth regulation or a bioassay system in which strategies for intervention can be tested.

Two distinct, mutually inclusive objectives are: (a) to assess interaction(s) between retroviral (v-*onc*) gene products and subversion of signal transduction in the normal mitotic pathways of prototype epithelial cells; and (b) to probe for undefined mitogenic activity secreted by low passage stromal cells derived from primary cultures of human tumor tissue. In addition to an existing BALB/MK model system, additional indicator cells were used to define target cell specificity and aid in detection and classification of mitogens.

Methods Employed:

A sensitive serum-free clonal growth assay which met the basal nutritional requirements of BALB/MK keratinocytes was developed to quantify responses to mitogens. This assay system also permits the extended incubation period sometimes required to fully discriminate an additive from synergistic response. Petri dishes were precoated with poly-D-lysine and/or fibronectin to augment adhesion and spreading, and commercially available hormones and growth factors used to supplement serum-free medium. Gene complementarity was assessed by systematically omitting growth factor(s). End points for these assays were: comparative increase in cell number, colony formation, or visual enumeration with a calibrated ocular micrometer. Stromal cells derived from human tumor samples (lung, breast and colon) were grown to confluence, serum-free medium conditioned (SFCM) by the monolayer, and the activity of novel growth factors in the SFCM tested with quiescent populations by a standard

thymidine incorporation assay. Additional epithelial cell strains or lines were used to detect mitogens to which BALB/MK might not respond.

Major Findings:

The BALB/MK keratinocytes require epithelial cell growth factor (EGF) for sustained proliferation in serum-containing medium. A chemically defined, serum-free medium was developed to assess the minimum growth factor requirements of BALB/MK keratinocytes and investigate whether v-*onc* genes alter cell requirements for growth. A combination of insulin, apparently acting as IGF-1, and EGF sustained growth to a degree comparable to medium containing serum and EGF. Both acidic and basic fibroblast growth factors (FGF) appear able to substitute for EGF but not insulin in proliferation assays. A similar pattern of growth factor activity was observed when quiescent populations were stimulated to enter S-phase by individual or combinations of factors. Infection with retroviruses containing v-*ras* oncogenes (v-H-*ras*, v-Ki-*ras*), oncogenes which encode growth factor receptors (v-*erb*B, v-*fms*) or v-*mos* permitted growth in defined medium containing insulin but lacking EGF. The v-*fgr* oncogene, a member of the *src* subfamily, was unique as it conferred independence from both insulin and EGF. In separate studies no evidence of a novel secreted mitogen was detected in SFCM generated by monolayers of the *fgr* transformant. These findings establish the utility of this biologic system for assay of epithelial cell growth factor requirements which are altered or complemented by known oncogenes.

To address the second objective we tested whether morphologically normal mesenchymal cells derived from human tumor tissue secrete undefined mitogen(s). These mitogens could exert a subtle, chronic mitogenic stimulus for neighboring epithelium and might contribute to tumorigenesis. Of particular interest were patients presenting with a familial predisposition, early age of onset or tumors at multiple sites. Usually the cell strains tested were established from patients 50 years of age or younger. A modest frequency of significant mitogenic activity (greater than fivefold background for BALB/MK keratinocytes) was seen with untreated SFCM from lung (38%) or breast fibroblasts (11%), while a greater frequency was observed with SFCM from colon (55%) and stomach (43%). If the frequency of intermediate activity (two- to fivefold background for BALB/MK cells) also is included as significant, lung (81%) and colon (66%)/stomach (71%) fibroblasts qualify as mesenchymal cells which elaborate significant mitogenic activity and conceivably stimulate adjacent epithelium in a paracrine fashion. Results with crude SFCM identified several lines which were appropriate for batch production of mitogen(s), but, to date, epithelial strains derived from tissues functionally or anatomically more similar to the tumor tissue than keratinocytes have not exhibited a tissue specificity. However, at least one epithelial strain responds to a new mitogen which binds to a heparin-sepharose column but exhibits little mitogenic activity for BALB/MK keratinocytes.

Publications:

Falco JP, Taylor WG, Di Fiore PP, Weissman BE, Aaronson SA. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of BALB/MK keratinocytes. Oncogene (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05467-03 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Cloning of Human c-fgr Proto-oncogene cDNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
Others:	S. Katamine	Fogarty International Fellow	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	T. Miki	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Genetics Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Normal human c-fgr cDNA clones were constructed by using normal peripheral blood mononuclear cell mRNA as a template. Nucleotide sequence analysis of two such clones revealed a 1,587-base-pair-long open reading frame which predicted the primary amino acid sequence of the c-fgr translational product. Homology of this protein with the v-fgr translational product stretched from codons 128 to 516, where 32 differences among 388 codons were observed. Sequence similarity with human c-src, c-yes and fyn translational products began at amino acid position 76 of the predicted c-fgr protein and extended nearly to its C-terminus. In contrast, the stretch of 75 amino acids at the N-terminus demonstrated a greatly reduced degree of relatedness to these same proteins. To verify the deduced amino acid sequence, antibodies were prepared against peptides representing amino- and carboxy-terminal regions of the predicted c-fgr translational product. Both antibodies specifically recognized a 55-kilodalton protein expressed in COS-1 cells transfected with a c-fgr cDNA expression plasmid. Moreover, the same protein was immunoprecipitated from an Epstein-Barr virus-infected Burkitt's lymphoma cell line which expressed c-fgr mRNA but not in its uninfected fgr mRNA-negative counterpart. These findings identified the 55-kilodalton protein as the product of the human fgr proto-oncogene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Profesional Personnel Engaged on this Project:

K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
S. Katamine	Fogarty International Fellow	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI

Objectives:

To assess structure and function of the human cellular counterpart of the *fgr* oncogene, which is in the *src* gene family, with tyrosine kinase activity. Initial studies are directed toward the cloning of human c-*fgr* cDNA.

Methods Employed:

A cDNA library constructed from human mononuclear cells according to the method of Okayama and Berg was screened by colony hybridization with ^{32}P-labeled v-*fgr* or c-*fgr* DNA fragment as a probe under stringent conditions. Nucleotide sequencing of cloned DNA was performed with cloning in the M13 vector and the dideoxy sequencing method.

Cloned cDNA in the Okayama-Berg expression vector was transfected into COS cells by the calcium phosphate method. Transfected cells were biosynthetically labeled with ^{35}S-methionine and their lysates were immunoprecipitated with antibodies against synthetic peptides representing amino- and carboxy-terminal regions of predicted c-*fgr* protein. Then immunoprecipitates were analyzed on SDS-polyacrylamide gels.

Major Findings:

We have isolated overlapping human c-*fgr* cDNA clones using normal peripheral blood mononuclear cells as a proven source for c-*fgr* mRNA. Nucleotide sequence analysis of two such clones revealed an open reading frame 529 codons in length. The region of homology with the v-*fgr* translational product, p70$^{gag\text{-}actin\text{-}fgr}$, stretched from codon 128 to 516, where 32 of 388 codon differences were observed. Thus, amino and carboxy terminal sequences of the proto-oncogene translational product are not present in the oncogene-encoded polyprotein.

Homology with c-*src* and c-*yes* translational products began at amino acid position 78 and nearly extended to their carboxy termini. The degree of relatedness was 77% and 76% amino acid sequence identity for *src* and *yes* proteins, respectively. In contrast, 77 amino acids at the N-terminus of the c-*fgr* protein demonstrated a much lower degree of relatedness to *src* and *yes* gene products.

As an approach toward verification of our cDNA open reading frame as well as identification of the c-*fgr* translational product, lysates of COS cells transfected with a construct containing the entire *fgr* cDNA open reading frame placed downstream of an SV40 promoter were tested by immunoprecipitation with antibodies against peptides representing amino- and carboxy-terminal regions of the predicted c-*fgr* protein. Both antibodies immunoprecipitated a 55-kd protein from lysates of DNA-transfected but not sham-transfected cells. Furthermore, homologous peptides blocked the ability of the antibodies to recognize the 55-kd protein. These findings verified the sequence predicted by the open reading frame and provided an approach to search for the *fgr* translational product in vivo. Thus, HL60 cells shown to express c-*fgr* mRNA upon retinoid treatment were subjected to immunoprecipitation analysis using the same antibodies. Lysates of induced but not uninduced HL60 cells contained a 55-kd protein that was specifically detectable with both amino and carboxyl region antisera. These results identify the product of the *fgr* proto-oncogene in vivo as a 55-kd protein, which we designated $p55^{c\text{-}fgr}$.

Publications:

Katamine S, Notario V, Rao CD, Miki T, Cheah MSC, Tronick SR, Robbins KC. Primary structure of the human *fgr* proto-oncogene product $p55^{c\text{-}fgr}$. Mol Cell Biol 1988;8:259-66.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05469-03 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Identification of New Tyrosine Kinase Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Kruh	Medical Staff Fellow	LCMB NCI
Others:	R. Perego	Guest Researcher	LCMB NCI
	S. Aaronson	Chief	LCMB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously described the characterization of genomic clones of a new gene, termed arg, with extensive homology to v-abl. The arg gene is a member of the tyrosine kinase family and is expressed in many tissues. arg is located on the long arm of chromosome 1 and the arg transcript is 12 kb.

Using the arg genomic clones as probes, cDNA clones were identified in poly A-primed as well as specifically primed cDNA libraries. Two overlapping cDNA clones containing the entire arg coding sequence were identified. Nucleotide sequence analysis of these two clones is currently being performed and will allow characterization of the complete arg coding sequence. Probes developed from the arg cDNA clones will be used to assess the role of arg in human tumors. Additionally, the identification of the cDNA clones will allow construction of an arg vector that will allow protein expression in eukaryotic systems.

Antibodies directed against arg peptides have been raised and are being used to identify the arg protein.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

G. Kruh	Medical Staff Fellow	LCMB	NCI
R. Perego	Guest Researcher	LCMB	NCI
S. Aaronson	Chief	LCMB	NCI

Objectives:

To characterize a new oncogene and understand its role in human neoplasia.

Methods Employed:

cDNA clones of the *arg* oncogene are identified in cDNA libraries by standard method. cDNA clones are characterized using the techniques of restriction enzyme analysis and Southern blotting. Nucleotide sequence analysis of the cDNA clones is accomplished by the dideoxy chain termination method. *Arg* expression is studied using the techniques of RNA extraction and Northern blot analysis.

Major Findings:

Two overlapping cDNA clones that contain the entire coding sequence of *arg* were identified. Current work involves nucleotide sequence analysis of these two clones to determine the predicted amino acid sequence.

Patents:

Kruh GD, Aaronson SA. US Patent (Pending): Definition of a Human Gene Related to but Distinct from the Abelson Proto-oncogene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05472-03 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structural Characterization of Putative Growth Factor Receptor Gene c-erbB-2

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	O. Segatto	Visiting Fellow	LCMB	NCI
	P. P. Di Fiore	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to study the molecular mechanisms involved in signal transduction and regulation of the catalytic activity of the erbB-2 receptor protein, a series of mutants in different structural domains of the gene product were generated. Mutated molecular clones were expressed into NIH/3T3 cells in order to assess their biologic activity in a focus assay. We found that two different classes of structural alterations, i.e., an NH-2 terminal deletion and a Val 659 to Glu or Val 659 to Asp mutation, are capable of upregulating the transforming potential of the erbB-2 product. Mutant proteins exhibit an increased level of in vitro tyrosine kinase activity as well as an increased level of in vivo phosphorylation on tyrosine residues. These results indicate that deregulated tyrosine kinase function is a major determinant of erbB-2 receptor oncogenic activity. We are currently investigating the role of tyrosine autophosphorylation in the modulation of the erbB-2 receptor catalytic activity by analyzing the biological and biochemical behavior of mutant proteins lacking one or more of the tyrosine residues which are believed to be targets of autophosphorylation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI

Objectives:

To study the mechanisms of signal transduction of the growth factor receptor *erb*B-2. Studies are directed toward assessing the structural domains of the mature protein involved in signal transduction, catalytic activity, and regulation of catalytic activity.

Methods Employed:

By means of site-directed mutagenesis techniques, we have generated a series of *erb*B-2 cDNA mutants in order to explore the biochemical pathways responsible for the oncogenic activity of the *erb*B-2 gene product. These mutant molecular clones have been expressed into NIH/3T3 cells in order to assess their biological activity. Assays have been developed with the aim of correlating differences in biological activities with biochemical parameters such as levels of protein expression, autokinase activity and tyrosine kinase activity.

Major Findings:

Two different classes of structural alterations of the *erb*B-2 receptor protein upregulate its transforming activity as well as its tyrosine kinase function. Activated *erb*B-2 proteins also show an increased content of phosphotyrosine in vivo. These results suggest that a deregulated tyrosine kinase function is a major determinant of the *erb*B-2 oncogene activity. Ongoing studies are aimed at defining the role of tyrosine autophosphorylation in the activation of *erb*B-2 receptor function.

Publications:

Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 1987;237:178-82.

Finzi E, Fleming T, Segatto O, Pennington CY, Bringman TS, Derynck R, Aaronson SA. The human TGFα coding sequence is not a direct acting oncogene when overexpressed in NIH/3T3 cells. Proc Natl Acad Sci 1987;84:3733-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05511-02 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Purification and Characterization of Epithelial Cell Mitogens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	J. S. Rubin	Biotechnology Fellow	LCMB	NCI
	P. W. Finch	Visiting Fellow	LCMB	NCI
	W. G. Taylor	Biologist	LCMB	NCI
	D. Bottaro	IRTA Fellow	LCMB	NCI
	A. C. Cech	Guest Researcher	LCMB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standerd unreduced type. Do not exceed the spece provided.)*

Using heparin-sepharose affinity chromatography and reversed-phase high pressure liquid chromatography, we have purified to homogeneity two novel epithelial cell mitogens from the conditioned media of M426 fibroblasts (derived from embryonic human lung tissue). The two factors differ in their target cell specificity, one being more potent on keratinocytes (BALB/MK cell line), while the other is more potent on a mammary epithelial cell line (B5/589). The first is a monomer with an estimated molecular weight of 25-30 kd, while the second is a homodimer with each chain having a molecular weight of approximately 32 kd. An amino-terminal protein sequence has been determined for both factors and they showed no significant homology to any previously identified molecules. As described in a related project report (Z01CP05512-02), oligonucleotide probes designed on the basis of the amino acid sequences have enabled us to identify and characterize cDNA clones containing the coding sequence of each factor. Monoclonal and polyclonal antibodies against both factors are now being prepared. We also have initiated studies to characterize the cell surface receptors of both growth factors.

Another mitogen has been partially purified from a commercial source of bovine pancreatic ribonuclease I. It appears to be an intermediate in the processing of transforming growth factor (TGF) alpha. Definitive proof must await further purifica-tion. Growth inhibitory substance(s) have been identified in the conditioned media from a variety of sources. We are investigating their relationship to the TGF beta family.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
J. S. Rubin	Biotechnology Fellow	LCMB	NCI
P. W. Finch	Visiting Fellow	LCMB	NCI
W. G. Taylor	Research Biologist	LCMB	NCI
D. Bottaro	IRTA Fellow	LCMB	NCI
A. C. Chech	Guest Researcher	LCMB	NCI

Objectives:

To purify and study growth regulatory factors that stimulate or inhibit the proliferatrion of epithelial cells; to determine whether the production of such factors influences the incidence of human malignancy.

Methods Employed:

Conventional methods of protein purification, including affinity chromatography, as well as fast protein liquid chromatography and high pressure liquid chromatography; mitogenic bioassay, which entails monitoring of ^{3}H-thymidine incorporation into DNA; iodination of proteins; competitive binding with iodinated protein derivatives; metabolic labelling with P^{32}-orthophosphate; Western blot analysis; enzyme-linked immunosorbent assays.

Major Findings:

1. Two novel epithelial cell growth factors have been purified to homogeneity from the conditioned media of M426 fibroblasts.

2. The first factor is a monomer with an estimated molecular weight of 25-30 kd; it is a potent mitogen on the BALB/MK cell line, with significant stimulation on other epithelial lines but no stimulation of fibroblast lines or endothelial cells.

3. The second factor is a homodimer with each chain having an estimated molecular weight of 32 kd; it exhibits a strong mitogenic effect on the B5/589 mammary epithelial cell line and significant stimulation on other epithelial lines, but has only marginal activity on NIH/3T3 fibroblasts.

4. Another mitogen has been partially purified from a commercial source of bovine pancreatic ribonuclease and appears to be related to TGFα.

5. Growth inhibitory substance(s) have been identified in the conditioned media from a variety of sources and may belong to the TGFβ family.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05512-02 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Cloning of Epithelial Growth Factor Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	P. W. Finch	Visiting Fellow	LCMB	NCI
	J. S. Rubin	Biotechnology Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Two novel epithelial cell growth factors (EpGF I and EpGF II) have been purified to homogeneity from media conditioned by the embryonic lung fibroblast cell line, M426. The purified factors were subjected to NH 2-terminal sequence analysis and oligonucleotide probes were designed on the basis of the determined sequences. Hybridization of these probes to an M426 cDNA library identified positive clones for each factor.

For each factor a representative set of clones was characterized by restriction mapping and DNA sequence analysis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB NCI
P. W. Finch	Visiting Fellow	LCMB NCI
J. S. Rubin	Biotechnology Fellow	LCMB NCI

Objectives:

To isolate full length cDNAs encoding epithelial cell growth factors. The clones will be overexpressed to determine the oncogene potential of the growth factors in NIH/3T3 transformation assays and to facilitate the isolation of large quantities of pure growth factor. The distribution of the growth factors in normal and tumorigenic human tissues will be studied.

Methods Employed:

Screening of cDNA libraries with mixed pools of oligonucleotides as hybridization probes; standard gene cloning and DNA sequencing techniques.

Major Findings:

1. The predicted amino acid sequence of EpGF I is related to the heparin binding growth factors, aFGF and bFGF, and the two related proteins encoded by the *int*-2 and *hst* genes. EpGF I cDNA hybridized to a 3.5-kb mRNA species from M426.

2. The predicted amino acid sequence of EpGF II does not show any homology with any reported growth factor sequence, and may represent a new class of growth factor.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05513-02 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Mechanisms of Transformation Induced by fgr and Related Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
Others:	S. Katamine	Guest Researcher	LCMB	NCI
	S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
	N. Popescu	Microbiologist	LB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*
Laboratory of Oral Biology and Physiology, NIDR (S. Gutkind): Division of Hematology/Oncology, Washington University, St. Louis, MO (T. Ley)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Efforts to determine normal functions for protein-tyrosine kinases encoded by human fgr and related proto-oncogenes have focused on the isolation of cDNA molecules representing their transcriptional units and complete coding sequences. We have isolated and sequenced human c-fgr and fyn cDNAs and have deduced the primary amino acid sequence of their encoded product. These findings have made it possible to identify the products of both genes, designated p55 c-fgr and p59 fyn. These gene products are protein-tyrosine kinases with conserved catalytic domains and unique amino terminal regions. We have shown that expression of the human c-fgr gene is limited to normal monocytes, granulocytes, macrophages and Epstein-Barr virus-infected B lymphocytes. Cultured granulocyte precursor cells express c-fgr mRNA only when induced to differentiate. Kinetic studies of p55 c-fgr expression in differentiating granulocytic cells imply that this protein functions on mature cells that no longer are capable of proliferating.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI
S. Katamine	Guest Researcher	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
N. Popescu	Microbiologist	LB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. To determine the mechanism of v-*fgr*-induced transformation.

2. To elucidate the functions of *fgr* and *fyn* proto-oncogenes.

3. To determine the role of *fyn* as an oncogene in model and naturally occurring systems.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; radioimmunoassays for qualitative and quantitative characterization of gene products; in vitro synthesis and immunoprecipitation; peptide synthesis; antibody production; fractionation of cellular components and protein purification to analyze gene products; molecular hybridization techniques to analyze genes; recombinant DNA techniques for the purification and amplification of genes; analysis of genetic structure using restriction endonuclease mapping, nucleotide sequencing, S1 nuclease mapping, primer extension, cDNA cloning, site-directed mutagenesis, and electron microscopy techniques; DNA transfection and construction of virus mutants for analysis of transforming activity.

Major Findings:

Normal human c-*fgr* cDNA clones were constructed by using normal peripheral blood mononuclear cell mRNA as a template. Nucleotide sequence analysis of two such clones revealed a 1,587-base-pair-long open reading frame which predicted the primary amino acid sequence of the c-*fgr* translational product. Homology of this protein with the v-*fgr* translational product stretched from codons 128 to 516, where 32 differences among 388 codons were observed. Sequence similarity with human c-*src*, c-*yes* and *fyn* translational products began at amino acid position 76 of the predicted c-*fgr* protein and extended nearly to its C-terminus. In contrast, the stretch of 75 amino acids at the N-terminus demonstrated a greatly reduced degree of relatedness to these same proteins. To verify the deduced amino acid sequence, antibodies were prepared

against peptides representing amino- and carboxy-terminal regions of the predicted c-*fgr* translational product. Both antibodies specifically recognized a 55-kilodalton protein expressed in COS-1 cells transfected with a c-*fgr* cDNA expression plasmid. Moreover, the same protein was immunoprecipitated from an Epstein-Barr virus-infected Burkitt's lymphoma cell line which expressed c-*fgr* mRNA but not in its uninfected *fgr* mRNA-negative counterpart. These findings identified the 55-kilodalton protein as the product of the human *fgr* proto-oncogene.

The *src* gene is the prototype for a family of closely related genes whose products have protein-tyrosine kinase activity. We have described a new member of this family, designated *fyn*, whose cDNA was isolated from normal human fibroblasts. To examine the possible role of *fyn* as an oncogene, we investigated the effects of *fyn* overexpression on NIH/3T3 cells. Our findings demonstrate that normal *fyn* overexpression induces morphologic transformation and anchorage-independent growth. In addition, at relatively low frequency, *fyn* acquired properties of a dominant-acting oncogene capable of inducing the fully tumorigenic phenotype. Genetic changes associated with the conversion of normal *fyn* cDNA into a potent oncogene were localized to the carboxy terminal region of its translational product.

A DNA probe representing *fyn* coding sequences was labeled by nick-translation and used to hybridize normal human lymphocyte chromosome preparations *in situ*. The specific location of silver grains was determined on banded chromosomes after hybridization and autoradiography. A total of 231 grains, scored on 103 cells, were localized to a 400-band ideogram. Ninety of these grains (39%) were found on chromosome 6, whereas grains not present on chromosome 6 were detected at random locations throughout the human genome. The largest number of grains on chromosome 6 was observed at 6q21, where we have assigned the location of the *fyn* gene.

Publications:

Katamine S, Notario V, Rao CD, Miki T, Cheah MSC, Tronick SR, Robbins KC. Primary structure of the *fgr* proto-oncogene product, $p55^{c\text{-}fgr}$. Mol Cell Biol 1988;8:259-66.

Kawakami T, Cheah MSC, Leal F, Igarashi H, Pennington CY, Robbins KC. Involvement of polypeptide growth factors and their receptors in the neoplastic process. In Valeriote F, Crissman J, Al-Sarraf M, eds. Head and neck cancer: scientific principles and management. Amsterdam: Elsevier (In Press)

Kawakami T, Kawakami Y, Aaronson SA, Robbins KC. Acquisition of transforming properties by *fyn*, a normal *src*-related human gene. Proc Natl Acad Sci USA (In Press)

Popescu NC, Kawakami T, Matsui T, Robbins KC. Chromosomal localization of the human *fyn* gene. Oncogene 1987;1:449-51.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05514-02 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*: Analysis of a Proto-oncogene Encoding a Putative Growth Factor Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	T. Matsui	Visiting Fellow	LCMB	NCI
	M. Heidaran	IRTA Fellow	LCMB	NCI
	T. Miki	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Using cloned v-fms or mouse platelet-derived growth factor receptor cDNA as a probe, we have isolated genomic DNA clones homologous to the proto-oncogene family. A novel putative kinase receptor gene has been identified and characterized by cDNA cloning. Its primary structure is similar to that of tyrosine kinase receptors thus far cloned and share a common pattern of cysteine distribution which is seen in the receptors for platelet-derived growth factor (PDGF) and colony stimulating factor (CSF-1). This new gene is overexpressed in several human sarcoma cells, suggesting that this gene may be involved in the neoplastic process of some tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
T. Matsui	Visiting Fellow	LCMB	NCI
M. A. Heidaran	IRTA Fellow	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI

Objectives:

To extend our knowledge of growth factor receptors as human proto-oncogenes, we are exploring growth factor receptor-related genes.

To analyze how growth factor receptor genes may contribute to cell transformation, we screened the abnormalities of the genes in samples derived from human tumors.

Methods Employed:

A major effort has been made to develop cDNA clones corresponding to the full length of the human PDGF receptor and its related gene mRNA. To isolate such clones, probes were generated from the genomic clone pT11 and used to screen libraries from normal human fibroblasts as well as human brain. Individual clones have been assembled into a complete cDNA. This cDNA has been used as a hybridization probe of the DNA and RNA of human tumor material and tumor cell lines in order to detect abnormalities of DNA or mRNA.

Major Findings:

1. Complete cDNA corresponding to the mRNA of human PDGF receptor-related gene has been isolated.

2. Overexpression without amplification of the PDGF receptor-related gene has been detected in tumor cell lines derived from human leiomyosarcomas and glioblastomas.

3. Complete cDNA corresponding to the mRNA of the human PDGF receptor gene has been isolated.

4. Expression of human PDGF-receptor cDNA in several cell lines which originally lacked receptor expression has been induced by using retroviral expression vectors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05546-01 LCMB

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Structural and Functional Characterization of v-sis Gene Product

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB NCI
Others:	N. A. Giese	IRTA Fellow	LCMB NCI

COOPERATING UNITS *(if any)*: None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The translational product of the v-sis oncogene is a disulfide-linked homodimer which is closely related to platelet-derived growth factor (PDGF). Deletion mutagenesis of the v-sis gene was used to identify an internal region of 89 codons comprising the minimal transforming domain. Oligonucleotide-directed mutagenesis within the minimal transforming domain was used to individually change each of the eight cysteine codons to serine codons. Analysis of these mutants revealed that all cysteine residues were necessary for disulfide-linked dimer formation. In contrast, only four of the cysteine residues were found to be essential for transforming activity. Deletion mutagenesis within the minimal transforming domain was used to map structurally important regions. Characterization of the proteins encoded by these mutants resulted in the identification of inactive homodimers. The v-sis gene has been overexpressed using the baculovirus vector system. The v-sis protein expressed in this system efficiently dimerizes and has high specific activity when tested for mitogenesis on NIH/3T3 cells. Therefore, this system will be used to express and further characterize mutant v-sis proteins.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB NCI
N. A. Giese	IRTA Fellow	LCMB NCI

Objectives:

The structural and functional characterization of the v-*sis*/PDGF-2 gene product.

Methods Employed:

Standard recombinant DNA techniques; DNA sequencing; oligonucleotide-directed mutagenesis; Bal 31 deletion mutagenesis; transfection assay; tissue culture; immunoprecipitation and SDS-PAGE analysis of proteins; COS cell assay for transient overexpression of proteins; and baculovirus vector system for protein overexpression.

Major Findings:

1. A minimal transforming region of 84 codons within the v-*sis* gene was defined.

2. Each of the eight cysteine codons within the minimal transforming region are essential for disulfide-linked dimer formation.

3. Of the eight cysteine codons, four are essential and four nonessential for transforming activity.

4. The essential cysteine residues are involved in intrachain disulfide bridges.

5. The v-*sis* protein overexpressed in insect cells using the baculovirus vector system efficiently dimerizes and is biologically active.

Publications:

Giese NA, Robbins KC, Aaronson SA. The role of individual cysteine residues in the structure and function of the v-*sis* gene product. Science 1987;326:1315-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05547-01 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of PDGF Expression in the Neoplastic Process

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Pech	Visiting Scientist	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI
	C. D. Rao	Visiting Associate	LCMB NCI
	K. C. Robbins	Chief, Molecular Genetics Section	LCMB NCI

COOPERATING UNITS *(if any)*
Children's Hospital Medical Center, Cincinnati, Ohio (G. Jones)

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Gene Structure Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human platelet-derived growth factor (PDGF) is one of several known growth factors involved in the regulation of cell proliferation. PDGF is composed of two polypeptide chains, PDGF-1 and PDGF-2. One or both of these chains can be found in a number of human tumor cells which also express the PDGF receptor. The deregulation of PDGF expression might confer a growth advantage to those cells by autocrine stimulation and contribute to the malignant phenotype. We have started to investigate which mechanisms regulate PDGF expression in tumor versus normal cells. In addition, we wanted to know how a high level of PDGF expression would affect normal diploid cells or the complete organism. We found that PDGF-1 differs in its expression pattern from PDGF-2. PDGF-2 expression is controlled at three different levels. The PDGF-2 promoter, which was identified and dissected into its different regulatory elements, is one target for control in normal and tumor cells; other levels of regulation are posttranscriptional and include inhibition of translation by secondary RNA structures. A high expression of PDGF-2 gave rise to polyclonal tumors in mice injected with a retrovirus containing the normal PDGF-2 gene. Experiments with transgenic mice have been initiated to introduce the human genes for PDGF-1 and -2 into the germ line and study effects of their overexpression.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Pech	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
C. D. Rao	Visiting Associate	LCMB	NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB	NCI

Objectives:

(1) To identify the regulatory mechanisms governing the expression of the human PDGF-2 protein. (2) To characterize PDGF proteins in human tumor cells. (3) To determine which changes in the regulatory pathway are responsible for the aberrant expression of the PDGF-2 protein in human malignancies. (4) To evaluate the role of PDGF-2 expression in the generation of tumors in vivo. (5) To investigate the developmental effects of PDGF overexpression and repression.

Methods Employed:

Standard recombinant DNA technology; cDNA cloning; nuclease S1 mapping and primer extension analysis of RNA; RNase A/T1 protection analysis of RNA; Northern blots of RNA and Southern blots of DNA; DNA and RNA sequencing; deletion mutagenesis; site-specific mutagenesis using synthetic oligonucleotides; cellular transformation and animal tumorigenicity assays; electroporation of established and primary cell lines; enzymatic assays for the transient expression of reporter genes; gel retardation and DNase 1 footprint assays; standard techniques for rescue and propagation of retroviruses; generation of antisera directed against synthetic peptides; immunoprecipitation and immunoblotting; generation of transgenic mice; mitogenic assays.

Major Findings:

The oncogene v-*sis* of the simian sarcoma virus encodes a protein, $p28^{v\text{-}sis}$, which is closely related to PDGF-2. The human gene for PDGF-2 has been cloned and characterized. Expression of the human PDGF-2 gene in NIH/3T3 cells has been shown to confer a transformed phenotype to these cells. Normal fibroblasts and glial cells show no easily detectable levels of PDGF-2 mRNA, whereas a large fraction of sarcoma and glioblastoma cell lines or tumor tissues express the PDGF-2 transcript. Since fibroblasts and glial cells possess PDGF receptors and can be stimulated by PDGF, the aberrant expression of PDGF-2 may be important in the generation of those human malignancies. In order to identify differences in PDGF-2 regulation in normal and tumor cells, the PDGF-2 transcriptional unit was defined, the promoter was characterized

and the regulation at a transcriptional and posttranscriptional level in different model systems was evaluated.

We investigated the induction of three PDGF mRNAs during the megakaryocytic differentiation of K562 cells. Two concomitantly induced PDGF-1 mRNA variants are regulated differently from PDGF-2 mRNA. We defined and analyzed the PDGF-2 promoter which is a target for regulation in K562 cells. Different regulatory sequence elements which also bind nuclear factors were identified. One element acts as a transcriptional silencer; two other regions are necessary for the maximum activity of the promoter in megakaryoblasts. A minimal promoter region was found to be sufficient for maximum activity in uninduced K562 cells. The upstream regulatory elements are the target for *trans*-activation and responsible for the higher level of PDGF-2 mRNA in a bladder carcinoma cell line. Beyond the regulation of promoter activity, other mechanisms of negative control must be involved to prevent the expression of PDGF-2 mRNA in uninduced K562 cells and normal fibroblasts.

The 5' untranslated region (UTS) of PDGF-2 mRNA has a very high G+C content. The level of PDGF-2 mRNA in glioblastomas and fibrosarcomas which can be detected in Northern blots contrasts with the very low level of protein in those tumor cells. We found that hairpin structures within the 5' UTS exert a strong inhibitory effect on translation of PDGF-2 or other genes.

A fragment which contained all the genomic PDGF-2 sequences, was inserted into a retroviral vector derived from the Abelson murine leukemia virus. The virus was injected subcutaneously, intramuscularly and intracerebrally into newborn mice. A total of 43 mice developed fibromas or fibrosarcomas. Detailed mapping experiments showed that the virus rescue had generated a provirus consistent with a faithfully processed PDGF-2 transcript.

In addition, the PDGF-2 cDNA was brought under the control of different eukaryotic promoters. Those constructs have been introduced into the mouse germline. Analysis of these transgenic mice is underway.

Publications:

Rao CD, Igarashi H, Pech M, Robbins KC, Aaronson SA. Oncogenic potential of the human platelet-derived growth factor. Cold Spring Harbor Symp. on Quant Biol 1988; 51:959-66.

Rao CD, Pech M, Robbins KC, Aaronson, SA. The 5' untranslated region of the c-*sis*/platelet-derived growth factor 2 transcript is a potent translational inhibitor. Mol Cell Biol 1988;8:284-92.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05548-01 LCMB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Expression Cloning System for Oncogene cDNAs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Miki	Visiting Scientist	LCMB	NCI
Others:	M. Crescenzi	Visiting Fellow	LCMB	NCI
	T. P. Fleming	IRTA Fellow	LCMB	NCI
	S. Blam	Guest Researcher	LCMB	NCI
	K. C. Robbins	Chief, Mol. Genetics Section	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are developing a cDNA expression cloning system for isolation of dominant as well as recessive oncogenes. In this system, cDNA libraries are constructed in eukaryotic expression vectors using poly(A)-selected RNAs from transformants or tumors. The library DNA can then be used to transfect NIH/3T3 cells. cDNA clones will be recovered from resulting foci, and their structures analyzed. In order to make expression cloning feasible for this purpose, cDNA libraries containing complete coding sequences will be necessary.

At this point, we have developed a high efficiency cDNA cloning system which can direct the orientation of inserts in eukaryotic expression vectors which possess large cloning capacities. Our lambda-plasmid composite vectors contain a retroviral long terminal repeat (LTR) promoter to express cDNA and a simian virus 40 (SV40) early promoter-driven neo gene as a eukaryotic cell selection marker. cDNA was synthesized from a linker-primer containing the site for SfiI, an infrequent cutter of DNA. An adaptor was ligated at both ends of the double-stranded cDNA molecules, cleaved by SfiI, and ligated with the lambda vector arms prepared by cutting at two different SfiI sites. Due to the directional cloning strategies and non-symmetrical structure of sticky ends of both the vector and insert DNAs, the efficiencies of 10 to 100 million plaque-forming units of phages were obtained from one microgram of poly(A)-selected RNA. Furthermore, we have shown that our libraries contain cDNAs for several growth factors and receptors that are nearly full length molecules of 2.5 to 6.5 kb, at high frequencies.

GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. Miki	Visiting Scientist	LCMB NCI
M. Crescenzi	Visiting Fellow	LCMB NCI
T. P. Flemming	IRTA Fellow	LCMB NCI
S. Blam	Guest Researcher	LCMB NCI
K. C. Robbins	Chief, Molecular Genetics Section	LCMB NCI
S. A. Aaronson	Chief	LCMB NCI

Objectives:

Recessive oncogene and certain novel dominant oncogene cDNAs are difficult to clone because of their size and because probes for their detection are not available. One approach toward cloning such oncogenes relies upon an efficient expression cloning system of cDNA. The use of expression cloning has been hampered by inefficient synthesis of long cDNAs and low efficiency of DNA-mediated gene transfer (DNA transfection). To overcome these problems, we have developed a high efficiency cDNA cloning system and are in the process of constructing new expression vectors designed to facilitate the isolation of novel oncogenes and other recessive genes which contribute to the malignant process.

Methods Employed:

Standard molecular cloning methods were used to construct vectors. DNA-mediated gene transfer (DNA transfection) was used to introduce cloned DNA into NIH/3T3 cells. RNA extraction and cDNA synthesis methods were used to construct cDNA libraries.

Major Findings:

1. Two expression vectors for cDNA cloning and expression have been developed. λpCEV9 and λpCEV15 vectors consist of a λ phage genome as well as a plasmid DNA which contains a retroviral LTR promoter to express cDNA, and SV40 early promoter-driven *neo* gene as a selectable marker. These vectors were designed to take advantage of the efficient λ packaging system. By combining λ DNA with a plasmid, cloned cDNAs identified by their ability to alter the growth properties of transformed cells can easily be rescued from eukaryotic cells by direct bacterial transformation. These vectors also contain the sites for the new cDNA cloning strategies described below.

2. A high efficiency cDNA cloning strategy is an important component of these vectors. cDNA is synthesized from a linker-primer containing an

SfiI site. An adaptor was ligated at both ends of the double-stranded cDNA molecules, cleaved by an infrequent cutter SfiI, and ligated with the λ vector arm prepared by cutting at two different SfiI sites. Due to the directional cloning strategies and non-symmetrical structure of sticky ends of both the vector and insert DNAs, the efficiencies of 10^7 - 10^8 plaque-forming units of phages were obtained from one μg of poly(A)$^+$ RNA.

3. To assess the efficacy of the method, cDNA libraries have been constructed. Screening of the libraries for several growth factors and receptors showed that nearly full-length cDNA molecules of 2.5 - 6.5 kb could be isolated at high frequency.

4. The following advantages for the new cDNA cloning system have been designed: (a) Cloning efficiency is very high, due to the non-symmetrical structure of ends of vector arms and cDNA inserts. Upon ligation, desired λ DNA concatemers with cDNA inserts are produced spontaneously to ensure efficient packaging; (b) almost all clones in the libraries contain cDNA insert when vector/insert molar ratio is adjusted appropriately before ligation; (c) cDNA molecules are inserted into the vectors in the orientation which allows the promoter to express cDNA inserts; (d) each clone possesses a single insert, obviating the need to identify the insert of interest; (e) relatively long cDNA fragments can be cloned. The vectors accommodate longer inserts than other λ vectors do without sacrificing efficiency; (f) plasmids carrying cDNA inserts can be released from λ DNA by NotI cleavage. This feature facilitates analysis of the cDNA structure, permits the generation of sublibraries in plasmid vectors, and makes it possible to recover the cDNA clones from eukaryotic cells; (g) since the SfiI adaptor can contain any sequence except for the 3' extension, useful sequences, such as multiple translation start signals or phage promoters for in vitro transcription, can be incorporated as needed; h) the protocol is straightforward. Since no alkaline phosphatase treatment is required, even the preparation of vector λ arms is relatively easy. No methylation reaction is needed after cDNA synthesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05549-01 LCMB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Phosphorylation in Growth Factor-mediated Cell Activation and Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	C. J. Molloy	Biotechnology Fellow	LCMB	NCI
	D. Bottaro	IRTA Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein phosphorylation appears to constitute a major mechanism through which growth factors and related transforming oncogenes influence intracellular events. In an effort to understand the role of specific protein phosphorylation events in growth factor-mediated cell activation and transformation, epidermal growth factor (EGF) was utilized in experiments with several cell lines expressing the EGF receptor tyrosine kinase. Tyrosine kinase activity elicited by EGF, which was manifested as receptor autophosphorylation as well as endogenous substrate phosphorylation, was characterized by both immunoblotting and immunoprecipitation techniques using specific antiphosphotyrosine antibodies. In the EGF-response cells expressing different levels of the EGF receptor protein, preliminary data suggest that EGF causes a dose-dependent increase in receptor tyrosine autophosphorylation which is linear and consistent with a monomeric intramolecular model of EGF receptor activation. Further experiments designed to identify specific tyrosine phosphorylated polypeptide substrates of the EGF receptor kinase were performed using one- and two-dimensional gel electrophoresis coupled with immunoblotting. The results show that several tyrosine phosphoproteins are induced following EGF stimulation. These include proteins of apparent molecular weights of 36, 40, 70, 80 and 150 kd. Similar experiments were carried out using oncogene-transformed cells in an attempt to identify common pathways of mitogenic signal transduction requiring tyrosine phosphorylation. Results suggest an important role of specific protein phosphorylations in cell growth and transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Aaronson	Chief	LCMB	NCI
C. Molloy	Biotechnology Fellow	LCMB	NCI
D. Bottaro	IRTA Fellow	LCMB	NCI

Objectives:

To study the role of tyrosine phosphorylation of proteins in growth factor-initiated cell activation and oncogene transformation.

To identify important endogenous polypeptide substrates of transforming and signal transducing protein kinases.

Methods Employed:

Standard cell culture techniques, including metabolic radiolabeling of cell proteins, protein extraction techniques, one- and two-dimensional polyacrylamide gel electrophoresis, protein immunoblotting and immunoprecipitation assays, standard in vitro enzyme assays, affinity chromatography of polypeptides using immobilized lectins and antibodies, and standard antibody production.

Major Findings:

In an effort to understand the role of tyrosine phosphorylation of proteins in growth factor-initiated cell activation, specific antisera directed against phosphotyrosine-containing polypeptides were generated. These antibodies were demonstrated to recognize autophosphorylated forms of the EGF receptor, PDGF receptor, as well as the *erb*B-2 oncogene protein by both immunoprecipitation and immunoblotting experiments. Using these antibodies, NIH/3T3 and NR6 cells transfected with a eukaryotic vector containing human EGF receptor cDNA were shown to express normal size receptors which became phosphorylated on tyrosine residues in response to exogenously added EGF. Using selected clones of these cells expressing different levels of the EGF receptor, the effect of EGF stimulation on receptor activation was studied. Preliminary evidence indicated that EGF induced a dose-dependent increase in receptor autophosphorylation on tyrosine that was linear and correlated with receptor density as well as the mitogenic response observed in the cells. These results support a monomeric, intramolecular model of EGF receptor activation.

Studies designed to identify potentially relevant tyrosine-phosphorylated polypeptides involved in growth factor signaling and oncogenic transformation were performed. Using one- and two-dimensional polyacrylamide gel electrophoresis coupled with protein immunoblotting using antiphosphotyrosine antibodies, several potential substrates of the activated EGF-receptor kinase were identified in EGF-

responsive cells. These included proteins of apparent molecular weights of 36, 40, 70, 80, and 150 kd. Similar experiments were carried out on whole cell lysates and subcellular fractions of various oncogene transformed cells in an attempt to identify common polypeptide substrates of tyrosine kinases in growth regulation. Prelimary data suggest that several membrane-associated proteins are phosphorylated during growth factor stimulation and transformation. Future studies will attempt to purify and further characterize these tyrosine phosphorylated proteins with the goal of identifying their specific function in cell growth and transformation.

Publications:

Moscat J, Molloy CJ, Fleming TP, Aaronson SA. Epidermal growth factor activates phosphoinositide turnover and protein kinase C in BALB/MK keratinocytes. Mol Endocrinol (In Press)

CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS:

STATE OF CALIFORNIA DEPARTMENT OF HEALTH SERVICES (NO1-CP-51001-35)

Title: Breeding and Production of 129/J and NFR Mice and Specified Services

Current Annual Level: $154,655

Man Years: 3:05

Objectives: To provide in vivo support for four major research efforts within the LCMB: (1) viral and cellular genes involved in malignant transformation; direct effect of specific oncogenes introduced into appropriate animals by viral recombinants; (2) analysis of genetically altered target cells by grafting into immunodeficient athymic nude host mice; (3) the role of host immune response in oncogene-induced tumors; and (4) heterotransplantation of human tumor cell-derived lines in athymic nude mice.

Major Contributions: The purpose of this contract is to provide support services for research conducted by LCMB; therefore, a discussion of major contributions will be found in the projects conducted by LCMB.

Proposed Course: This contract has been negotiated to run from March 1, 1985 through September 30, 1989.

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Molecular Oncology plans and conducts research defining the molecular and genetic elements responsible for the development and expression of the malignant phenotype in humans and animals. Towards this end, the Laboratory of Molecular Oncology (1) applies the skills of molecular biology, recombinant DNA technology and the methods of immunology to identify and isolate cellular transforming onc genes from malignant and normal cells and tissues, as well as to characterize the product(s) encoded by these genes; (2) identifies, isolates, characterizes and determines the function(s) of these onc genes as determined by the expression and functional analysis of their specified oncoproteins; (3) develops the molecular probes and methodologies required to identify the target(s) of the onc gene product(s), relative to the process of malignant transformation and the normal mode(s) of cellular action; (4) determines and evaluates the organizational structures and function(s) of the normal cellular homologs (proto-onc genes) of the acute transforming viral onc genes by expression in normal eukaryotic and prokaryotic cellular systems; and (5) examines the molecular controlling elements and mechanisms regulating prokaryotic and eukaryotic gene expression, aiming to adapt, modify and apply this understanding to the expression and control of the neoplastic processes. The Laboratory of Molecular Oncology conducts research on the molecular elements responsible for the development and expression of malignant phenotypes in humans and animals. The Laboratory applies skills in molecular biology, recombinant DNA technology and hybridoma-monoclonal antibody production in a comprehensive program to identify and isolate cellular transforming genes and to characterize products expressed by these genes. This mission is accomplished by bringing together expertise in the diverse disciplines of eukaryotic and prokaryotic virology, molecular biology and genetics. The Carcinogenesis Regulation Section studies the relationship between oncogenic gene expression and the conversion of cells from the normal to the transformed, malignant state. Specific regions of molecularly cloned, acute transforming retrovirus genomes are tested for transforming activity and to determine the molecular mechanisms by which their oncogene products act in concert with cellular factors to activate the neoplastic process. This will be accomplished by examining the molecular organization and products of normal essential cellular proto-onc gene(s) and the mechanism(s) by which these genes are subverted into transforming genes, as well as by comparing them to the organization and expression of their corresponding malignant counterparts. The Microbiology Section conducts research aimed at identifying genes involved in the oncogenic transformation of normal cells, and in characterizing their mechanisms of action at a molecular level. These studies are accomplished by using a variety of gene transfer techniques to identify such genes present in human tumors and tumor-derived cell lines, and to introduce such recombinant DNA-cloned genes or their retroviral vector homologs into well-characterized cells in vitro in order to study their effects. Model cell culture systems are employed to determine how specific transforming genes can alter

normal cell functions, and to identify those normal functions which may serve as primary targets of oncogene action. Since oncogene-induced changes often involve alterations in the structure and/or expression of cellular genes, presumably at the level of DNA gene structure, the effects of oncogenes and their products are an area of particular interest. Transformation studies are also augmented using monoclonal antibodies prepared against various homologous cellular proteins. Additionally, this section develops systems to assay and analyze the mechanism of malignant transformation in human primary and established lines of cells using sequences derived from viral and genomic cellular DNA that may have oncogenic potential. Such investigations should lead to a more complete understanding of the mechanisms by which such altered genes are able to transform normal cells to their malignant phenotype. The Transgenic Analysis Section designs and carries out an integrated research program to characterize and identify the regulatory and molecular elements involved in expression and function of genes controlling critical cellular processes; develops new assays to test the function of genes introduced into living tissue and cells employing a variety of methods, including microinjection, electroporation and calcium phosphate-mediated gene transfection procedures; and evaluates genes introduced into intact animals and tissue for phenotypic and genotypic expression by a variety of molecular and cell biological techniques employing recombinant DNA methodology. The Office of the Chief, in addition to coordinating the administrative responsibilities of the Laboratory and its sections, conducts research to investigate the molecular structure and function and biochemical properties of select oncogenes, including the ras family of oncogenes and the p21 ras onco-protein(s), as well as on other oncogenes. Such studies are directed towards a well-defined molecular and biochemical description of malignant transformation by select oncogenes and their products, as well as of their normal cellular counterparts in differentiative and proliferative stages of the cell cycle.

The major portion of the present and future emphases of this Laboratory concerns the identification, isolation and analysis of oncogenic sequences by molecular cloning techniques, as well as their oncogene protein products, in order to evaluate their relationship to the malignant transformation process. We have pursued studies into several major areas. We are analyzing the structural and biological properties of specific retroviral onc genes present in both avian and mammalian acute transforming retroviruses. We are identifying, isolating and characterizing their normal cellular homologs, the proto-onc genes, from their species of origin, as well as from the human genome. Through combined efforts of the sections within the Laboratory of Molecular Oncology, we have developed strategies and protocols to address the mechanisms by which cellular proto-oncogenes can become transduced, activated and expressed, and have attempted to delineate the pleiotropic molecular changes affected by these concerted events. We have also capitalized on our research emanating from investigations performed on acute transforming viruses. In particular, the Carcinogenesis Regulation Section has found that the avian leukemia virus, E26, has homologous sequences in mammalian species which are dispersed to two different chromosomal loci that have distinctive domains; these we have termed ets-1 and ets-2 and they correspond to the 5′ and 3′ regions of the v-ets oncogene of the replication-defective avian erythroblastosis virus, E26. This virus induces a mixed erythroid/myeloid leukemia in chickens. E26 includes elements from two proto-oncogenes, chicken proto-myb and chicken proto-ets, and gag from the viral gag gene. In humans, the ets loci have been mapped to chromosome 11 for ets-1, and chromosome 21 for ets-2 by somatic cell hybrid studies and direct in situ analysis using isotopically-labeled probes. In particular, we have, by in situ hybridization of an ets-2

clone to normal human chromosome preparations, confirmed the assignment of human ets-2 to chromosome 21 and localized it to the HSA 21q22.1-22.3 portion of the human chromosome in a region known to be involved in Down's syndrome (DS) and nearby the amyloid protein gene associated with Alzheimer's disease (AD).

The human genomic clones homologous to the ets region were cloned in our laboratory and shown to be related to the v-ets region by partial sequence analysis. The human ets-1 gene encodes a single mRNA of 6.8 kb; the human ets-2 gene encodes three mRNAs of 4.7 kb, 3.2 kb and 2.7 kb. These Hu-ets-1 and Hu-ets-2 genes have recently been shown to be transposed in certain leukemias. Because of the significance of ets in neoplasia, we embarked on a search for other human genes closely related to ets. A cDNA library was prepared from a human COLO 320 cell line which expresses very high levels of ets-specific transcripts. Two cDNA clones reactive with the Hu-ets-2 probe were isolated. Characterization of these clones by restriction mapping and sequence analysis revealed that they represented the complete coding sequence of a novel human gene named erg (ets-related gene). The erg gene shows a homology of 40% and 70% to two domains of the 5′ and 3′ regions of the v-ets oncogene. Like ets-2 in human, the erg gene has also been localized on human chromosome 21. The precise location of the ets-2 and ets-related genes and the analysis of their loci in different human cancers, Down's syndrome and Alzheimer's disease should make it possible to determine if amplification, translocation or rearrangement of this ets family of genes can be linked to the manifestation of any of these diseases.

Recently, the functional relationship between the onc genes of the transforming retroviruses and their cellular prototypes has been facilitated by structural comparisons at the nucleic acid and predicted protein levels. We have determined the complete nucleotide sequence of the chicken protein-ets gene and compared it to the ets gene of E26. E26 is a genetic hybrid with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The chicken ets gene is present as a single locus with v-ets homologous sequences found in nine regions over 60 kb of genomic DNA. In addition, the cellular gene contains unique 5′ and 3′ sequences. Thus, the E26 virus demonstrates: (1) substitution of viral genes for parts of normal cellular genes; (2) truncation of the gene; and (3) acquisition of non-cellular coding proto-ets sequences. These structural differences may be responsible for the oncogenic potential of this retrovirus. We have previously determined that the mammalian homologs of v-ets are located on different chromosomes. The mammalian ets-2 genes from man and mouse encode for nearly identical amino acids and are over 90% conserved relative to the chicken ets gene. The ets-2 gene appears to have mitogenic activity upon transfected cells. The human ets-1 gene product is over 95% identical to the chicken gene from where the virus transduced ets sequences. Alignment of the predicted ets proteins with v-ets suggests that three domains exist. The domain closest to the carboxyl-termini is highly conserved in all genes characterized from species ranging from human to Drosophila. The domain located at the amino-terminal end of ets-2 is less homologous to v-ets, while the ets-1 gene and the v-ets gene are similar in this region. The central domain of v-ets is found to be similar only to that of ets-1, being very divergent in ets-2. Recombinant DNA technology will be used to generate mutants to evaluate the function of these three domains.

Our Laboratory, having determined the localization of human ETS genes at the 11q23 and 21q22 regions, suggested that there was a possible involvement of these genes in both constitutional and acquired (neoplasia) diseases associated with a

known cytogenetic abnormality. The 11q23 region is involved in a number of chromosome abnormalities peculiar to acute leukemias of the myelomonocytic lineage. In two of these abnormalities, the translocations (4;11)(q21;q23) and (9;11)(p21;q23), transpositions of the ETS1 gene from its normal position on chromosome 11 to chromosomes 4 and 9, is evident. The 21q22 region is also relevant both in human and cancer genetics; in an acquired cytogenetic abnormality specific to AML-M2 leukemias, we found ETS2 transposed from chromosome 21 to chromosome 8. Despite the repositioning of the ETS genes, neither one was found structurally involved by the chromosome rearrangements. With respect to the ETS2 gene, part of the section's work was aimed at demonstrating whether or not it belongs to the obligate genetic region necessary for the expression of the constitutional aneuploidy known as Down's syndrome. Our work using the proto-ets-2 as a probe led to the preliminary observation that there were three copies of ETS alleles in the rare, cryptic trisomic patient occurring in the 21q22 region. This finding makes it possible to conclude that the ETS2 gene may belong to the set of genes needed for the expression of the multitrait Down's syndrome. To provide a basis of understanding for the functional relationship between the oncogenes of transforming retroviruses and their cellular homologs, molecular structural comparisons at the nucleic acid and predicted protein levels have been conducted. Towards this end, we have determined the nucleotide sequences of the proto-ets genes in a number of organisms and compared it to the v-ets gene of E26.

Organisms from humans to Drosophila have been found to contain cellular sequences and transcripts that were homologous to viral-onc genes. The normal function of the genes encoded for by these cellular sequences is presently unknown. Only by understanding the normal function of these cellular proto-ets genes will we have a possible means to understand how they can become transforming upon transduction by the virus. With this goal in mind, we undertook the investigation of Drosophila, sea urchin and Xenopus species.

Cellular sequences homologous to the ets region of the chicken retrovirus E-26 have been found in Drosophila in this laboratory. The characterized portion of this gene corresponds to the last two exons of the chicken c-ets-1 gene, and has over 90% homology at the predicted amino acid level. It is designated D-ets-2 and has been localized on chromosome 3R at position 58 A/B. This gene produces a single transcript of 4.7 Kb in all developmental stages. Low stringency hybridization of Drosophila genomic DNA shows several other bands that also hybridize with a viral ets probe. Hybridization of a cDNA library under these conditions led to the isolation of a cDNA clone that showed considerable homology to v-ets, but is not D-ets-2. This gene, called D-elg (for Drosophila ets-like gene), has ~60% homology with D-ets-2 and is located on chromosome 3R at 97D. It produces two transcripts of 2.3 and 2.0 Kb in embryo, pupae, and adult stages. Drosophila appears to have conserved the 3' region of the ets gene very well in at least two different genes. It is now hoped that Drosophila will also provide a system to determine the function of these genes, by utilizing transgenic embryos or reintroduction of modified ets genes using recombinant P-elements of Drosophila.

Using a cDNA library prepared from X. laevis, oocyte RNA was screened with avian virus E26 v-ets DNA as a probe. From one million plaques, 23 positive clones were obtained; one of these contained a 2.5 kb DNA ets-2 homologous insert. The DNA sequence of this clone, as determined using the dideoxynucleotide chain termination method, contained a single major open reading frame capable of

encoding 467 amino acid residues. This cDNA of Xenopus is more closely related to the c-ets-2 sequences from human, mouse, and chicken than to c-ets-1 or v-ets sequences. The X. laevis ets-2 sequence contained extensive homology with the other vertebrate ets-2 sequences throughout its entire length and was coterminal with these c-ets-2 sequences. The methods for fertilization in vitro and isolation of different stages of oocytes have been established. The pattern of RNA expression of the c-ets-2 gene in X. laevis was examined by RNA gel blot analysis of RNAs from oocytes and embryos at different stages of development. For each stage, a single 3.2 kb ets-2-specific mRNA was observed. The maximum level of ets-2 expression occurred in the early stage of oocyte development.

The proto-ets gene was discovered in our Laboratory to be present in a variety of organisms. We have found that this gene codes for nuclear proteins against which a number of monoclonal antibodies have been raised. These antibodies were used to check for ets protein expression in bacterial (E. coli w3110) and yeast (S288C haploid) cells. The antibody directed against the 56 Kd ets-2 protein was found to give a positive signal in yeast cells, as well as in the COLO 320 DM cells when used as a control, but failed to do so in the bacterial cells. In addition, a new monoclonal antibody tested against an ets-2 peptide was found to react by immunoprecipitation analyses with both the yeast and the human COLO 320 DM cellular extracts. Thus, there may be a possible ets-like product present in very primitive eukaryotic cells.

The presence of putative erg proteins encoded by the erg (ets-related gene) locus has been examined in specific human cell lines. To probe the erg proteins, polyclonal antisera have been prepared against synthetic polypeptides representing amino acid sequences deduced from the DNA sequence of the erg gene locus. To facilitate further analysis of the putative erg proteins; we have purified a bacterially-expressed erg protein and are using this highly purified protein to raise polyclonal erg antiserum. In immunoprecipitates from the nuclear fractions of ^{35}S-methionine-labeled COLO 320 cells we detected two proteins, 56 and 63 Kd, which reacted specifically with the specific erg-R1 antiserum. There is the possibility that these proteins represent the cellular erg gene products.

Previous studies in our Laboratory have suggested that fish have oncogenic sequences homologous to those found in mammalian and avian species. We were the first to confirm the presence of fish oncogenes by isolating and sequencing the c-myc gene from rainbow trout. Other fish genes homologous to known mammalian oncogenes have been identified by Southern blot hybridization. In order to examine the role of fish oncogenes, Southern blots were prepared using DNA digests from tumor and normal tissue. Hybridization to known oncogene sequences did not detect rearrangements or gene amplifications. Therefore, in order to detect the presence of other oncogenes, we developed a transfection system in which fish DNA could be transfected into NIH 3T3 cells. The transforming ability of fish tumor DNA was examined by standard focus assay, nude mouse assay, and colony selection assay. DNA from diethylnitrosamine-induced mesothelioma in the fish, medaka, was the most efficient DNA in transforming NIH 3T3 cells. Secondary transfectants caused tumor formation in nude mice within 1-1/2 weeks following transduction. Southern blot analysis of these transfectant DNAs hybridized to medaka genomic DNA probe showed specific bands present in tumor-induced transfectants; no such bands were present in DNA from NIH 3T3 controls and cells transfected with non-tumorigenic medaka DNA. This finding suggested the presence of specific fish sequences in transformants that may contribute to

tumorigenesis. These unique sequences did not appear to be homologous to any known oncogenes such as K-ras, H-ras, N-ras, c-myc, m-met or v-erbB. This study will continue to focus on the transforming gene isolate from fish, in order to determine its molecular nature and role in tumorigenesis.

A key role for the c-myc oncogene in cellular proliferation events has been postulated by many workers. C-myc may act by modulating the expression of other cellular genes whose products directly control proliferation. Permanent cell lines (in which the endogenous c-myc gene is tightly regulated by growth factors and cell/cell contact) have been constructed in our laboratory in which expression of an exogenously transfected c-myc gene is controlled by the Drosophila heat shock 70 promoter. Transcription and subsequent translation of the exogenous c-myc gene can be specifically induced by mild heat shock. The endogenous c-myc gene is not expressed under these conditions. Compared to heat-shocked cell lines which contain constructs lacking c-myc, several changes in cellular gene expression have been observed: (1) two-dimensional analysis of the proteins from c-myc-containing cell lines shows the induction of eight protein species and the repression of five protein species relative to cell lines lacking c-myc; (2) the transcripts of two genes (3CH77 and 3CH92) previously identified as serum-inducible are induced when c-myc is expressed; and (3) the endogenous heat shock 70 gene may be specifically induced in response to c-myc. Therefore, regulated c-myc expression is seen to alter the expression of other cellular genes, including the induction of some known to be expressed only in proliferating cells.

Research work in the Transgenic Analysis Section has focused upon the proto-oncogenes, c-myc and c-ets (1 and 2), which are cellular homologs of the oncogenes carried by the avian myelocytomatosis virus, MC29, and the avian acute leukemia virus, E26, respectively. These genes are suspected to play some role(s) in the pathogenesis of certain types of human malignancy. Production of monoclonal antibodies against products of these genes has been applied towards the biological and biochemical characterization of these products. The c-myc and c-ets (1 and 2) genes are cellular homologs of the oncogenes carried by the avian myelocytomatosis virus, MC29, and the avian acute leukemia virus, E26, respectively. These genes are thought to play some role in the pathogenic effect of these viruses, and antibodies to the products of these genes has been generated for the biological and biochemical characterization of these proteins. Thus far, a monoclonal antibody against the myc gene products and three monoclonal antibodies against the ets-2 protein have been prepared; the monoclonal antibodies against the ets-2 protein have been used to characterize the c-ets protein in a number of cells and tissues. By subcellular fractionation and immunocytochemical technique, the human ets-2 protein was demonstrated to be located in the nucleus, and a minor amount of the protein was also found in the cytoplasm. Pulse-chase experiments and in vivo labeling with [32P] showed that the ets-2 protein is a short-lived phosphoprotein. Tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG), both of which activate protein kinase C, stabilized the ets-2 protein, thereby quickly elevating its steady-state level. These results point to the possibility that the ets-2 protein has a role in the protein kinase C pathway of signal transduction as a nuclear effector.

A peptide antibody corresponding to a hydrophilic and highly conserved 3' amino acid sequence of the human ets-2 protein was shown to specifically react with all known ets proteins (p135 v-ets, ets-1, ets-2, erg-1, erg-2), as well as several

other uncharacterized ets-related antigens. The ets-2 protein was identified by use of monoclonal antibodies prepared against a bacterially expressed ets-2 protein. The ets-2 protein was shown to have several properties in common with other known nuclear oncogenes, including low abundance, fast turnover, nuclear localization and response to mitogenic stimuli. By contrast, the ets-1 protein appears to be a cytoplasmic phosphoprotein. Two-dimensional gel electrophoresis of these proteins show that they are their negatively charged proteins with an apparent isoelectric point of less than pH 6.0.

The ets-2 mRNA has been shown to be induced in regenerating mouse liver, suggesting that the ets-2 gene expression may be involved in cell proliferation. In order to test the expression of ets-2 on growth properties of mouse fibroblasts, we have constructed expression vectors capable of producing ets-2 protein in NIH 3T3 cells. The mouse and human ets-2 cDNAs were cloned into an expression vector carrying an inducible promoter. The NIH 3T3 cells transfected with the construct carrying mouse c-ets-2 genes showed discrete foci of densely-growing cells when maintained in low-serum or serum-free medium. These transfected cells formed colonies in soft agar and also induced tumors in nude mice. The control cells did not grow in serum-free medium and in the presence of low-serum, they did not show characteristic foci observed with ets-2-transfected cells. Our results suggest that the ets-2 proto-oncogene expression induces growth and morphological alterations in NIH 3T3 cells, and abolishes their serum requirement and is tumorgenic in nude mice. These properties provide an assay system to study the functions of ets-2 and its related genes in cell proliferation.

The ets-1 and ets-2 loci are transcriptionally active in humans and express a single 6.8 Kb ets-1-specific and three 4.7, 3.2 and 2.7 Kb ets-2-specific mRNAs, respectively. The c-ets-1 (P51) and the c-ets-2 (P56)-related proteins have been identified in the human cell lines Daudi, as well as COLO 320 DM, but they are expressed at extremely low levels.

In order to express the ets protein in large quantities, to study the biochemical properties and also to produce ets-specific antiserum, we have constructed several expression vectors capable of producing complete v-ets and its deletion mutants and defined human c-ets-1 and c-ets-2 gene products in E. coli.

The vectors with smaller inserts produced high levels of the ets products representing greater than 5% of the total bacterial proteins. In contrast, the vectors with larger inserts produced low levels representing less than 0.5% of the total protein. The bacterial ets proteins were characterized by immunoblotting on a Western blot using the ets-specific antibodies. In E. coli, the ets proteins aggregate and exist in the form of inclusion bodies and are insoluble. However, this property allowed us to purify the ets proteins to greater than 95% homogeneity by extracting the insoluble pellet with different solvents. The purified proteins were utilized to obtain the ets-1 and the ets-2-specific polyclonal and monoclonal antiserum. These polyclonal and monoclonal antibodies are expected to be useful in identifying and studying the biochemical and biological functions of the ets-1 and the ets-2 proteins.

The ets gene expression (ets-1, ets-2 and erg) was examined in fetal thymocytes at different stages of development in isolated subsets of adult thymocytes, and in peripheral T lymphocytes. The ets-1 gene expression was first detected at day 18 in fetal thymocytes, corresponding to the first appearance of CD4-positive

(CD4-positive, CD8-negative) thymocytes, and reaches maximal/plateau levels of expression in the thymus at 1-2 days after birth. The ets-2 gene expression is detected at least one day earlier, coinciding with the presence of both double-positive (CD4-positive, CD8-positive) and double-negative (CD4-negative, CD8-negative) blast thymocytes and reaches maximal/plateau levels one day before birth.

In the adult thymus, ets-1 and ets-2 mRNA expression is ten- and eight-fold higher, respectively, in the CD4-positive subset than in the other subsets examined. Higher levels of p55 ets-1 protein were also shown to exist in the CD4-positive subset. Both the CD4-positive and the CD8-positive T-cell subsets had lower ets RNA levels than the CD4-positive thymocytes. These results suggest that ets-2 and, more particularly, ets-1 gene products play an important role in T-cell development and differentiation and are not simply associated with proliferating cells. Proliferating cells are generally observed at a higher frequency in fetal thymocytes, or dull Ly-1 (low CD5-positive), and double-negative adult thymocytes. The ets-1 and ets-2 mRNA level does not change during erythroid differentiation.

The role of ets gene products in maturation of helper function and in T-cell activation and differentiation are presently being studied. The expression of the ets gene will also be studied in specific thymocyte and T-cell subsets derived from tumor tissues.

Laboratory work in the Office of the Chief has focused efforts on understanding the functional roles that oncogenes may play in normal and malignant systems. One major focus of this has been to investigate the molecular biology and biochemistry of the ras oncogenes and the ras p21 proteins. The long-range objective is to elucidate molecular mechanism of cell transformation induced by these genes and their products.

We have studied biochemical properties and biological activities of ras gene mutants of Harvey murine sarcoma viruses. Mutations with amino acid substitutions were constructed by oligonucleotide-directed mutagenesis; mutations of p21 were made at the novel site(s) phosphorylated by protein kinases A and C. A novel p21 phosphorylation was found in cells expressing high levels of this product of c-H- and c-K-ras gene. In vivo phosphorylation was stimulated by both PKC and PKA activators. The same serine residues were phosphorylated by protein kinases A and C in vitro. By peptide mapping, it was found that these protein kinases phosphorylated p21 at the same site(s) as was found in vivo. Mutations were made on H-ras p21 serine-177, allowing identification of this residue as the major phosphorylation site. This novel site lies in the hypervariable region of p21, which links the globular catalytic domain to the membrane-anchoring site at the C-terminus. Mutations were also made at the guanine base-binding site and the N-terminal glycine-rich region important for guanosine triphosphatase (GTPase) activity and proto-oncogene activation. The present results indicate that the GTP-binding region is critical for ras gene function. The ras product, p21 (21,000-dalton protein), binds guanine nucleotides and exhibits GTPase activity. The viral transforming and cellular ras proteins differ at positions 12 and 59. The point mutations at these positions affect the above activities. By analogy to other G-proteins, the ras gene poduct, p21, may act as a regulatory component of the signal transduction. There have been reports on the involvement of active protein kinase C for the mitogenic activity of ras p21 and also ras protein increases the levels of diacylglycerol (DAG), a protein kinase C

activator in some cells. Our laboratory showed that the ras gene product was phosphorylated by phorbol esters in certain cell lines.

Oligonucleotide-directed, site-specific mutagenesis has been used to dissect the biochemical basis of oncogenic activation and enzymatic activity of the ras oncogene. We have constructed point mutations of p21 proteins by oligo-nucleotide-directed mutagenesis of the v-ras-H oncogene, which substituted amino acid residues within the presumptive nucleotide-binding consensus sequence, GXGGXGK. When the glycine residue at positions 10, 13, or 15 was substituted with valine, the viral ras-H product, p21, lost its GTP-binding and autokinase activities. Other substitutions at position 22, 33, 51 or 59 did not impair its binding activity. G418-resistant NIH 3T3 cell lines were derived by transfection with constructs obtained by inserting the mutant proviral DNA into the pSV2neo plasmid. Clones with valine mutation at positions 13 or 15 were incapable of morphologically transforming cells, while all other mutants with GTP-binding activity were competent. Ras, with a valine mutation at glycine-10, which had lost its ability to bind GTP and its autokinase activity in vitro and in vivo, was fully capable of transforming NIH 3T3 cells. These cells grew in soft agar and formed tumors in nude mice. The p21 of cell lines derived from tumor explants still lacked the autokinase activity. In contrast, cells transfected with position-valine or a position-116 tyrosine ras mutant were tumorigenic in nude mice, but not immunocompetent in Balb/c mice. Cells transfected with the 15 valine mutant, however, did not consistently induce tumors in nude mice.

In studies designed to understand events related to gene regulation and expression our Laboratory is working on the regulation of the enzyme RNaseIII in prokaryotes. RNaseIII is a double-strand specific endoribonuclease that has different functions in E. coli. It also processes rRNA precursors for efficient maturation into ribosomes. It processes some mRNA either to activate gene expression or to reduce gene expression. Additionally, it regulates mRNA degradation. The int gene of phage λ is transcribed from two promoters yielding different mRNA transcripts. Int expression from one promoter is reduced by RNaseIII; from the other, expression is enhanced. In both cases, control of expression by RNaseIII occurs from a single site beyond the gene. This form of control is named retroregulation. The site present on the RNA is able to form a special stem and loop structure that is recognized by RNaseIII. This site is also a transcription termination signal for RNA polymerase. In order to understand how RNaseIII levels in the cells are modulated, its gene in E. coli, rnc, has been cloned into λ vectors and into pBR322 plasmid. Sequence analysis indicates a second gene in an operon with rnc. This gene produces a protein with significant homologies to the yeast ras genes and is called era (E. coli ras). Both rnc and era have been placed on expression vectors and their proteins have been purified and antibodies have been made. Era is an essential gene in E. coli. The purified protein binds GTP. E. coli mutants have been isolated that are conditionally lethal because of mutations in rnc and era. Suppression mutants that restore growth are being analyzed to determine proteins that may interact or compensate for the products of these genes. E. coli mutants have been isolated that are conditionally lethal because of insertion mutations blocking expression of era. Rnc has been demonstrated not to be an essential gene in E. coli. The purified rnc protein binds adenosine triphosphate (ATP). A separate insertion mutation that abolishes rnc function, but permits era expression, is being analyzed. The regulation of the operon is being investigated, and preliminary results suggest a system of autoregulation.

The Microbiology Section has constructed a replication-defective murine retrovirus, ME26, by inserting the avian gag-myb-ets sequences derived from the cloned avian acute leukemia virus, E26, into an Abelson murine leukemia virus (MuLV)-derived retroviral vector. Both ME26 DNA transfected non-producer cells and ME26-infected cells expressed a 135 Kd gag-myb-ets fusion protein, termed p135m. p135m is localized primarily in the nucleus and can easily be identified with a low-salt buffer containing detergents. NIH 3T3 cells infected with ME26 exhibit morphological changes and show an increase in proliferation; they also form small colonies in soft agar. ME26 induces an increased incidence of leukemia, primarily erythroid and myeloid following a long latency period after injection into newborn mice. Analysis of frameshift and deletion mutants is consistent with the evidence that v-ets sequences are necessary to mitogenically stimulate NIH 3T3 cell proliferation in culture.

Members of this section have analyzed the DNA sequence of a portion of the ovc oncogene, a human transforming sequence activated during DNA transfection and derived from the human ovarian carcinoma cell line, OVCAR-3. Comparison of this sequence with sequences contained in the GenBank data base reveals no significant homologies, suggesting that, consistent with previous hybridization data, ovc represents a previously unidentified human oncogene. Analysis of poly-A+ RNA from several normal and transformed human cell lines indicates that ovc sequences are expressed in the OVCAR-3 parent cell line and three other human adenocarcinomas.

We have shown that treatment of mouse fibroblasts with tunicamycin, a glycosylation inhibitor, renders them susceptible to infection by RD114 cat endogeneous virus and its pseudotypes. We have now been able to show that several other inhibitors of glycosylation, which act at different points along the glycosylation pathway, also have a similar effect. In addition, mouse lymphoid cells exhibit similar properties when exposed to tunicamycin. This suggests that this retroviral susceptibility may represent a common feature of mouse cells.

We have tested the ability of cell DNA from two cell lines derived from a methylcholanthrene-induced mouse fibrosarcoma (TN-10), and which exhibit differing metastatic potentials, to transform NIH 3T3 cells. Foci are induced following transfection at high efficiencies, and transformed cells have acquired the ability to grow in the absence of serum or protein growth factors. No rearrangements of several well-characterized oncogenes are detected in transfected cells, but foci appear to contain amplified copies of the murine K-ras oncogene.

Accumulating data suggest that due to their influence on the topological state of the DNA, topoisomerases I and II (topo I, II) may control various biological processes. Because of their involvement in the control of such fundamental biological activities, it is tempting to speculate that the deranged expression of these enzymes may play an important role in neoplastic transformation. Therefore, in order to elucidate this possibility, we have searched for the possible in vivo interrelationships between these enzymes and activated oncogenes. The enzymatic activities of the topo II in oncogene-transfected cells differed from those observed in untransfected cells, mainly on the degree of dependence on ATP, spermidine and Mg+2. Normal rat kidney (NRK) cells infected with a temperature-sensitive mutant (ts110) of Moloney murine sarcoma virus (Mo-MuSV) had a very slight topo I activity at the permissive temperature (34°),

while at the non-permissive temperature (39°) they exhibit a normal topo I activity. These cells produced p85gag-mos proteins at 34°C, but not at 39°C. Moreover, these cells were resistant to topo I inhibitor effects at 34°C, but were sensitive at 39°C. Further experiments are needed to determine whether the low topo I activity and the absence of inhibition of topo I activity, is a direct effect of the p85 gag-mos protein.

As part of the Institute's effort to understand the basic molecular mechanisms of human retroviral diseases, and acquired immune deficiency syndrome (AIDS) in particular, the LMO has launched a major effort to clone specific segments of the human immunodeficiency virus (HIV) gene and utilize these segments in the construction of prokaryotic expression vectors for the production of high levels of HIV-1- and HTLV-I-specific proteins. Using these systems, eight HIV-envelope-specific polypeptides have been expressed at high levels and have been purified to near homogeneity. Using these purified proteins as antigens, it has been possible to elicit specific polyclonal and monoclonal antibodies against these specific retroviral proteins that are able to react against the authentic cellular proteins in vivo. One clone, 566, expresses a protein which is highly immunoreactive after a 100,000-fold dilution of AIDS-positive human sera; further, this antigen is able to recognize all HIV-positive sera by enzyme-linked immunosorbent assay (ELISA) testing and is as sensitive in these assays as the natural gp41 viral env-encoded protein. We have also expressed a portion of the HIV genome encoding the non-structural proteins 3′-orf and sor in our prokaryotic vector systems. We have been able to obtain specific antibodies, using these reagent-antigens, that recognize the native viral product in HIV-infected cellular systems. Our monoclonal antibody against the HIV-1 3′-orf also recognizes the bacterially expressed HIV-2 3′-orf polypeptide and preliminary studies using the expressed 3′-orf for biochemical analysis shows that the HIV-2 3′-orf is capable of binding GTP. Our bacterially expressed sor protein has been used to screen a panel of sera from homosexual men that showed no reactivity to commercial HIV-antigens; five out of nine individuals tested prior to seroconversion were positive in a Western immunoblot assay using the bacterially expressed sor product. These results would suggest that sor may be a valuable reagent to employ for the immunodiagnosis of HIV reactivity during preclinical stages of infection. A recombinant derived tat gene product of HIV-1 is being introduced into lymphoidal $CD4^+$ cells under HIV-long terminal repeat (LTR) regulatory controls in order to assess the order and nature of molecular events that subsequently ensue following HIV-1 infection. In particular, use of such a controlled inducible system will enable investigations as to which specific cellular genes are derepressed or inhibited following HIV-1 tat expression. The transactivator of the transcription gene product (tat) of HIV-1 may modulate cellular gene expression. Infection of H9 cells with HIV-1 is demonstrated to produce changes in the level of expression of some serum inducible genes, although oncogene expression appears to be unaffected. Currently, uncharacterized genes (cDNA clones), whose expression may be repressed, have been isolated by differential hybridization of infected vs. uninfected H9 cell cDNA libraries. A construct in which functional tat can be expressed under control of the heat shock promoter is being used to construct permanent cell lines. Therefore, to study the influence of the HIV tat gene on the expression of cellular genes, we have placed this gene into the retroviral vector pGV1 under the transcriptional control of the HIV LTR. The recombinant plasmid was transfected into psi2 cells so as to produce an ecotropic viral stock. This virus was used to infect psiAM cells so as to induce G418-resistant colonies. At present these colonies are being purified and tested for the production of

amphotropic helper-free retrovirus. These viruses will be used to infect human lymphoid cells so as to establish cell lines constitutively expressing the tat gene. Such lines will be useful for the identification of cellular genes whose expression is controlled by the tat gene.

HIV infection has been shown by several laboratories to use a complex set of RNA synthesis and processing events in order to control viral expression. In order to determine if messages exist that have not been previously characterized, we have begun to directly analyze cDNA copies of HIV messages by Southern hybridization and DNA sequence analysis.

A cDNA library in lambda gt10 has been prepared from RNA isolated from HIV-infected H9 cells. A large number of phages from this library were found to hybridize to viral sequences. A total of 19 such phages were plaque-purified and analyzed by Southern hybridization using labeled oligonucleotides or restriction fragments derived from various locations on the HIV genome. The specific regions include: (A) 5'-end of tat; (B) 5'-end of trs; (C) 5'-portion of env (excluding tat and trs region); (D) pol/sor region. Clones with the following hybridization pattern have been isolated: class I, A+B-C-D+; class II, A-B-C+D+; class III, A-B-C-D+. We have purified several of these phages and have subcloned their insert DNA into pIBI vectors. Several of these phages have been characterized by Southern blot hybridization. We are currently sequencing these DNA segments to determine if they represent novel HIV messages and, thus, might reveal new exons or splice sites.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04899-16 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transforming Genes of Avian RNA Tumor Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. S. Papas	Chief	LMO	NCI
Others:	D. K. Watson	Research Microbiologist	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI
	J. A. Lautenberger	Research Chemist	LMO	NCI
	C. S. Schweinfest	Staff Fellow	LMO	NCI
	N. Sacchi	Visiting Scientist	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
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SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A major effort by the LMO has been to elucidate the processes by which specific retroviral oncogenes, as well as their cellular homologs, are able to impact on critical cellular events. Using the avian retroviral oncogenes, ets and myc, as probes, we have detected, isolated, cloned and sequenced the cellular homologs of these genes from evolutionarily diverse organisms such as humans, mice, fish, sea urchin, Xenopus and Drosophila. There is a family of these proto-ets genes where specific regions of these cellular genes are retained at very high levels of homology; each of these proto-ets oncogenes was compared to their viral homologs. In all cases, the proto-oncogenes were much larger than their corresponding viral oncogenes. The consistent truncation of the viral oncogene and consequent alteration of its products implicates this type of damage as a general event that results in the disruption of the function of these highly conserved genes. We have also developed and exploited several expression vector systems, both prokaryotic and eukaryotic, to produce oncogene-specific products in quantity. These expressed products were used to purify, characterize and develop immunologic reagents to locate and characterize the cellular proto-oncogene products. Such reagents have also been used to probe for the expression of oncogene-specific products in normal and malignant tissues and related them to specific human pathologies. We have been able to isolate and characterize the gene products of ets and determine some relevant properties of this gene that implicate its function in the signal transduction pathway and in T4 cell maturation. It also seems that these proto-ets genes may play a significant role in Down's syndrome (DS), and may possibly be related to the higher than normal incidence of leukemic disease seen in DS patients.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
C. W. Schweinfest	Staff Fellow	LMO	NCI
N. Sacchi	Visiting Scientist	LMO	NCI
R. Ascione	Research Chemist	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI

Objectives:

To investigate the processes by which retroviruses are able to exert their subversive influences, via genetic expression of their viral oncogenes, to manifest malignant transformation in vitro and neoplastic diseases in vivo. To investigate and determine the molecular interrelationship of viral oncogenes to their normal cellular counterparts, the proto-oncogenes, and to comprehend their structural similarities, differences and functional natures, respectively. To utilize the methods of recombinant DNA technology and monoclonal-hybridoma research in order to identify and understand the role of select viral oncogenes and cellular proto-oncogenes in the normal and malignant cellular growth and in developing and differentiating processes in a wide variety of cells and tissues. To utilize the expression vector systems designed in our laboratory to produce specific retroviral genome-encoded proteins; to purify these and obtain a number of polyclonal and monoclonal antibodies; to characterize the authentic proto-oncogene proteins in nontransformed cells and compare them to the oncogene products of the malignantly transformed cell.

Methods Employed:

1. Preparation of high molecular weight nucleic acids from eukaryotic cells was performed by standard methods (as described in Molecular Cloning, A Laboratory Manual, 1982) and modified according to our needs relative to phage, plasmid or prokaryotic cloning systems.

2. Nucleic acid analysis by standard techniques described for restriction enzyme analysis, resolution of components by agarose and/or polyacrylamide gel elecrophoretic systems; sequencing analysis by Maxam-Gilbert (Methods Enzymol. 1980;65:499-560); and techniques incorporating the hybridizing methods of Southern blot and Northern transfer protocols (as described in Molecular Cloning, A Laboratory Manual, 1982).

3. Construction of recombinant phage libraries and plasmids by restriction/ligation of isolated fragments of eukaryotic DNA homologous to onc genes; subcloning, as required, into pBR322, pBR325 or pBR328 or into cosmid or λ vectors, followed by production of phage by in vitro packaging.

4. Construction of cDNA library. Double-stranded cDNA was prepared from poly A^+ RNA using cloned reverse transcriptase enzymes and ligated into λgt10 and λgt11 vectors or bacterial vectors for amplification.

5. Immunoprecipitation of labeled cell lysates with onc-specific antisera and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The sepcific onc gene-related polypeptide was purified for further analysis by electroelution and high pressure liquid chromatography (HPLC).

6. S^{35}-methionine and -cysteine labeling of expressed protein in bacteria utilizing the plasmid, pJL6, and its derivatives.

7. Radiolabeling of bacterially expressed proteins or eukaryotic cellular proteins was performed using ^{35}S-methionine or -cysteine isotopes, followed by cellular fractionation and purification protocols, and subsequent identification of labeled protein products by SDS-PAGE analysis, visualized by autoradiography and/or Western blot (immunoblot electrophoresis) methods.

8. Immunologic analyses were performed using polyclonal or monoclonal antibodies prepared against oligopeptide-specific antigens constructed from the deduced, conserved nucleic acid sequences of specific onc genes or from bacterially expressed products. These antibodies were purified and used as antigens, and were analyzed by immunoprecipitation and Western blot hybridization procedures.

9. Protein, polypeptide and peptide analyses were performed using a combination of HPLC and fast protein liquid chromatography (FPLC) techniques, and reversed phase and gel filtration chromatography, in conjunction with ion-exchange methods, on a preparative or analytical scale. Confirmation of protein analysis was performed by microsequencing techniques using an automated gas-phase amino acid microsequencer in conjunction with high performance liquid chromatography.

10. Sea urchin embryo cultures from adult sea urchins of the species Strongylocentrotrus purpuratus were obtained from Marianus, Inc. (Long Beach, CA). Collection of eggs and sperm by intracoelomic injection of 0.5 M KCl and in vitro fertilizations were performed as described by Venkatesan et al. (Proc. Natl. Acad. Sci. USA 1986;83:3351-5). Embryo cultures were incubated at 15°C with stirring until the pluteus stage.

11. RNA isolation and Northern blot analysis of sea urchin and Drosophila tissues and embryos were performed on total RNA extracted from sea urchin tissue, whole Drosophila or embryos.

12. Computer analysis of DNA and protein sequences. A wide variety of sequencing analyses were performed in connection with the studies described in Major Findings. Extensive use was made of the University of Wisconsin software package implemented on the VAX computer of the Advanced Scientific Computer Laboratory (ASCL) for such applications as data entry, restriction mapping, protein sequence prediction, and alignment of multiple protein sequences. Other analyses required the various data base search procedures run on the Cray-XMP supercomputer of the ASCL. Specialized applications required the development of new programs.

13. Chromosomal in situ hybridization of Drosophila was performed using polytene chromosomes from D. melanogaster salivary glands.

Major Findings:

To understand the molecular process of cellular transformation, our Laboratory has been pursuing the study of the structure and function of the normal cellular proto-onc genes and its encoded product(s), comparing these to the homologous viral oncogenes and its encoded product(s). In particular, our approach focused on the ets family of genes, which are related to a sequence originally identified as a second cellular-derived sequence present in the genome of the avian leukemia virus, E26.

To characterize the difference between the viral and cellular ets genes and better understand their functions, we have analyzed these molecular structures and transcription patterns of the avian and mammalian proto-ets genes. The chicken cellular ets gene is present on a single chromosomal locus spanning over 60 kb of genomic DNA. The mammalian ets genes, by contrast, are dispersed onto separate, discontiguous chromosomal loci, recognizable as ets-1 and ets-2, which are each transcriptionally active and differentially regulated. More importantly, we have cloned and compared the ets-2 gene primary nucleotide sequence structure and its predicted products, those deduced from human and mouse cDNA; these genes are highly homologous to one another. Direct comparison of the predicted proteins derived from the human and mouse cDNA sequences, together with the encoded products of chicken, Xenopus, Drosophila and sea urchin ets genes, as well as with the other ets-family genes such as human erg, and the Drosophila-elg, have also been performed. The results of this comparison can be summarized as follows: Cellular ets sequences homologous to v-ets of the avian leukemia virus, E26, are very highly conserved. The open reading frame (ORF) coding for the putative human ets proteins, have been isolated and sequenced; the ets-2 cDNA clones obtained from both human and mouse are highly homologous and contain open reading frames capable of encoding proteins of 469 and 468 residues, respectively. The predicted human and mouse ets-2 proteins possess three recognizably distinct domains when compared to the chicken proto-ets gene-encoded product. Two of these ets-2 domains are homologous to the chicken proto-oncogene. The one close to the carboxyl-termini is very highly conserved (>90%) and this conservation is seen to be widely preserved throughout evolution. The other domain, located at the amino-terminal end of ets-2, is less homologous to the chicken proto-oncogene. Thus far, this region has not been identified in species below chicken. The third domain, which is located centrally, is totally diverged, and this region is missing in the human ets-related gene (erg). This domain appears to be highly conserved in the ets-1 gene that is localized on human chromosome 11.

Although the mammalian ets genes are present on two chromosomes, the chicken ets sequence is present as a single locus with v-ets homologous sequences found in nine regions over about 60 kb of genomic DNA. The major sequence difference between the v-ets and c-ets is found at the 3′ end, resulting in different carboxyl termini of p135 and the chicken proto-ets product. The chicken locus is primarily expressed in normal thymus cells as a 7.5 kb mRNA. The first two viral homologous regions are not found in the c-ets transcript or any other minor species, suggesting that they may be cryptic exons. Thus, these results

demonstrate that two major structural differences may have occurred during the transduction of proto-ets sequences by the virus: (1) truncation of sequences present at the 5′-and 3′-ends of the gene; and (2) acquisition of non-coding proto-ets sequences into the virus. Either or both of these differences may be, in part, responsible for the oncogenic potential of this retrovirus.

To better understand the function of the ets gene products, we isolated and purified the proto-ets gene-specific proteins in human cells and tissues. The human ets-2 gene product codes for a 56 KD nuclear protein. We have found that the ets-2 protein is phosphorylated and has a rapid turnover with a half-life of 20 min. When the human lymphocytic CEM cells were treated with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), the level of the ets-2 protein was quickly elevated by five- to 20-fold. The effect of TPA was mimicked by a synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG) and was blocked by the protein kinase C inhibitor, H7, indicating that protein kinase C is involved in the induction process. The increase in the ets-2 protein appeared to be due to its stabilization, because the protein had a half-life of more than 2h in the presence of TPA, and the ets-2 mRNA level did not increase significantly upon TPA treatment. The protein synthesis inhibitor, cyclo- heximide, enhanced the effect of TPA on the ets-2 protein and could slow the turnover of the protein by itself. The properties of the ets-2 protein, such as nuclear localization, phosphorylation, rapid turnover and response to protein kinase C, indicate that it belongs to a group of oncogene proteins which are generally thought to have regulatory functions in the nucleus (e.g., fos, myc, myb and p53). Our results suggest that protein kinase C, either directly or indirectly, regulates the level of the ets-2 protein by post-translational mechanisms.

Having shown that specific regions of the ets-gene are highly conserved in insects, invertebrates and higher mammals, we have observed that ets-2 is expressed in a wide variety of proliferating cells, as well as in a model murine hepatic regenerating system. We observed that there is a transient accumulation of ets-2 messenger RNA during the early stages of liver regeneration. Also in the differentiating Drosophila and sea urchin developmental stages, the ets-2 expression reaches a maxima during early stages of embryo development. By contrast we have shown that the ets-1 gene was more highly expressed in the mouse thymus than in other tissue and their level did not change between 1 week and several months of age. To determine whether ets-1 and ets-2 gene expression could be detected in earlier stages of thymocyte development, we studied their expression in fetal, neonatal and young thymocyte. The ets-1 expression coincides with the initial appearance of mature $CD4^+(CD8^-)$ thymocytes and is developmentally regulated. Thymocytes are a heterogeneous population of cells and therefore we decided to see if the ets gene expression corresponded to the appearance of specific subsets. We separated thymocytes into DN, ($CD4^-CD8^-$), dLy-1,($CD4^-CD8^-$low CD5), $CD4^+(CD8^-)$, and $CD8^+$ ($CD4^-$) subsets. The ets-1 mRNA level is about 10-fold higher in $CD4^+$ ($CD8^-$) thymocytes than in the DN, dLy-1, or $CD8^+$ ($CD4^-$) subsets. Moreover, the level of ets-1 RNA found in unfractionated thymocytes could be almost entirely accounted for in the $CD4^+$ ($CD8^-$)subset. The ets-2 RNA was also found to be more highly expressed in the $CD4^+$ ($CD8^-$)subset (about 8-fold greater) than that found in the other three thymocyte subsets examined. Although both the ets-1 and ets-2 mRNAs were present in the $CD4^+(CD8^-)$ subset, normalization of the mRNA amounts demonstrates that the ets-1 mRNA level is 10-fold higher than ets-2 in this subset. Thus, these results show that the

elevated ets expression in $CD4^+$ thymocytes agrees well with our earlier results obtained from developing thymocytes. However, the expression of the ets-1 gene is much lower in peripheral $CD4^+$ ($CD8^-$) T-cells as compared to $CD4^+$ ($CD8^-$) thymocytes.

During the past year, a major genetic linkage analysis of human chromosome 21 in the region q22, specifically involved by the A(8;21) breakpoint of acute myelogenous leukemia (AML-M2), has been carried out. The results obtained have shown that the DNA sequences affected by this rearrangement is proximal to both ETS2 and the ETS2-related gene, ERG, on chromosome 21. We have found that the breakpoint is at least 17 cM away from the centromere relative to the ETS genes. This study reveals that the ETS genes are not directly involved. At least one transcriptionally-active gene separates the ETS genes from the breakpoint. The result of this study has allowed researchers to focus upon other genes or regulatory regions proximal to ETS that may be directly disrupted by the translocation.

Dosage analysis using specific fibroblast cell strains, presenting aneuploidies for different chromosome 21 segments, as well as fibroblasts, lymphocytes and brain tissue from patients with complete trisomy 21 have been carefully quantitated. We have, in particular, examined tissues from sporadic and familial Alzheimer's diseases which have positively indicated that the ets-2 gene in the 21q22 region could be a possible "candidate" gene of relevance as it is in the genetic region of DS. What we found was that the ets-2 is not either rearranged or triplicated in every instance where tissue from Alzheimer's patients were probed using various ETS family genes (both with the sporadic and the familial form of the disease). Our data thus excludes, with a great deal of certainty, that ets-2 is involved in Alzheimer's disease pathogenesis.

The predicted ets-2 gene product possesses three distinct domains when compared to v-ets. The domain closest to the carboxyl-terminus appears to be very highly preserved in all species examined, and the gene (ets-2) has been isolated, cloned and recently sequenced in our laboratory from the amphibian, Xenopus, the invertebrate species, Drosophila, and the sea urchin. There is a high (>90%) degree of conservation, which implies a functional importance for the proteins encoded from the proto-ets gene derived from such evolutionarily distinct organisms. Using polytene salivary chromosomes of Drosophila we have been able to localize the D-ets and ets-like gene, elg, to specifically distinct chromosomal-banded regions. These gene products are differentially expressed during early stages of Drosophila development as they are during sea urchin and Xenopus embryogenesis. Thus, it would appear that these proto-ets genes may have some important functional role that would justify their preservation througout evolution.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04963-12 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Toward a Molecular Description of Malignant Transformation by p21 ras Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Y. Shih	Research Chemist	LMO	NCI
Others:	L. S. Ulsh	Microbiologist	LMO	NCI
	P. Saikumar	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have studied biochemical properties and biological activities of ras gene mutants of Harvey murine sarcoma viruses. Mutations with amino acid substitutions were constructed by oligonucleotide-directed mutagenesis of the proviral DNA clone, pH-1. Mutations of p21 were made at the novel site(s) phosphorylated by protein kinases A and C. Novel p21 phosphorylation was found in cells expressing high levels of this product of c-H- and c-K-ras gene. In vivo phosphorylation was stimulated by both protein kinase C (PKC) and protein kinase A (PKA) activators. The same serine residues were phosphorylated by protein kinases A and C in vitro. By peptide mapping, it was found that these protein kinases phosphorylated p21 at the same site(s) as was found in vivo. Mutations were made on H-ras p21 serine-177, which allow identification of this residue as the major phosphorylation site. The novel site lies in the hypervariable region of p21, which links the globular catalytic domain to the membraneanchoring site at the C-terminus. Mutations were also made at the guanine base binding site and the N-terminal glycine-rich region important for guanidine triphosphatase (GTPase) activity and proto-oncogene activation. The present results indicate that the GTP-binding region is critical for ras gene function.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

T. Y. Shih	Research Chemist	LMO NCI
L. S. Ulsh	Microbiologist	LMO NCI
P. Saikumar	Visiting Fellow	LMO NCI

Objectives:

The major focus of this project is to investigate the molecular biology and biochemistry of the ras oncogenes and their p21 products. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these gene products, with the purpose of contributing to our understanding of the roles of oncogenes in human carcinogenesis, and to develop strategies for human tumor detection, monitoring and intervention. Earlier work on this project has contributed to the identification of the ras oncogenes and their product, p21, in Harvey and Kirsten murine sarcoma viruses (J Virol 1978;25:238-52; Virology 1979;96:64-79), and characterization of the major properties of ras p21, i.e., activities associated with guanine nucleotide binding (Nature 1980; 287:686-91). These observations have led to the current concept that ras proteins function as cellular signal transducers, relaying extracellular growth control signals to intracellular effectors in control of cell proliferation. Many other studies have implicated the roles of ras oncogenes in various aspects of the multistep and multifactor process of human carcinogenesis (Ann Rev Biochem 1987;56:779-827). In collaboration with Drs. Papas and Lautenberger (see Projects Z01CP04899 and Z01CP05120), biochemically-active ras proteins have been overproduced in E. coli, yielding a major research avenue to elucidate, in detail, the structure and function of p21 proteins (Science 1983;221: 858-60). The major emphasis of the current study is to investigate the structure-function relationship of the ras proteins and their roles in cellular signal transduction pathways important for the control of cell growth and differentiation.

Methods Employed:

1. Purification of p21 from E. coli. A recombinant p21 was overproduced in E. coli carrying the plasmid, pJLcIIras I, by raising the temperature from 31°C to 41°C. p21 was then purified to over 95% purity under nondenaturing conditions. Alternatively, p21 was isolated by cell lysis with lysozyme and NP40. After centrifugation, the protein was extracted from pellets with 8 M urea.

2. Phosphorylation of the recombinant p21 by protein kinases. Protein kinase C was purified from rat brain, and p21 was phosphorylated by incubation with protein kinases and gamma-^{32}P-adenosine triphosphate (ATP).

3. In vivo phosphorylation of p21. Cells were labeled with ^{32}P-orthophosphate and p21 was immunoprecipitated with antibodies. Cells were treated with phorbol dibutyrate to activate protein kinase C, or 8-bromo-cAMP to stimulate protein kinase A.

4. Peptide mapping of p21. To study the phosphorylation sites, the phosphorylated p21 was digested with trypsin and peptides were mapped by 2° electrophoresis and chromatography on thin-layer plates.

5. Site-directed mutagenesis. The H-ras oncogene of the proviral pH-1 DNA was cloned into the single-stranded M13 phage. The specific amino acid of ras protein was changed by oligonucleotide-directed mutagenesis of the M13 template DNA. The mutant ras was either reconstructed into the pH-1 clones for transfection assays or inserted into the pJL6 vector for expression of mutant ras proteins in E. coli.

6. Transfection of NIH 3T3 cells. Transforming activities of mutant ras genes were evaluated by transfection into NIH 3T3 cells and selected with the neo^{R} gene marker. Tumorigenicity was evaluated by growth in soft agar and induction of tumors in nude and Balb/c mice.

7. Guanine nucleotide binding assays. p21 was incubated with ^{3}H-guanosine diphosphate (GDP); the binary complex was retained by nitrocellulose filters, and radioactivity was counted.

Major Findings:

It is generally believed that ras proteins couple transduction of receptor-mediated proliferation or differentiation signals through the cell membrane to intracellular effectors. The precise nature of ras-mediated signals and effectors remains to be determined. In yeast, Saccharomyces cerevisiae, RAS2 protein regulates adenylate cyclase activity. In mammalian cells, ras elevates 1,2-diacylglycerol, an activator of protein kinase C in some cell lines, but the direct targets are being delineated. It is well known that viral ras p21 of Harvey and Kirsten sarcoma viruses is autophosphorylated at the threonine-59 site. In this study, we found that cellular ras, which lacks this autokinase site, is also phosphorylated in vivo on serine residues. This novel phosphorylation was stimulated not only by phorbol ester, which activates protein kinase C (PKC), but interestingly, also by permeable cAMP derivatives, which stimulate cAMP-dependent protein kinase A (PKA). This in vivo phosphorylation was mimicked by in vitro phosphorylation of both Harvey and Kirsten ras p21s by PKC and PKA. Peptide mapping indicated that these protein kinases phosphorylated

p21 in vitro at the same site(s) as was found in vivo. By phosphopeptide sequencing and construction of mutants by site-directed mutagenesis, we identified serine-177 of Harvey p21 as the major phosphorylation site for both PKC and PKA. This novel phosphorylation site lies in the hypervariable region of p21, which in the three-dimensional structure determined for Harvey p21 links the globular catalytic domain of p21 to the membrane-anchoring site at the C-terminus. Location of this phosphorylation site at this flexible joint region of p21 suggests that this novel phosphorylation plays a role in transmitting signals from interaction of p21 with membrane components, such as receptor molecules to the globular catalytic domain of p21 molecules, and thus regulates the coupling function of p21 to intracellular effector molecules.

Publications:

Clanton DJ, Lu Y, Blair DG, Shih TY. Structural significance of the GTP-binding domain of ras p21 studied by site-directed mutagenesis. Mol Cell Biol 1987;7: 3092-7.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05120-09 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression of Retroviral and Oncogene Proteins in Bacteria

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO NCI
Others:	Z. Q. Chen	Visiting Associate	LMO NCI
	L. Burdett	IRTA Fellow	LMO NCI
	T. S. Papas	Chief	LMO NCI
	F. Wong-Staal	Biologist	LTCB NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We recently have expressed a segment of the Xenopus laevis ets-2 gene in bacteria. The gene is related to the v-ets gene on the oncogenic avian retrovirus, E26. The expressed protein will be used to raise antibodies that will be used for studies on the ets-related proteins in that organism. Since Xenopus has been extensively studied by developmental biologists, this study should give insight into the function of the ets genes.

The bacterially-expressed human immunodeficiency virus (HIV) sor protein (r-sor) produced by LMO has been shown to be of potential use in the diagnosis of latent HIV infection, since antibodies against r-sor appear before enzyme-linked immunosorbent assay (ELISA) seroconversion in many patients (Ranki et al. 1987;2:589-93). Antibodies raised against r-sor have been used to verify the absence of the HIV p23 sor protein in cells infected with viruses with mutant sor genes and to demonstrate differences in the level of sor expression in different virus-infected cell lines (Fisher et al., Science, 1987;237:888-93).

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO	NCI
Z. Q. Chen	Visiting Associate	LMO	NCI
L. Burdett	IRTA Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI
F. Wong-Staal	Biologist	LTCB	NCI

Objectives:

We are using bacterial expression systems for three major applications: (i) production of biochemically active proteins; (ii) production of selected antigens for detecting specific antibodies in sera; (iii) as immunogens in raising antisera that can be used for the characterization of "native" proteins. Currently, we are applying the system to applications concerning the v-ets oncogene, the c-ets proto-oncogene, and proteins from human retroviruses.

Methods Employed:

1. Recombinant DNA procedures. The recombinant DNA procedures used were described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

2. Purification of heterologous proteins expressed in bacteria. Plasmids containing DNAs inserted into the pJL6 vector (Lautenberger et al., Gene 1983;23:75-84) or one of its derivatives were introduced in Escherichia coli cells carrying a temperature-sensitive allele of the phage lambda repressor (cI857) on a defective prophage. Such bacteria were grown at 32° to an optical density at 590 nm of 0.5 and the culture temperature was then shifted to 42°. After shaking for 1 hr at the elevated temperature, the cells were pelleted and proteins were extracted as described by Krippl et al. (Proc. Natl. Acad. Sci. USA 1984;81:6988-92. Generally, the expressed heterologous protein is found in the KSCN pellet and can be solubilized in 7 M urea or 7 M guanidinium thiocyanate.

3. Reverse phase high-performance liquid chromatography of proteins. The guanidinium hydrochloride extract of the KSCN pellet fractions was brought to a concentration of 0.1% trifluoroacetic acid and applied to a Waters Associated C_{18} Bonda Pak column pre-equilibrated with 0.1% trifluoroacetic acid in water. The protein was eluted from this Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) column with a linear gradient of 0-80% acetonitrile which also contained 0.1% trifluoroacetic acid. Protein was detected in the column fractions by Coomassie blue staining of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) lanes or by immunoblotting.

4. Immunoblot detection of expressed heterologous proteins. Protein fractions were subjected to electrophoresis on 10% SDS-PAGE and immobilized on nitrocellulose filters. Strips containing the transferred proteins were

detected by incubation of the washed strips with 125-I Staphlococcus aureus protein A followed by autoradiography.

5. Preparation of antisera directed against expressed heterologous proteins. Rabbits were immunized against 100 μg of bacterially expressed protein purified by RP-HPLC. The initial immunization was followed by two 100 μg booster doses 10 and 20 days later. Response to the immunogen is monitored by immunoblot analysis using RP-HPLC purified protein as antigen. Such sera may be used to detect proteins in radiolabeled cell extracts by immuno- precipitation (Kan et al., 1986;231:1553-55).

Major Findings:

1. Expression of Xenopus laevis ets-2 amino acid sequences in bacteria. A 394 bp segment of the Xenopus ets-2 gene has been introduced into the plasmid pJLA16 derivative of pJL6. The inserted gene was expressed as described in "Methods Employed." A 17 kDa protein was observed in induced cells containing the insert, but not in uninduced cells or in induced cells containing the plasmid in the opposite orientation. The size of this protein is consisent with the size of the inserted DNA segment. The protein reacts with anti-ets peptide serum B (Fujiwara et al., Oncogene 1988;2:99-103) in a reaction that can be completely inhibited by competing peptide. This indicates the expressed protein contains ets-related epitopes. The expressed Xenopus ets-2 protein will be used to raise antibodies in rabbits. Such antibodies will be useful in identifying the ets-2 gene product in Xenopus (see project Z01CP05484-03 LMO). Since Xenopus has been widely studied by developmental biologists, characterization of ets-related proteins in this organism may provide clues to the function of the ets proto-oncogene.

2. Diagnosis of latent HIV-1 infection using bacterially-expressed sor product. The bacterially-expressed HIV sor protein (r-sor; Kan et al., Science 1986;231:1553-5) produced by LMO has been used in a recent study published by workers in the NCI Laboratory of Tumor Cell Biology (LTCB) and collaborators (Ranki et al., Lancet 1987;2:589-93). They surveyed stored serum samples from homosexual men that had been collected prior to seroconversion (as defined by commercial ELISA assay). In five out of nine such individuals, pre-seroconversion sera reacted with the r-sor in an immunoblot assay. This indicates that the r-sor has potential usefulness for diagnosis of latent HIV-1 infection.

3. Analysis of the HIV sor gene product using sera against r-sor. Fisher et al. (Science 1987;237:888-93), used antibodies produced by LMO that had been raised against r-sor to detect the p23 HIV sor gene product. They found that this protein was absent in MOLT-3 cells infected with viruses with mutant sor genes, but present in cells infected with control wild-type virus. They also found that the quantity of sor protein varied between different host cell lines, even if they were infected by the same virus. For example, infected MOLT-3 cells contained much more sor than infected H9 cells.

Publications:

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Samuel KP, Konopka A, Seth A, Lautenberger JA, Papas TS. The 3′-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Lett 1988;218:81-6.

Van Beneden RJ, Watson DK, Chen TT, Lautenberger JA, Papas TS. Teleost oncogenes: evolutionary comparison to other vertebrate oncogenes and possible roles in teleost neoplasms. Marine Environ Res 1988;24:339-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05238-07 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	D. Blair	Supv. Res. Chemist	LMO	NCI
	G. Mavrothalassitis	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

Developmental Genetics Lab., Johns Hopkins Hospital, Baltimore, MD (R. Reeves); Dept. Molecular Biology, U. California, Berkeley, CA (P. H. Duesberg); Program Resources, Inc., Frederick, MD (S. Showalter, M. J. McWilliams)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	1.6	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The functional relationship between the onc genes of transforming retroviruses and their cellular prototypes has been facilitated by structural comparisons at the nucleic acid and predicted protein levels. We have determined the complete nucleotide sequence of the chicken protein-ets gene and compared it to the ets gene of E26. E26 is a genetic hybrid with sequences derived from viral structural genes and parts of essential cellular proto-onc genes. The chicken ets gene is present as a single locus with v-ets homologous sequences found in nine regions over 60 kb of genomic DNA. In addition, the cellular gene contains unique 5′ and 3′ sequences. Thus, the E26 virus demonstrates (1) substitution of viral genes for parts of normal cellular genes, (2) truncation of the gene, and (3) acquisition of non-cellular coding proto-ets sequences. These structural differences may be responsible for the oncogenic potential of this retrovirus. We have previously determined that the mammalian homologs of v-ets are located on different chromosomes. The mammalian ets-2 genes from man and mouse encode for nearly identical amino acids and are over 90% conserved relative to the chicken ets gene. The ets-2 gene appears to have mitogenic activity upon transfected cells. The human ets-1 gene product is over 95% identical to the chicken gene from where the virus transduced ets sequences. Alignment of the predicted ets proteins with v-ets suggests that three domains exist. The domain closest to the carboxyl-termini is highly conserved in all genes characterized from species ranging from human to Drosophila. The domain located at the amino-terminal end of ets-2 is less homologous to v-ets, while the ets-1 gene and the v-ets gene are similar in this region. The central domain of v-ets is found to be similar only to that of ets-1, being very divergent in ets-2. Recombinant DNA technology will be used to generate mutants to evaluate the function of these three domains.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO NCI
T. S. Papas	Chief	LMO NCI
A. Seth	Visiting Scientist	LMO NCI
D. G. Blair	Supv. Res. Chemist	LMO NCI
G. Mavrothalassitis	Visiting Fellow	LMO NCI

Objectives:

The purpose of this investigation is to determine the relationship between onc genes and their normal cellular homologues. Structural analysis of these genes will allow us to better understand their biological functions. In addition, we plan to evaluate the involvement of proto-onc genes in human malignancy.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction of low melting agarose.

3. Preparation of DNA probes using purified onc-specific DNA by nick-translation using E. coli DNA polymerase and DNaseI.

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of onc probes.

5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to cosmid or λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of onc-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (Proc Natl Acad Sci USA 1975;72:3961-5).

7. Subcloning of isolated DNA fragments into appropriate plasmid vectors, as required.

8. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560) and/or Sanger (Proc Natl Acad Sci USA 1977; 75:5463-7). In addition, uniquely labeled DNA will be sequenced following RNA-directed primer extension.

9. Total cellular RNA from cultured cells or tissues was prepared by the guanidine isothiocyanate method. In addition, RNA was prepared after cellular fractionation in the presence of RNase inhibitors. Separation of polyA+ and polyA- RNA by two cycles of purification through oligo(dt) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λgt10 vector DNA for amplification.

11. Cloning of onc-genes and proto-oncogenes into prokaryotic vectors capable of overexpression of inserted DNA. Isolation of proteins used for antigens and for functional studies.

12. Controlled expression of oncogenes in eukaryotic vectors, using constructions with regulatable promoters. Transfection of eukaryotic vectors in mammalian cell lines for identification of expressed product(s) and for analysis of possible biological activity associated with the construct.

13. Identification for promoter and regulatory sequences present in genomic clones of the cellular homologs of oncogenes. To include nucleotide sequence analysis and functional assays using expression of defined reported genes, such as chloramphenicol acetyltransferase (CAT).

14. Nuclease protection assays to define the 5′ and 3′ ends of transcripts. Verification by sequence analysis of cDNA clones and by primer-extension of mRNA.

Major Findings:

1. Sequences related to ets (one of the two cellular genes present in the avian retrovirus, E26) have been detected by restriction enzyme digestion and Southern blot analysis using genomic DNA derived from vertebrates (avian and mammalian species) and invertebrates (Drosophila).

2. Chicken, mouse and human genomic libraries were screened and specific clones have been isolated. In addition, human, chicken and mouse cDNA clones have been isolated and subjected to nucleotide sequence analysis.

3. Chicken DNA segments homologous to the ets region were molecularly cloned and shown to be almost identical to v-ets by sequence analysis. The chicken ets sequence is present as a single locus with v-ets homologous sequences found in nine regions over about 60 kb of genomic DNA. Each region is bordered by consensus splice signals.

4. The chicken and viral ets genes are not homologous at the 3' end; thus, the transforming protein of E26, p135 and the cellular ets gene product have different carboxyl termini.

5. Northern analysis of chicken thymus RNA, using probes from the first two viral homologous regions, failed to detect the major chicken c-ets transcript (7.5 kb).

6. Chicken thymus RNA was used to prepare a complementary DNA library for isolation of an ets cDNA clone. Sequence analysis of an ets cDNA defines the complete open reading frame as including 441 amino acids. With the exception of 27 amino and 13 carboxyl terminal amino acids, the chicken proto-ets gene is identical to the v-ets.

7. cDNA clones for human and mouse ets-1 and ets-2 loci have been isolated and sequenced. This analysis demonstrated a strong conservation of amino acids (over 90%), suggesting that these genes perform an important function. The predicted amino acids encoded by the 469 (human) and 468 (mouse) ets-2 genes are similar (91% identity).

8. The predicted ets-2 proteins possess three distinct domains when compared to v-ets. The domain closest to the carboxyl-termini is highly conserved (>90%) and this conservation is seen to be widely preserved throughout evolution, including Drosophila. The domain located at the amino-terminal end of ets-2 is less homologous to the virus/chicken proto-oncogene and, thus far, this region has not been identified in lower eukaryotes. The third domain, which is located centrally, is diverged in ets-2 genes, but is conserved in the ets-1 gene.

9. The predicted ets-1 protein is nearly identical to the 441 amino acids of the chicken proto-ets gene, having over 95% amino acid in common.

10. Both genetic loci are transcriptionally active in birds and mammals, yielding distinct products.

11. The expression of ets-1 and ets-2 genes in human cell lines has been assayed by Northern blot analyses. The pattern seen suggests that these two genes are independently regulated.

12. Viral ets fragments, and human ets exons and cDNA have been placed in appropriate systems for protein overexpression and these proteins have been utilized to elicit antibody response.

13. A large coding segment of human ets-2 has been inserted into a eukaryotic expression vector under the transcriptional control of the heat shock promoter. Such constructs will be microinjected into fertilized mouse eggs to assess the role of ets-2 during development and to establish cell lines with inducible ets-2. The entire human and mouse cDNA open reading frame has been inserted into another vector under control by the metallothionein promoter. Transfection of NIH cells with this construct yields foci.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05295-07 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Activation of Oncogenes in Viruses and Human Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. G. Blair	Supv. Research Chemist	LMO	NCI
Others:	K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI
	Y. Lu	Visiting Fellow	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	E. Priel	Visiting Scientist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

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LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have analyzed the DNA sequence of a portion of the ovc oncogene, a human transforming sequence activated during DNA transfection and derived from the human ovarian carcinoma cell line, OVCAR-3. Comparison of this sequence with sequences contained in the GenBank data base reveals no significant homologies, suggesting that, consistent with previous hybridization data, ovc represents a previously unidentified human oncogene. Analysis of poly-A+ RNA from several normal and transformed human cell lines indicates that ovc sequences are expressed in the OVCAR-3 parent cell line and three other human adenocarcinomas.

We had previously shown that treatment of mouse fibroblasts with tunicamycin, a glycosylation inhibitor, renders them susceptible to infection by RD114 cat endogeneous virus and its pseudotypes. We have now shown that several other inhibitors of glycosylation, which act at different points along the glycosylation pathway, also have a similar effect. In addition, mouse lymphoid cells exhibit similar properties when exposed to tunicamycin. This suggests that this may represent a common feature of mouse cells.

We have tested the ability of cell DNA from two cell lines derived from a methylcholanthrene-induced mouse fibrosarcoma (TN-10), and which exhibit differing metastatic potentials, to transform NIH 3T3 cells. Foci are induced following transfection at high efficiencies, and transformed cells have acquired the ability to grow in the absence of serum or protein growth factors. No rearrangements of several well-characterized oncogenes are detected in transfected cells, but foci appear to contain amplified copies of the murine K-ras oncogene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. G. Blair	Supv. Research Chemist	LMO	NCI
K. J. Dunn	Bio. Lab. Tech. (Micro.)	LMO	NCI
Y. Lu	Visiting Fellow	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI
A. Seth	Visiting Scientist	LMO	NCI
E. Priel	Visiting Scientist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses (MSV) and the function of specific gene products of MSV in this process.

To develop screening and selection systems to identify and isolate human transforming DNA sequences from primary human tumors and tumor cell line DNAs. To identify, isolate and characterize such sequences, and their gene products. To characterize the normal cellular homologs of such sequences, and to determine the mechanism by which their oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA transfection, measurement of tumorgenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect the expression of specific cellular proteins.

Major Findings:

1. The ovc oncogene sequences, localized on human chromosome 9, represent a unique set of transforming sequences which are expressed in the human ovarian tumor line, OVCAR-3, and three adenocarcinoma-derived cell lines, but not in several other human tumor lines or normal human fibroblasts. We had previously identified a set of transforming human DNA sequences, generated by a transfection-induced gene fusion of two human genetic loci, which would transform NIH 3T3 cells to a morphologically-transformed, serum-independent, tumorigenic state. We haved cloned a portion of those transforming sequences and identified a 2.2 kb fragment which hybridizes to unique sequences located on human chromosome 9. Sequence has been obtained for approximately 1.1 kb of the chromosome 9 specific fragment. Comparison of this sequence with the GenBank data base reveals no significant homologies. This strengthens our previous hybridization analysis which indicated that the ovc sequence represents a novel human oncogenic sequence.

We have also analyzed poly A+ RNA from several normal and transformed human cell lines for expression of the ovc sequences. We have detected expression of two RNA species of 2.0 and 3.7 kb homologous to the 2.2 kb ovc fragment in

OVCAR-3, the parent tumor line from which ovc was isolated, and three other human adenocarcinoma-derived cell lines. Several other human tumor-derived cells, as well as normal human fibroblasts, did not express detectable ovc RNA.

2. The glycosylation inhibitors, 2-deoxy glucose, swainsinone and castanospermin, as well as tunicamycin, enhance the susceptibility of mouse fibroblasts and lymphoid cells to infection by viruses containing envelope components derived from the cat endogenous virus RD114. We had previously determined that tunicamycin, an inhibitor which blocks the first step of N-linked glycosylation in mammalian cells, renders mouse fibroblasts susceptible to infection by pseudotypes of RD114, a cat endogenous retrovirus. In an attempt to understand the mechanism of this effect, we tested the effects of several other inhibitors which affect glycosylation at an early stage (2-deoxy glucose) or a late stage (swainsinone and castanospermin) in the pathway. Cells treated with each of the inhibitors tested became susceptible to RD114 virus infection within 24 hours of treatment, and each showed a different optimum concentration for maximal susceptibility. The two late-stage inhibitors were less efficient at inducing susceptibility, but treated cells were still 500-fold more sensitive than untreated controls. In addition, two mouse lymphoid cell lines, MEL and NCS-1, also became susceptible when treated with tunicamycin, suggesting that the effect is a property of mouse cells in general. It suggests that alterations in cell glycosylation either alters the specificity of existing viral receptors, or exposes previously masked or non-functioning receptors to the RD114 virus.

3. Cadmium induction of cells transformed by v-mos under the control of a metallothionein promoter is toxic. We had previously linked v-mos to the mouse metallothionein promoter, which is inducible by heavy metals or glucocorticoids, in order to express high levels of mos protein in mammalian cells in culture. Cells expressing mos fused at the amino terminus to human growth hormone or tissue plasminogen activator-derived sequences, and induced by cadmium, expressed high levels of mos fusion protein, but showed only low levels of cell toxicity. In contrast, cells expressing an unfused mos product showed high levels of cell death when exposed to even low levels of cadmium. These results are consistent with the hypothesis that overexpression of large amounts of normal v-mos is toxic to mouse fibroblasts, and provides a system in which to investigate the mechanism of that toxicity.

4. DNA from methylcholanthrene-induced mouse fibrosarcomas contain a transfectable oncogene sequence which may be K-ras. Two cell lines, IC9 and IE7, which differed in their metastatic potential, had been isolated from the mouse methylcholanthrene-induced sarcoma, TN-10. Transfection of DNAs isolated from both cell lines reproducibly induced foci at high efficiency on NIH 3T3 cells, and transfected cells formed tumors efficiently in nude mice. Cells from individual foci were able to grow in defined media (QBSF-51) in the absence of serum or protein growth factors. DNA from primary foci could also induce foci in transfected Rat-1 fibroblasts, as well as NIH 3T3 cells. Southern analysis of DNA from primary or secondary transfectants showed no rearranged sequences homologous to the oncogenes H-,K- or N-ras, mos, met, raf, trk, fms, or fgfIII. The transforming activity was inactivated by each of seven restriction enzymes. Transfectants did show the presence of amplified

copies of an unrearranged murine K-ras. Our data suggests that this may be responsible for the transfectable transforming activity, and analyses of the nature of the activating modification are in progress.

5. Primate c-mos is biologically active. The c-mos proto-oncogene of an Old World monkey was linked to Moloney murine sarcoma virus-derived long terminal repeat (LTR) sequences and transfected into NIH 3T3 cells. Constructs in which the LTR was linked to monkey c-mos upstream of an overlapping open reading frame present in the locus were inactive in focus formation, but constructs in which the LTR was inserted immediately 5′ to the mos open reading frame induced transformed foci at low efficiency. The transforming efficiencies were similar to those observed previously for human c-mos. The results are consistent with a model in which, like previously suggested for the human mos gene, the transforming ability of monkey mos is low in mouse cells, and the 5′ open reading frame acts to reduce or eliminate the transforming potential.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05440-04 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Site-Directed Mutagenesis of ras Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. J. Clanton	Senior Staff Fellow	LMO	NCI
Others:	T. Y. Shih	Research Chemist	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	Y. Lu	Visiting Fellow	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI

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1.6	1.4	0.2

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Oligonucleotide-directed, site-specific mutagenesis is used to dissect the biochemical basis of oncogenic activation and enzymatic activity of the ras oncogene.

Point mutations of p21 proteins were constructed by oligonucleotide-directed mutagenesis of the v-ras-H oncogene, which substituted amino acid residues within the presumptive nucleotide-binding consensus sequence, GXGGXGK. When the glycine residue at positions 10, 13, or 15 was substituted with valine, the viral ras-H product, p21, lost its guanosine triphosphate (GTP)-binding and autokinase activities. Other substitutions at position 22, 33, 51 or 59 did not impair its binding activity. G418-resistant NIH 3T3 cell lines were derived by transfection with constructs obtained by inserting the mutant proviral DNA into the pSV2neo plasmid. Clones with valine mutation at positions 13 or 15 were incapable of morphologically transforming cells, while all other mutants with GTP-binding activity were competent. Ras, with a valine mutation at glycine-10, which had lost its ability to bind GTP and its autokinase activity in vitro and in vivo, was fully capable of transforming NIH 3T3 cells. These cells grew in soft agar and formed tumors in nude mice. The p21 of cell lines derived from tumor explants still lacked the autokinase activity. In contrast, cells transfected with position-valine or a position-116 tyrosine ras mutant were tumorigenic in nude mice, but not immunocompetent in Balb/c mice. Cells transfected with the 15 valine mutant, however, did not consistently induce tumors in nude mice.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. J. Clanton	Senior Staff Fellow	LMO NCI
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L. S. Ulsh	Microbiologist	LMO NCI
Y. Lu	Visiting Fellow	LMO NCI
D. G. Blair	Supv. Research Chemist	LMO NCI

Objectives:

The objective of this project is to study the biochemical basis of oncogenic activation and enzymatic activity of the ras oncogenes and their products by site-directed mutagenesis. Mutant proteins developed in this way can be expressed in a bacterial expression system and the comparative biochemistry of the mutant proteins studied. The oncogenicity of the mutant viruses thus generated can be assessed by in vitro NIH 3T3 cell transfection and by in vivo animal tumor formation.

Methods Employed:

Oligonucleotides of 17 bases containing single base changes from the v-ras^H gene are used for site-directed mutagenesis as described by Zoller and Smith (DNA 1984;3:479-88).

We have also constructed a plasmid which contains the entire genome of Harvey murine sarcoma virus linked to the neomycin resistance gene of pSV2neo, which confers resistance to the drug G418 (J Mol Appl Genet 1982;1:327-41). This system will allow us to select for ras-transfected cells by a phenotype unrelated to the transforming ability of the ras gene. Therefore, mutations which may abrogate the ability to transform cells can be easily detected and isolated.

A number of other laboratory techniques are utilized for these studies. Ras genes (as well as other DNA fragments) are isolated by gel electrophoresis and identified by blot hybridization. Proteins expressed by this system, purified by column chromatography, and analyzed by polyacrylamide gel electrophoresis and Western blotting show the GTP-binding domain of p21 is crucial for its cellular function. Biochemical studies of the mutated ras proteins employ filter binding assays and immunoprecipitation by specific monoclonal antibodies. Tumorigenicity is measured either by colony formation in agarose or by injection of cells into nude mice.

Major Findings:

Some ras mutants which fail to bind GTP or induce morphological transformation in cells in culture induce a tumorigenic phenotype when introduced into NIH 3T3 cells. We constructed a series of mutants of the Harvey v-ras oncogene which introduced altered amino acids at positions 10(V), 13(V), 15(V), 22(K), 33(H),

51(S), and 116(Y). Previously, we had shown that the 10(V) mutant, which had lost its GTP-binding capacity, was still capable of inducing morphological transformation and tumorigenicity when introduced into NIH 3T3 cells. We have extended the study to include the whole range of mutants, three of which [13(V), 15(V) and 116(Y)] do not show appreciable GTP-binding activity and do not morphologically transform NIH 3T3 cells. Cells transfected with wt-ras or with the 51(S), 33(H), 10(V) and 22(K) mutant constructs are morphologically transformed and form tumors in nude mice within 1-2 weeks of injection, and all but 22(K) are also tumorigenic in immunocompetent Balb/c mice. Cells transfected with 13(V) and 116(Y) are morphologically normal and induce tumors with a latency of 5-6 weeks in nude mice. Cells transfected with 15(V) occasionally formed tumors at 7-9 weeks, while control cells transfected with pSV2neo alone formed tumors only after 10 weeks. Cells transfected with 22(K), 13(V), 15(V), 116(Y) and pSV2neo were not tumorigenic in Balb/c mice. These results suggest that neither GTP-binding nor morphological transformation are necessary for the induction of tumors by viral H-ras, and suggest that second site mutations in v-ras, which already contains activating mutations, can attenuate the expression of the v-ras-induced phenotype in NIH 3T3 cells.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05441-04 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Gene Products of the c-myc Locus and the c-ets Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	S. Fujiwara	Visiting Associate	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	A. De Klein	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

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Transgenic Analysis Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.8	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets gene family consists of a group of genes which are highly homologous to the 3′ domain of the oncogene carried by the avian acute leukemia virus, E26. A peptide antibody corresponding to a hydrophilic and highly conserved 3′ amino acid sequence of the human ets-2 protein was shown to specifically react with all known ets proteins (p135 v-ets, ets-1, ets-2, erg-1, erg-2), as well as several other uncharacterized ets-related antigens. The ets-2 protein was identified by use of monoclonal antibodies prepared against a bacterially expressed ets-2 protein. The ets-2 protein was shown to have several properties in common with other known nuclear oncogenes, including low abundance, fast turnover, nuclear localization and response to mitogenic stimuli. By contrast, the ets-1 protein is a cytoplasmic phosphoprotein. Two-dimensional gel electrophoresis of these proteins show their negative charge with an apparent isoelectric point of less than pH 6.0.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI
T. S. Papas	Chief	LMO	NCI
A. Seth	Visiting Scientist	LMO	NCI
A. De Klein	Visiting Fellow	LMO	NCI

Objectives:

The objective of this project is to characterize the protein products of the human proto-oncogenes, c-myc, c-ets-1 and c-ets-2. The characterization includes identification, subcellular localization, and determination of function for the normal gene products.

Methods Employed:

Biological Materials: Cell lines are used for enriched sources for the ets and ets-related proteins, including the human adenocarcinoma line, COLO 320, the human T-cell leukemia line, CEM and the Burkitt lymphoma line, Daudi. The COLO 320 cells are a rich source of myc proteins, ets-2 and erg proteins. Daudi cells are a source of ets-1 and CEM cells produce both ets-1 and ets-2. Normal tissues, such as mouse thymus or calf thymus, are used as an enriched source of c-ets proteins.

Protein Isolation: We have found three methods to extract the ets-2 proteins from cells. The cells or tissues are subfractionated into nuclear and cytoplasmic fractions. The nuclei (containing the majority of the ets-2) are extracted with urea, low ionic strength buffers or with 0.42 M NaCl. Our initial studies used urea-denatured extracts which allowed the isolation of an ets-related protein using fast protein liquid chromatography (FPLC) or high pressure liquid chromatography (HPLC) and preparative sodium dodecyl sulfate (SDS) gel electrophoresis. This was done to determine the N-terminal amino acid sequence using electroblotting and gas-phase microsequencing techniques. Two-dimensional gel electrophoresis was used to determine the purity of the preparations and to compare with the established two-dimensional maps of nuclear proteins.

Protein Sequence Analysis: The Advanced Scientific Computer Laboratory (ASCL) VAX and CRAY supercomputer facility is used to analyze amino sequences of the ets and ets-related proteins.

Major Findings:

Antibodies: Our work with the characterization of the anti-peptide antibody to the sequence, FKLSDPDEVARRW, led to the realization that this antibody recognized all of the known ets proteins, including ets-1, ets-2, erg-1, erg-2, v-ets, as well as other cellular proteins which may be defined as ets-related antigens. This antibody is a pan-ets reagent. Monoclonal antibodies were

developed against the bacterially expressed ets-2 protein and these recognize a subset of the p56 proteins previously identified with the **pan-ets** antibodies. Two-dimensional gel electrophoresis of proteins immunoprecipitated from 35-S-methionine-labelled COLO 320 or CEM cells allowed the distinction between the ets related antigens identified with the **pan-ets** antibody and the ets-2 protein identified with the ets-2 monoclonal antibody.

Characterization of the ets-2 protein: We have shown that the nuclear ets-2 protein is phosphorylated, has a 20-minute half-life, is an acidic protein, and responds to the mitogenic activation by protein kinase C by increasing its half-life from 20 minutes to greater than 2 hours. These properties suggest that the ets-2 protein is a nuclear regulatory protein with properties similar to those of fos, myc, myb, and p53.

Characterization of ets-related proteins: We have found several ets-related proteins with the **pan-ets** antibody. These proteins are also nuclear proteins, but are positively charged and at least a p31 and p37 co-purify with hnRNP proteins. The positively-charged p56, ets-related protein co-extracts with the hnRNP and snRNP proteins, but does not seem to be physically associated with these proteins. The erg proteins have also been identified using the **pan-ets** antibody; they seem to be localized as nuclear proteins only in those specific cell lines in which they are found expressed.

ets Proteins in Tissues: The ets proteins are highly expressed in mouse thymus. The ets proteins correlate with the high expression of ets mRNA found in the $CD4^+$ ($CD8^-$) thymocyte subset, indicating that these proteins may have an important role in T-cell development, as well as in thymocyte proliferation.

Immunohistochemistry of the ets Proteins: The **pan-ets** antibody has been shown to react with the nuclei of COLO 320 cells, using biotinylated second antibodies and avidin D, coupled with horseradish peroxidase. We now have preliminary data suggesting that the pan-ets and monoclonal ets antibodies react at the periphery of the nucleus using an immunoelectronmicroscopy technique.

In situ hybridization of ets mRNA: We have used v-ets antisense probes to hybridize with methanol-acetone and paraformaldehyde fixed thin sections of mouse thymus. It has been found that the v-ets probe selectively hybridizes with cells in the cortical region of the mouse thymus.

Publications:

Bhat NK, Komschlies K, Fujiwara S, Mathieson BJ, Young H, Fisher RJ, Papas TS. Ets gene expression in murine thymocyte subsets. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;178.

Fujiwara S, Fisher RJ, Bhat NK, Papas TS. Human ets-2 protein: nuclear location, phosphorylation, rapid turnover and induction by TPA. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;107.

Fujiwara S, Fisher RJ, Seth A, Bhat N, Showalter SD, Zweig M, Papas TS. Characterization and localization of the products of the human homologs of the v-ets oncogene. Oncogene 1988;2:99-103.

Papas TS, Bhat NK, Chen TT, Dubois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human diseases. In: Peschle C, eds. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences, 1987;171-91.

Papas TS, Bhat NK, Fujiwara S, Ascione R, Fisher RJ. Temporal regulation of ets gene expression in murine tissue. In: Waymouth C.,ed. Modern cell biology: molecular mechanisms in the regulation of cell behavior. New York: Alan Liss, Inc., 1987;5:123-9.

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Seth A, Blair DG, Dunn KJ, Fisher RJ, Vande Woude GF. High-level expression of human growth hormone-mos ($p58^{hGH\text{-}mos}$) product in C-127 cells. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;112.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05442-04 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human ETS Genes in Human and Cancer Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Sacchi	Visiting Scientist	LMO	NCI
Other:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)* Dept. Neurogenetics, Harvard Univ., Boston, MA (J. F. Gusella); Eleanor Roosevelt Cancer Inst., Denver, CO (H. D. Drabkin); Dept. Biology & Genetics, Medical Faculty, Milan, Italy (L. Weintraub); Down Syndrome Ctr., Genova, Italy (L. Perroni)

LAB/BRANCH

Laboratory of Molecular Oncology

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The localization of human ETS genes at the 11q23 and 21q22 regions suggested a possible involvement of these genes in both constitutional and acquired (neoplasia) diseases, presenting a known cytogenetic abnormality. The 11q23 region is involved in a number of chromosome abnormalities peculiar to acute leukemias of the myelomonocytic lineage. In two of these abnormalities, the translocations (4;11)(q21;q23) and (9;11)(p21;q23), transpositions of the ETS1 gene from its normal position on chromosome 11 to chromosomes 4 and 9, is evident. On the other hand, the 21q22 region is relevant both in human and cancer genetics. In an acquired cytogenetic abnormality specific to AML-M2 leukemias, we found ETS2 transposed from chromosome 21 to chromosome 8. Despite the repositioning of the ETS genes, neither one was found to be structurally involved by the chromosome rearrangements. The role of these genes in the pathogenesis of these leukemias is, therefore, not directly demonstrated, even if "position effect," well known to affect gene regulation at a distance, may somehow alter their expression. The real "cancer genes" involved by the above-mentioned abnormalities, therefore, have to be identified.

As far as the ETS2 gene is concerned, part of the work was aimed at demonstrating whether or not it does belong to the obligate genetic region necessary for the expression of the constitutional aneuploidy known as Down's syndrome. This work led to a preliminary observation, based on a singular patient sample (only very rare Down's syndrome patients are informative), that there are three copies of ETS alleles in the region. It is, therefore, possible that the ETS2 gene belongs to the set of genes needed for the expression of the multitrait Down's syndrome clinical picture.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Sacchi	Visiting Scientist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To extend the genetic and molecular analysis of the chromosome region 21q22>21qter both to analyze the nature--transcriptional vs. nontranscriptional--of the chromosome 21 sequence involved by the t(8;21) rearrangement and to improve the understanding of the minimal genetic region of the complex multitrait pathology of Down's syndrome (DS).

Methods Employed:

1. Employing long-range restriction mapping techniques.

2. Cloning from cosmid library sequences adjacent to chromosome 21 probes known for being proximal and distal to the chromosome junctions $8q^-$ and $21q^+$ breakpoint.

3. Analyzing genetic recombinations of polymorphic loci of the telomere region of chromosome 21 in selected families (parental age) with Down's syndrome occurrence.

Major Findings:

1. Over the past months a major genetic linkage analysis of the region q22 specifically involved by the t(8;21) breakpoint of acute myelogenous leukemia (AML-M2) has been carried out. The results obtained show that the DNA sequences involved in the rearrangement is distal from the centromere and proximal to both the ETS2 and the ETS2-related gene, ERG, located on chromosome 21. The breakpoint is at least 17,000 kilobases away from the centromere relative to the ETS genes, indicating, therefore, that they are not directly involved. At least one transcriptionally active gene separates the ETS genes from the breakpoint. The result of this study has been submitted for publication (Sacchi et al., Genomics).

2. Densitometric analysis on various fibroblastic cell lines containing a variety of chromosome 21 aneuploidies were performed. Additionally, other cell types, such as lymphocytes and brain tissue from patients having a complete trisomy 21, were examined, especially those having a history of sporadic and familial Alzheimer's disease (AD). Other workers had suggested that the ETS2 gene located in the relevant genetic region of DS could be a possible "candidate" gene associated also with AD. Careful quantitation demonstrated that the ETS2 gene was not found either rearranged or triplicated in every Alzheimer's patient (both with the sporadic and the familial form of the disease). Our data exclude, with a great deal of reliability, that ETS2 is

involved in Alzheimer's disease pathogenesis (Sacchi et al., Proc Natl Acad Sci USA, submitted), thus contradicting recent reports (J. M. Delabar et al., Am Genet 1986;29:226-8; Delabar et al., Science 1987;235:1390; Blauquet et al., IXth International Workshop on Human Gene Mapping, 1987, Abstr. 734).

Publications:

Cheng SV, Nadeau JH, Tanzi RE, Watkins PC, Sacchi N, Gusella JF. Synteny in man and mouse of DNA markers from the chromosomal region linked to familial Alzheimer's disease and Down syndrome. Proc Natl Acad Sci USA (In Press)

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Anal Biochem 1987; 162:156.

Dagna Bricarelli F, Pierluigi M, Perroni L, Grasso M, Arslanian A, Sacchi N. High efficiency in the attribution of parental origin of nondisjunction in trisomy 21 by both cytogenetic and molecular polymorphisms. Hum Genet (In Press)

Papas TS, Bhat NK, Chen TT, DuBois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human diseases. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences, 1987;171-91.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Curran T, Skalka A, eds. The oncogene handbook. New York: Elsevier (In Press)

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Sacchi N, Nalbantoglu J, Sergovich FR, Papas TS. The ETS2 gene in the Down syndrome genetic region is not rearranged in Alzheimer's disease. Proc Natl Acad Sci USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05443-04 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oncogene Expression During Cell Differentiation and Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. S. Papas	Chief	LMO	NCI
Others:	R. J. Fisher	Expert	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (N. K. Bhat); Biological Carcinogenesis and Development Program, Frederick, MD (K. L. Komschlies); Biological Response Modifiers Program, Frederick, MD (B. J. Mathieson, H. A. Young)

LAB/BRANCH

Laboratory of Molecular Oncology

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NCI, NIH, Frederick, Maryland 21701-1013

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets gene expression (ets-1, ets-2 and erg) was examined in fetal thymocytes from different stages of development in isolated subsets of adult thymocytes and in peripheral T-lymphocytes. The ets-1 gene expression was first detected at day 18 in fetal thymocytes, corresponding to the first appearance of CD4-positive (CD4-positive, CD8-negative) thymocytes, and reaches maximal/plateau levels of expression in the thymus at 1-2 days after birth. The ets-2 gene expression is detected at least one day earlier, coinciding with the presence of both double-positive (CD4-positive, CD8-positive) and double-negative (CD4-negative, CD8-negative) blast thymocytes and reaches maximal/plateau levels one day before birth.

In the adult thymus, ets-1 and ets-2 mRNA expression is ten- to eight-fold higher, respectively, in the CD4-positive subset than in the other subsets examined. Higher levels of p55 ets-1 protein were also shown to exist in the CD4-positive subset. Both the CD4-positive and the CD8-positive T cell subsets had lower ets RNA levels than the CD4$^-$positive thymocytes. These results suggest that ets-2 and, more particularly, ets-1 gene products play an important role in T-cell development and differentiation and are not simply associated with proliferating cells, which are observed at a higher frequency in fetal thymocytes, or dull Ly-1 (low CD5-positive), and double-negative adult thymocytes. The ets-1 and ets-2 mRNA level did not change during erythroid differentiation.

The role of ets gene products in maturation of helper function and in T-cell activation and differentiation are under investigation. The ets gene expression will also be studied in specific thymocyte and T-cell subsets derived from tumor tissues.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO NCI
R. J. Fisher	Expert	LMO NCI
S. Fujiwara	Visiting Associate	LMO NCI
R. Ascione	Research Chemist	LMO NCI

Objectives:

To determine the role of nuclear oncogene products, specifically the role of the ets gene products in cell proliferation and differentiation, and to understand the molecular mechanisms involved in the regulation of ets gene expression; and to develop a mammalian expression vector system to express genes of special interest.

Methods Employed:

1. Isolation of Nucleic Acids, and RNA and DNA Blot Transfer Analyses. Isolation of high molecular weight DNA, total poly A^+ RNA, nucleic acid fractionation on agarose gels, transfer of nucleic acids to membranes, probe preparation, hybridization and washing of filters were done as described by Maniatis et al. (Molecular Cloning, A Laboratory Manual, 1982).

2. Cloning. Restriction enzyme digestion, isolation of DNA fragments from gels by electroelution and elutip-d columm chromatography, ligation, transformation of competent cells by plasmids, plasmid isolation and characterization were carried out as described by Maniatis et al. Appropriate viral and cellular ets DNA fragments were subcloned in Germini vectors to get a higher yield of plasmids and to prepare labeled riboprobes.

3. Isolation of thymocyte and lymph node subsets. Thymocyte subsets were isolated as described (Brownell et al., Mol Cell Biol 1987;1304-8) with the following modifications. The double-negative (DN) subset was isolated from the cells remaining after the positive selection of the $CD8^+$ and $CD4^+$ subsets by adherence to anti-Ig-coated plastic dishes. Non-adherent cells were retreated with a mixture of the monoclonal antibodies (MAbs) anti-Ly-2.2 (mouse IgG2a; clone 19/178) and anti-L3T4 (rat IgM; clone RL 172.4). The dLy-1 (low CD5, DN) subset was isolated using the same method described for DN cells, except for the use of an additional MAb to Ly-1 (CD5), either anti-Ly-1.1 (mouse IgG3; clone 39/3) or anti-Ly-1.2 (mouse IgM; clone C3PO), depending on the mouse strain used. Complement-dependent lysis followed the MAb treatment and viable cells were selected over a discontinuous Ficoll gradient. The $CD4^+$ and $CD8^+$ lymph node cell subsets were isolated by the following method of Salup et al., J Immunol 1987;138:3635-40. After cell separations, subsets were monitored for purity using flow cytometric analysis in conjunction with immunofluorescence.

Major Findings:

1. The mouse c-ets-1 and ets-2 mRNA are expressed at a high level in thymus.

2. The ets-1 and ets-2 mRNA levels are higher in postnatal thymocytes compared to early fetal thymocytes.

3. The ets-1 expression observed in day 18 of gestation coincides with the initial appearance of mature $CD4^+$ thymocytes, whereas ets-2 gene expression is present at constitutive levels from early thymic development.

4. Both ets-1 and ets-2 mRNA levels are very high in $CD4^+$, $CD8^-$ thymocytes than in double-negative ($CD4^-$, $CD8^-$), dLy-1 or $CD8^+$, $CD4^-$ thymocytes. Most of the ets-1 mRNA found in total thymocytes is due to $CD4^+$, $CD8^-$ thymocytes.

5. Both ets-1 and ets-2 proteins are also present at higher levels in $CD4^+$, $CD8^-$ thymocytes, which agrees well with the mRNA data (see above).

6. Both ets-1 and ets-2 mRNA level is lower in peripheral T-cells compared to thymocytes. Also, the ets-1 and ets-2 mRNA levels are lower in $CD4^+$, $CD8^-$ T-cells compared to $CD4^+$, $CD8^-$ thymocytes.

7. There is no gross change in ets-gene methylation pattern in different thymic subsets.

8. In macrophages, ets-2 (but not ets-1) genes are expressed at high levels.

9. When U937 (monocytes) cells are allowed to differentiate into macrophages by the addition of 12-O-tetradecanoyl-phorbol-13-acetate (TPA), the ets-2 mRNA level increased within 4 hours.

10. The ets-1 and ets-2 mRNA level did not change when human erythroleukemic cells or mouse erythroleukemic cells are allowed to differentiate into erythroid cells by the addition of N,N'-Hexamethylene-bisacetamide (HMBA).

Publications:

Bhat NK, Komschlies K, Fujiwara S, Mathieson BJ, Young H, Fisher RJ, Papas TS. Ets gene expression in murine thymocyte subsets. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;178.

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Fujiwara S, Fisher RJ, Seth A, Bhat NK, Showalter SD, Zweig M, Papas TS. Characterization and localization of the products of the human homologs of the v-ets oncogene. Oncogene 1988;2:99-103.

Papas TS, Bhat NK, Chen TT, DuBois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human diseases. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences, 1987;171-91.

Papas TS, Bhat NK, Fujiwara S, Ascione R, Fisher RJ. Temporal regulation of ets gene expression in murine tissue. In: Waymouth C, ed. Modern cell biology: molecular mechanisms in the regulation of cell behavior. New York: Alan R Liss, Inc, 1987;123-9.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Curran T, Skalka A, eds. The oncogene handbook. New York: Elsevier (In Press)

Zhou XM, Hsu GW, Chen YQ, Gu JR, Bhat NK, Watson DK, Ascione R, Papas TS. Expression of ets-2 and IGF-II mRNA in human primary hepatic cancer. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;114.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05483-03 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
RNA Processing, Transcription Termination and Gene Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	H. E. Takiff	Expert	LMO	NCI
Others:	S-M. Chen	Visiting Associate	LMO	NCI

COOPERATING UNITS *(if any)* Bionetics Research, Inc. (D. Court, J. Bardwell); Dept. of Gen. & Mol. Biol., Cent. de Invest. y de Estudios Avanzados Del IPN, Mexico City, Mexico (G. Guarneros; Inst. of Medical Science, University of Tokyo, Tokyo, Japan (Y. Nakamura)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

RNaseIII is a double-strand specific endoribonuclease that has different functions in E. coli. It processes rRNA precursors for efficient maturation into ribosomes. It processes some mRNA either to activate gene expression or to reduce gene expression. It regulates mRNA degradation. The int gene of phage λ is transcribed from two promoters yielding different mRNA transcripts. Int expression from one is reduced by RNaseIII; from the other, expression is enhanced. In both cases, control of expression by RNaseIII occurs from a single site beyond the gene. This form of control is named retroregulation. The site present on the RNA is able to form a special stem and loop structure that is recognized by RNaseIII. This site is also a transcription termination signal for RNA polymerase. In order to understand how RNaseIII levels in the cells are modulated, its gene in E. coli, rnc, has been cloned on λ vectors and on pBR322 plasmid. Sequence analysis indicates a second gene in an operon with rnc. This gene produces a protein with significant homologies to the yeast ras genes and is called era (E. coli ras). Both rnc and era have been placed on expression vectors and their proteins have been purified and antibodies have been made. Era is an essential gene in E. coli. The purified protein binds guanosine triphosphate (GTP). E. coli mutants have been isolated that are conditionally lethal bcause of mutations in rnc and era. Suppression mutants that restore growth are being analyzed to determine proteins that may interact or compensate for the products of these genes. E. coli mutants have been isolated that are conditionally lethal because of insertion mutations blocking expression of era. Rnc has been demonstrated not to be an essential gene in E. coli. The purified rnc protein binds adenosine triphosphate (ATP). A separate insertion mutation that abolishes rnc function, but permits era expression, is being analyzed. The regulation of the operon is being investigated, and preliminary results suggest a system of autoregulation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

H. E. Takiff	Expert	LMO	NCI
S-M. Chen	Visiting Associate	LMO	NCI

Objectives:

RNaseIII exerts retroregulation on λ int gene expression. To understand RNaseIII levels and the degree to which they can modulate gene expression by RNA processing, RNaseIII's own gene regulation must be studied. How does cell growth and global regulatory factors like cAMP, ppGpp, and others affect rnc gene expression? Antibodies to both RNaseIII and the era product have been made and will be used to measure levels of the proteins in cells. Transcriptional and translational activity, and their regulation will be studied both by gene fusion to galK and directly by mRNA labeling and hybridization studies.

E. coli genes of related function are frequently joined in operons. The relationship between the functions of rnc and era will be examined genetically and biochemically. Other laboratories will be examining the enzymological properties of the purified proteins, i.e., RNA binding, processing of the rnc/era message, GTP binding, etc.

Methods Employed:

Standard microbial, genetic, biochemical, and recombinant DNA techniques are used. RNaseIII is assayed by degradation-labeled poly A-U duplexes to trichloroacetic acid (TCA) solubility. Using ammonium sulfate washes and column chromatography, both RNaseIII and the era product were purified from cells where the protein was produced by the expression vector, pJL6, at levels equal to 10-20% of total protein.

Antibodies will be used to detect endogenous cellular levels by immuno-precipitation and also by Western blotting.

DNA sequencing from plasmids will be used to determine the exact location of the insertion mutations in the rnc/era operon and why one mini-Tn10 insertion renders the bacteria tetracycline dependent.

Standard methods will be used to construct a series of galK fusions to the rnc/era operon to study its regulation. Fusion results can be confirmed by RNA hybridizations to rnc and era genes using RNA probes made with in vitro transcription by T7 and/or SP6 polymerase.

Major Findings:

1. Retroregulation of λ int gene expression by RNaseIII has been extensively characterized.

2. The rnc gene, encoding RNaseIII, has been cloned on λ and pBR322 vectors from genomic libraries of E. coli.

3. In our clones of rnc, a second, era, has been detected in the same operon. Sequence analysis indicates a relationship of this E. coli protein to yeast ras protein, and perhaps to EFTU.

4. A complete genetic map of the rnc era region of the coli chromosome has been nearly completed using transposition by mini-tn10 and PI transduction to localize rnc era and nearby genetic markers: glyA, nadB, purI.

5. Insertion of the gene for chloramphenicol acetyl transferase into the middle of era on λ has been carried out. This mutant era cannot be transferred to the chromosome to replace the normal gene, indicating that era is essential to E. coli.

6. The era gene product has been purified to homogeneity and its GTP binding and GTPase properties analyzed.

7. The gene for kanamycin resistance was inserted into the rnc gene on λ clone. The mutant rnc can be transferred to the chromosome if the downstream era gene is supplied on a plasmid, indicating that rnc is not essential to E. coli.

8. Rnc has been found to bind ATP.

9. Insertion mutations upstream of rnc have a polar effect on the expression of era, indicating that they are in the same operon and probably coordinately transcribed and regulated.

10. The rnc105 mutation has been cloned on a plasmid.

11. By immunoprecipitation, more RNaseIII and era product is made from a rnc105 plasmid in a rnc105 host than with intact rnc host and plasmid, suggesting that the operon is autoregulated.

Publications:

Bahl H, Echols H, Straus D, Court D, Crowl R, Georgopoulos CP. Induction of the heat shock response of E. coli through stabilization of σ^{32} by the phage λ cIII protein. Genes Dev 1987;1:57-64.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05484-03 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Proto-oncogene ets in Sea Urchin and Xenopus laevis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Z.-Q. Chen	Visiting Associate	LMO	NCI
Others:	J. A. Lautenberger	Research Chemist	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	L. A. Burdett	IRTA Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cDNA library (a gift of Dr. D. Melton, Harvard University) prepared from X. laevis oocyte RNA was screened with avian virus E26 v-ets DNA as a probe. From one million plaques, 23 positive clones were obtained. One of these contained a 2.5 kb DNA insert. The DNA sequence of this clone, as determined by the dideoxynucleotide chain termination method, was found to contain a single major open reading frame capable of coding for at least 467 amino acid residues. Since it is more closely related to c-ets-2 sequences from human, mouse, and chicken than to c-ets-1 or v-ets sequences, this cDNA is derived from a transcript of the X. laevis c-ets-2 gene. The open reading frame begins at the extreme 5′-end of the clone with homology starting at residue 6 of the other ets-2 genes. The X. laevis ets-2 sequence contains extensive homology with the ets-2 sequences throughout its length and it is coterminal with these c-ets-2 sequences. The methods for fertilization in vitro, and the isolation of RNA and proteins from different staged oocytes, have been established. The pattern of RNA expression of the c-ets-2 gene in X. laevis was examined by RNA gel blot analysis of RNAs from oocytes and embryos at different stages of development. For each stage, a single 3.2 kb mRNA species was observed. The maximum level of expression was found in the early stage oocytes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Z.-Q. Chen	Visiting Associate	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
R. Ascione	Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
L. A. Burdett	IRTA Fellow	LMO	NCI

Objectives:

To define the role and biological function in cellular growth and differentiation of the proto-oncogene, ets, and its evolutionary relationship from invertebrates to man.

Methods Employed:

1. Isolation of DNA and RNA from sea urchin and Xenopus laevis. Sea urchin and Xenopus laevis eggs were fertilized in vitro and staged at various times during embryogeneis. After harvesting, nucleic acids were fractionated and analyzed according to standard procedures (Molecular Cloning, A Laboratory Manual, 1982).

2. Isolation and identification of Xenopus laevis proto-ets sequences. Screening a cDNA library with v-ets as a probe; purification of a positive clone; restriction enzyme analysis; isolation of DNA fragments from gels by electroelution after agarose electrophoresis; elutip and chromatographic separation; ligation; transformation into competent cells by plasmid constructs; plasmid preparation, isolation, and labeled probes were performed as described by Maniatis et al. (1982). Sequencing of DNA in M13 using the chain-terminator method for DNA analysis (Proc Natl Acad Sci USA 1977;74:5463-7). Probes: Viral ets-2 DNA (1.28 kb). Clones were nick-translated using ^{32}P-labeled nucleotides and were used to identify and detect Xenopus laevis proto-oncogene homologous to the ets-2 gene.

3. Expression of Xenopus laevis proto-ets-2 gene. Isolation and selection of Xenopus laevis mRNA by oligo dT column chromatography and analysis by Northern transfer (Northern blot) electrophoresis. Analysis of ets-2-related protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and specific antibody against ets-2 predicted oligopeptide. Analysis by immunoelectrophoresis (Western blot) to identify the ets product during different stages of embryogenesis.

4. Microinjection: Using J. B. Gurdon and M. P. Wickens' method (Methods Enzymol 1983;101:370), microinjected the antibody (anti-ets-2) or antisense (anti-ets-2 mRNA) into oocytes and fertilized egg and observed the developmental course.

5. In situ hybridization: Used antibody or antisense as probe to detect the location of ets-2 protein or mRNA in oocytes or embryos (Jamsich et al., EMBO J. 1984;3:1939).

Major Findings:

Screening an oocyte cDNA library with the v-ets (E26) gene as a probe. We have identified a homolog of Xenopus laevis with the v-ets-2 gene. The sequence was found to contain a single major open reading frame capable of coding for at least 467 amino acid residues. The 5′ and 3′ portions of the sequence are strikingly homologous to ets-2 of virus (E26) and human. The homologs for the middle sequence between the 5′ and 3′ portions of X. laevis ets-2 are divergent. The open reading frame begins at the extreme 5′-end of the clone, with homology starting at residue 6 of the other ets-2 genes. The X. laevis ets-2 sequence contains extensive homology with the ets-2 sequences throughout its length, and it is coterminal with these c-ets-2 sequences. The expression of ets-2-related mRNA occurred at an early stage during X. laevis embryogenesis. A single 3.2 kb mRNA species was observed. The maximum level of expression was found in the oocyte stage.

Publications:

Chen Z-Q, Kan NC, Pribyl L, Lautenberger JA, Moudrianakis E, Papas TS. Molecular cloning of the ets proto-oncogene of the sea urchin and analysis of its developmental expression. Dev Biol 1988;125:432-40.

Shih TY, Clanton DJ, Hattori S, Ulsh LS, Chen Z-Q. Structure and function of p21 ras proteins: immunochemical, biochemical and site-directed mutagenesis studies. In: Colburn NH, Moses HL, Stanbridge EJ, Fox CF, eds. Growth factors, tumor promoters, and cancer genes. New York: Alan R Liss, 1987;321-32.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05485-03 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Application of Monoclonal Antibodies to the Study of Oncogene Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. Fujiwara	Visiting Associate	LMO	NCI
Others:	R. J. Fisher	Expert	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid and Protein Synthesis Laboratory, Program Resources Inc., Frederick, MD (N. K. Bhat)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

C-myc and c-ets (1 and 2) genes are cellular homologues of the oncogenes carried by the avian myelocytomatosis virus, MC29, and the avian acute leukemia virus, E26, respectively. These genes are thought to have some role in the pathogenesis of these viruses, and antibodies to the products of these genes are planned for the biological and biochemical characterization of these proteins. So far, a monoclonal antibody against the myc gene products and three monoclonal antibodies against the ets-2 protein have been generated and described in previous reports. This year, the monoclonal antibodies against the ets-2 protein have been used to characterize the c-ets protein. By subcellular fractionation and immunocytochemical technique, the human ets-2 protein was demonstrated in the nucleus, and a minor amount of the protein was also found in the cytoplasm. Pulse-chase experiments and in vivo labeling with [32P] showed that the ets-2 protein is a short-lived phosphoprotein. Tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and synthetic diacylglycerol, 1-oleoyl-2-acetyl glycerol (OAG), both of which activate protein kinase C, stabilized the ets-2 protein, thereby quickly elevating its steady-state level. These results point to the possibility that the ets-2 protein has a role in the protein kinase C pathway of signal transduction as a nuclear effector.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Fujiwara	Visiting Associate	LMO	NCI
R. J. Fisher	Expert	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The immediate objective of this project is to generate highly specific, immunological probes for the proteins encoded by human myc, ets-1 and ets-2 genes. These probes are to be applied to biological and biochemical characterization of the gene products in search of their normal functions. Furthermore, as a specific probe for a single epitope, a monoclonal antibody is particularly useful in investigating protein structure at individual domain levels. Detailed analysis of the structure of these human oncogene products performed in relation to functional significances should give insights into molecular mechanisms by which these proteins mediate malignant cellular transformation. Practically, monoclonal antibodies are used in the detection of the oncogene products by immunoprecipitation, immunoblot and immunocytochemistry. They are also utilized as probes in the screening of cDNA libraries prepared by the λgt11 expression vector. In addition, we are currently trying to purify the human ets-2 protein by affinity chromatography using a monoclonal antibody against the protein.

Methods Employed:

The antigens used are produced from c-myc, ets-1 or ets-2 genes which have been expressed in E. coli or from oligopeptides synthesized from the predicted amino acid sequences of these genes. BALB/c mice are immunized with these antigens and hybridomas are generated using standard procedures, including polyethylene glycol-mediated cell fusion and hypoxanthine, aminopterine-thymidine (HAT) selection of hybrid cells. Hybridoma culture fluids are screened for specific antibodies by the enzyme-linked immunoblot techniques. Proteolytic fragment profiles of the detected proteins are obtained by digestion with staphylococcal V8 protease, chymotrypsin, or trypsin, followed by one-dimensional or two-dimensional electrophoresis analysis. The proteins detected by monoclonal antibodies are purified by high performance liquid chromatography (HPLC).

Major Findings:

This year we have been focusing on the characterization of the protein encoded by the human ets-2 gene by using polyclonal and monoclonal antibodies. Subcellular fractionation and immunocytochemical techniques showed that the ets-2 protein is localized in the nucleus, although a minor amount of the protein is also present in the cytoplasm. Pulse-chase experiments indicated that the ets-2 protein has an extremely short half-life of 20 minutes, and in vivo labeling with [32P] orthophosphate demonstrated that it is a phosphoprotein. When the human

lymphocytic CEM cells were treated with the tumor promoter, TPA, the level of the ets-2 protein was quickly elevated by five- to 20-fold. This effect of TPA was mimicked by a synthetic diacylglycerol, OAG, and was blocked by the inhibitor of protein kinase C (PKC), H7, indicating the involvement of the enzyme. The increase in the ets-2 protein is due to its stabilization, because the protein has a half-life of more than 2h in the presence of TPA and the ets-2 mRNA level did not increase upon TPA treatment. The protein synthesis inhibitor, cycloheximide, enhanced the stabilizing effect of TPA on the ets-2 protein and delayed the turnover of the protein by itself. Similar induction of the ets-2 protein was also demonstrated as an early event in the TPA-induced macrophage differentiation of human HL-60 cells. The properties of the ets-2 protein, such as nuclear localization, phosphorylation, rapid turnover and response to PKC, indicate that it belongs to a group of oncogene proteins which are generally thought to have regulatory functions in the nucleus (e.g., myc, fos, myb and p53). Our results further suggest that PKC, either directly or indirectly, regulates the level of the ets-2 protein by a post-translational mechanism and this raises the possibility that the protein is involved in the PKC pathway of signal transduction as a nuclear effector.

Publications:

Bhat NK, Komschlies K, Fujiwara S, Mathieson BJ, Young H, Fisher RJ, Papas TS. Ets gene expression in murine thymocyte subsets. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;178.

Fujiwara S, Fisher RJ, Bhat NK, Papas TS. Human ets-2 protein: nuclear location, phosphorylation, rapid turnover and induction by TPA. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;107.

Fujiwara S, Fisher RJ, Seth A, Bhat NK, Showalter SD, Zweig M, Papas TS. Characterization and localization of the products of the human homologs of the v-ets oncogene. Oncogene 1988;2:99-103.

Papas TS, Bhat NK, Chen TT, DuBois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human diseases. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences, 1987;171-91.

Papas TS, Bhat NK, Fujiwara S, Ascione R, Fisher RJ. Temporal regulation of ets gene expression in murine tissue. In Waymouth C, ed. Molecular mechanisms in the regulation of cell behavior. New York: Alan R Liss, 1987;123-9.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Curran T, Skalka A, eds. The oncogene handbook. New York: Elsevier (In Press)

Schweinfest CW, Fujiwara S, Lau LF, Papas TS. C-myc can induce the expression of G_0/G_1 transition genes. Mol Cell Biol (In Press)

Schweinfest CW, Fujiwara S, Papas TS. c-myc protein expression can induce the expression of G_0/G_1 transition genes. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/ Washington DC: IRL Press, 1988;113.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05516-02 LMO

PERIOD COVERED: October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization of Normal and Oncogenic ras Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. Y. Shih	Research Chemist	LMO	NCI
Others:	P. Saikumar	Visiting Fellow	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study of the molecular basis of cellular transformation by activated ras oncogenes in relation to the function of its proto-oncogenes has been the major emphasis of this project. The ras gene product, p21 (21,000-dalton protein), binds guanine nucleotides and exhibits quanosine triphosphate (GTPase) activity. The viral transforming and cellular ras proteins differ at positions 12 and 59. The point mutations at these positions affect the above activities. By analogy to other G-proteins the ras gene product, p21, may act as a regulatory component of the signal transduction. There have been reports on the involvement of active protein kinase C for the mitogenic activity of ras p21 and also ras protein increases the levels of diacylglycerol (DAG), a protein kinase C activator in some cells. Our laboratory earlier showed that the ras gene product was phosphorylated by phorbol esters in certain cell lines. We have further shown that ras p21 could be phosphorylated by protein kinase C and protein kinase A, both in vivo and in vitro. By peptide mapping, sequencing and oligonucleotide-directed mutagenesis, the phosphorylation site for protein kinases has been identified as 177 serine at the carboxyl terminus of p21. Our studies are continuing to understand the biological significance of this phosphorylation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. Y. Shih	Research Chemist	LMO	NCI
P. Saikumar	Visiting Fellow	LMO	NCI
L. S. Ulsh	Microbiologist	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI

Objectives:

To understand the molecular principles involved in the cellular transformation by ras oncogenes, with respect to the normal functions of ras proto-oncogenes during regular cell growth and differentiation.

Methods Employed:

1. Site-directed mutagenesis. Oligonucleotides of 17-21 bases containing single or two-base changes from the H-ras gene were used. The ras coding gene was cloned into M13 phage and the mutant oligonucleotide was annealed to the single-strand recombinant DNA and synthesized to the second strand with oligonucleotide as the primer. The newly synthesized replicative form (RF) DNA was used to transfect the E. coli cells. The plaques were screened for the mutation by hybridizing with the labeled oligonucleotide.

2. Construction of Expression Vectors. The 0.88 kb HindIII fragment containing the coding sequences for p21 was cloned into the bacterial expression vector, pJL6.

The SstII-PstI fragment containing the p21 genome was modified to give XhoI ends. This XhoI fragment was cloned into pMAM/neo, an inducible mammalian expression vector containing the mouse mammary tumor virus promoter and neomycin gene as a selectable marker.

3. Transfection of NIH 3T3 Cells. The DNA transfections of NIH 3T3 cells were done by the calcium phosphate precipitation method. The transformants were selected by G418 drug resistance.

4. Purification of p21 from E. coli. The recombinant p21 was overproduced in the E. coli-carrying plasmid, pJL6, containing the ras gene by raising the temperature of the culture from 32°C to 42°C. The p21 was then purified either under non-denaturing conditions or by 8M urea extraction.

5. Phosphorylation of p21. The purified p21 is phosphorylated in vitro by incubating with purified kinases and γ-^{32}P-adenosine triphosphate (ATP).

For in vivo phosphorylation, the cells were treated with appropriate activators and labeled with ^{32}P orthophosphate. Using the antibodies, the p21 was immunoprecipitated.

6. Guanine Nucleotide Binding Assay. The quanosine diphosphate (GDP)-binding activity of the purified proteins was assayed by incubating with ^{3}H-GDP and filtering onto nitrocellulose filters. The amount of binary complex formed was measured by counting the radioactivity. The guanine nucleotide exchange was started by the addition of cold nucleotide. The GTPase activity was measured by the release of inorganic phosphate after incubating with γ-^{32}P-GTP.

Major Findings:

1. The effect of point mutations at positions 12 and 59 of ras p21 on its biochemical activities have been characterized. It seems that oncogenic p21 mutants insure the maintenance of the active GTP-p21 complex either by lowering GTPase activity or by increasing the exchange of guanine nucleotides, or by both.

2. It has been reported that ras proteins appear to elevate 1,2-diacylglycerol, an activator of protein kinase C (PKC) in some cell lines, and also the active PKC is required for the mitogenic activity of ras p21 proteins. Novel phosphorylation of H-ras by protein kinase C and protein kinase A, both in vivo and in vitro, has been characterized. We identified 177 serine of H-ras p21 as the major site of phosphorylation by protein kinases. We have also constructed point mutations which substituted alanine and cysteine for 177 serine. These mutations were not phosphorylated by both kinases. The studies are continuing to understand the biological significance of this phosphorylation in our laboratory.

Publications:

Saikumar P, Ulsh LS, Clanton DJ, Huang K-P, Shih TY. Novel phosphorylation of c-ras p21 by protein kinases. Oncogene Res (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05563-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Introduction of the HIV tat Gene into Lymphoid Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Bionetics Research, Inc., Frederick, MD (B. Felber, G. Pavlakis)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to study the influence of the human immunodeficiency virus (HIV) tat gene on the expression of cellular genes, this gene has been inserted into the retroviral vector, pGV1, under the transcriptional control of the HIV long terminal repeat (LTR). The recombinant plasmid was transfected into psi2 cells to produce an ecotropic viral stock. This virus was used to infect psiAM cells to induce G418-resistant colonies. Presently, these colonies are being purified and tested for the production of amphotropic helper-free retrovirus. These viruses will be used to infect human lymphoid cells to establish cell lines constitutively expressing the tat gene. Such lines will be useful for the identification of cellular genes whose expression is controlled by the tat gene.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The HIV tat gene has been shown to regulate transcription initiated at the viral LTR and possibly regulate translation of viral proteins. Since tat has not been shown to bind HIV DNA, this protein may induce the expression of cellular genes. We plan to investigate this possibility by the methods of differential hybridization and subtractive hybridization. Each of these methods require RNA from both tat^+ and tat^- cells that are otherwise isogenic. For these purposes, it is also desirable that the cells used be of lymphoid origin since $CD4^+$ lymphocytes are a major target of HIV. Because it is difficult to introduce DNA into lymphocytes by many of the conventional techniques, we have chosen to use recombinant retroviruses to achieve this goal.

Methods Employed:

1. Construction of recombinant plasmids. Plasmid pMVL3ctat was digested with restriction enzymes, Asp718I and BamHI, and fragments were resolved by agarose gel electrophoresis. An 840 bp fragment containing the HIV-1 LTR and tat sequences was isolated from the gel by the glass bead absorption method (Bio101, Inc.). The ends of this fragment were made blunt by filling them in with Klenow polymerase and the fragment was ligated to BamHI linkers. The ligation reaction product was digested with BamHI to remove excess linkers, and the fragment, now containing BamHI termini, was repurified by agarose gel electrophoresis. This DNA was ligated to plasmid pGV1 DNA (see "Major Findings" for description) that had been linearized by digestion with BamHI. Transformation competent Escherichia coli strain HB101 cells (BRL) were transformed by the ligation reaction product and selected for kanamycin resistance. Resistant colonies were screened for the presence of recombinant plasmids by restriction enzyme analysis of small-scale plasmid preparations.

2. Recombinant retrovirus preparation. The method of Felber and Pavlakis (Science 1988;239:184-7) was used. Plasmids containing HIV LTR and tat sequences inserted into pGV1 were transfected into cell line psi2 (Mann et al., Cell 1983;33:153) which contains a packaging-defective Moloney sarcoma virus (MSV). Forty-eight hours after transfection, the medium of the psi2 cells containing recombinant helper-free retrovirus was collected and used to infect psiAM cells (Cone and Mulligan, Proc Natl Acad Sci USA 1984;81:6349-53), which contain a packaging-defective MSV retrovirus carrying an amphotropic env coat, allowing infection of human cells. The infected psiAM cells were treated with G418, and resistant psiAM colonies containing integrated recombinant proviruses were tested for virus production on HeLa cells. Several independent G418-resistant colonies of psiAM cells that generated 10^2 to 10^4 infectious

viral particles per milliliter were identified. These clones can be used for viral production and infection of human cell lines.

Major Findings:

Construction of recombinant retroviruses expressing the HIV tat gene. The retroviral vector, pGV1 (Jhappan et al., J Virol 1986;60:750), contains a Moloney sarcoma virus LTR and packaging signal, a colE1 origin of replication, the SV40 origin of replication and major early promoter, the neomycin resistance gene from transposon tn5 (via the plasmid pSV2neo), and a multiple cloning site. Recombinant plasmids containing an inserted fragment with HIV LTR and tat sequences in each orientation have been constructed (plasmids pGV1LTRtat-s and pGV1LTRtat-a). The recombinant plasmids are being used to introduce the HIV tat gene into lymphoid cells under HIV LTR control as described under "Methods Employed." Presently, it has been demonstrated that the recombinant plasmids can induce psi2 cells to produce a virus that can generate G418-resistant colonies in psiAM cells. The recombinant plasmid also has been transfected into LTR-cat cells (Felber and Pavlakis, Science 1988;239:184-7) and the cell extracts have been assayed for CAT activity. It was found that the pGV1-LTR-tat plasmids greatly stimulated CAT production in the LTR-cat cells, confirming that the LTR and tat gene in these plasmids are active.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05564-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of HIV Messenger RNA Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO NCI
Others:	C. Schweinfest	Staff Fellow	LMO NCI
	C. Jorcyk	Biologist	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*

Bionetics Research, Inc., Frederick, MD (B. Felber, G. Pavlakis)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human immunodeficiency virus (HIV) infection has been shown by several laboratories to use a complex set of RNA synthesis and processing events in order to control viral expression. In order to determine if messages exist that have not been previously characterized, we have begun to directly analyze cDNA copies of HIV messages by Southern hybridization and DNA sequence analysis.

A cDNA library in lambda gt10 has been prepared from RNA isolated from HIV-infected H9 cells. A large number of phages from this library were found to hybridize to viral sequences. A total of 19 such phages were plaque-purified and analyzed by Southern hybridization using labeled oligonucleotides or restriction fragments derived from various locations on the HIV genome. The specific regions include: (A) 5′-end of tat; (B) 5′-end of trs; (C) 5′-portion of env (excluding tat and trs region); (D) pol/sor region. Clones with the following hybridization pattern have been isolated: class I, A+B$^-$C-D+; class II, A-B-C$^+$D+; class III, A-B-C-D+. We have purified several of these phages and have subcloned their insert DNA into pIBI vectors. Several of these phages have been characterized by Southern blot hybridization. We are currently sequencing these DNA segments to determine if they represent novel HIV messages and, thus, might reveal new exons or splice sites.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO	NCI
C. Schweinfest	Staff Fellow	LMO	NCI
C. Jorcyk	Biologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

HIV infection has been shown by several laboratories to use a complex set of RNA synthesis and processing events in order to control viral expression. In order to determine if messages exist that have not been previously characterized, we have begun to directly analyze cDNA copies of HIV messages by Southern hybridization and DNA sequence analysis.

Methods Employed:

1. cDNA library preparation. RNA was isolated from virus-infected cells by the guanidinium thiocyanate method of Chirgwin et al. (Biochemistry 1979;18:5294-9). The library was prepared according to the protocol supplied by the Boehringer-Mannheim Biochemicals' cDNA cloning kit. The library was plated on Escherichia coli C600 Hfl cells and screened by using nick-translated cloned HIV proviral DNA as a probe.

2. Southern blot hybridization and recombinant DNA techniques. The methods used for Southern blot hybridization and recombinant DNA procedures have been described by Maniatus et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

Major Findings:

Characterization of potential novel HIV cDNA clones. A cDNA library in lambda gt10 has been prepared from RNA isolated from HIV-infected H9 cells. A large number of phages from this library were found to hybridize to viral sequences. A total of 19 such phages were plaque-purified and analyzed by Southern hybridization using labeled oligonucleotides or restriction fragments derived from various locations on the HIV genome. The specific regions include: (A) 5'- end of tat; (B) 5'-end of trs; (C) 5'-portion of env (excluding tat and trs region); (D) pol/sor region. Clones with the following hybridization pattern have been isolated: class I, $A^+B^-C^-D^+$; class II, $A^-B^-C^+D^+$; class III, $A^-B^-C^-D^+$. We have purified several of these phages and have subcloned their insert DNA into pIBI vectors. Several of these phages have been characterized by Southern blot hybridization. We are currently sequencing these DNA segments to determine if they represent novel HIV messages and, thus, might reveal new exons or splice sites.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05565-01 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Biochemical and Functional Properties of the ets Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO	NCI
Others:	D. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. K. Watson	Research Microbiologist	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Transgenic Analysis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets-1 and ets-2 loci are transcriptionally active in humans and express a single 6.8 Kb ets-1-specific and three 4.7, 3.2 and 2.7 Kb ets-2-specific mRNAs, respectively. The c-ets-1 (P51) and the c-ets-2 (P56)-related proteins have been identified in the human cell line, Daudi, as well as COLO 320 DM, but they are expressed at extremely low levels.

In order to express the ets protein in large quantities, to study the biochemical properties and also to produce ets-specific antiserum, we have constructed several expression vectors capable of producing complete v-ets and its deletion mutants and defined human c-ets-1 and c-ets-2 gene products in E. coli.

The vectors with smaller inserts produced high levels of the ets products representing greater than 5% of the total bacterial proteins. In contrast, the vectors with larger inserts produced low levels representing less than 0.5% of the total protein. The bacterial ets proteins were characterized by immunoblotting on a Western blot using the ets-specific antibodies. In E. coli, the ets proteins aggregate and exist in the form of inclusion bodies and are insoluble. However, this property allowed us to purify the ets proteins to greater than 95% homogeneity by extracting the insoluble pellet with different solvents. The purified proteins were utilized to obtain the ets-1 and the ets-2-specific polyclonal and monoclonal antiserum. These polyclonal and monoclonal antibodies will be useful in identifying and studying the biochemical and biological functions of the ets-1 and the ets-2 proteins.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

A. Seth	Visiting Scientist	LMO	NCI
D. Thompson	Biologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI

Objectives:

Expression of the viral and human ets-1 and ets-2 proto-oncogene products in E. coli.

Methods Employed:

The restriction enzyme digestions, gel electrophoresis, isolation of DNA insert from gels by electroelution, ligation of insert to vector DNA, bacterial transformation, screening of clones by in situ colony hybridization, DNA probes by nick-translation, double-strand DNA sequencing, small-scale DNA preparation and analysis of the DNA by gel electrophoresis were according to published procedures.

The procedures for thermal induction of proteins, purification of bacterially-expressed proteins, sodium dodecyl sulfate (SDS)-gel electrophoresis, protein labeling with ^{35}S-methionine, protein extraction, immunoprecipitation, Western blotting and production of monoclonal and polyclonal antibodies have been described elsewhere.

Major Findings:

1. Construction of the ets expression vectors. We have constructed several expression vectors capable of producing complete v-ets and its deletion mutants, and portions of the c-ets-1 and the c-ets-2 products in E. coli.

2. Expression of the v-ets, ets-1 and ets-2 proto-oncogene products in E. coli. E. coli harboring the various ets expression plasmids (pTSP-8, pPST-33, pSPT-6, pRSAL-10, pAVA-10, pBAN-150) were analyzed for the production of the ets protein before and after the induction by SDS-polyacrylamide gel electrophoresis. As expected, all the expression vectors produced the predicted size proteins.

3. Characterization of the ets proteins expressed in E. coli. The bacterially expressed ets proteins were characterized by immunoblotting on Western blots using the ets-specific antibodies.

4. Purification of the ets products. The ets proteins were purified to greater than 95% homogeneity by extracting the bacterial protein pellet with different solvents. The availability of sufficient quantities of purified ets proteins will allow us to crystallize and study the biochemical properties associated with them.

5. Production of monoclonal and polyclonal ets-specific antibodies. The purified ets proteins were utilized to generate ets-1 and ets-2-specific antibodies. These antibodies should prove useful to identify and study the properties of ets-1 and ets-2 proto-oncogene products.

6. By comparison of the amino acid sequence of the ets protein with known oncogenes, we have localized a region of homology with consensus adenosine triphosphate (ATP)-binding domain (GXGXXG-14aa-K) of src family of kinases. The bacterially expressed proteins will be used to test the ATP-binding activity.

Publications:

Fujiwara S, Fisher RJ, Seth A, Bhat NK, Papas TS. Human ets-1 and ets-2 proteins: identification and intracellular localization. In: Voellmy R, Ahmad F, Black S, Burgess DR, Rotundo R, Scott W, Whelan W., eds. Advances in gene technology: the molecular biology of development. Cambridge: Cambridge University Press, 1987;7:77.

Fujiwara S, Fisher RJ, Seth A, Bhat NK, Papas TS. The products of the human homologs of the v-ets oncogene: ets-1, a cytoplasmic 51 Kd protein, and ets-2, a nuclear 56 Kd protein. Oncogene 1988;2:99-103.

Papas TS, Bhat NK, Chen TT, DuBois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human disease. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York, New York Academy of Sciences, 1987;171-91.

Samuel KP, Seth A, Konopka A, Lautenberger JA, Papas TS. The 3′-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Lett. 1987;218:81-6.

Samuel KP, Seth A, Zweig M, Showalter SD, Papas TS. Bacterial expression and characterization of nine polypeptides encoded by segments of the envelope gene of human immunodeficiency virus. Gene 1988;64:121-34.

Seth A, Blair DG, Dunn KJ, Fisher RJ, Vande Woude GF. High level expression of human growth hormone mos ($p58^{hGH\text{-}mos}$) product in C-127 cells. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC, IRL Press, 1988:112.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05566-01 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Biological & Biochemical Functions of ets-1 and ets-2 Proto-oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO	NCI
Others:	D. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. K. Watson	Research Microbiologist	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Transgenic Analysis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.9	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The c-ets-2 proto-oncogene encodes a phosphoprotein of approximately 56-64 Kd and is localized in the nucleus. The ets-2 mRNA has been shown to be induced in regenerating mouse liver, suggesting that the ets-2 gene expression may be involved in cell proliferation. In order to test the expression of ets-2 on growth properties of mouse fibroblasts, we have constructed expression vectors capable of producing ets-2 protein in NIH 3T3 cells. The mouse and human ets-2 cDNAs were cloned into an expression vector carrying an inducible promoter. The NIH 3T3 cells transfected with the construct carrying mouse c-ets-2 genes showed discrete foci of densely-growing cells when maintained in low-serum or serum-free medium. These transfected cells formed colonies in soft agar and also induced tumors in nude mice. The control cells did not grow in serum-free medium and in the presence of low-serum they did not show characteristic foci observed with ets-2-transfected cells. Our results suggest that the ets-2 proto-oncogene expression induces growth and morphological alterations in NIH 3T3 cells, and abolishes their serum requirement and is tumorgenic in nude mice. These properties provide an assay system to study the functions of ets-2 and its related genes in cell proliferation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Seth	Visiting Scientist	LMO	NCI
D. Thompson	Biologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
D. K. Watson	Research Microbiologist	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI

Objectives:

Transforming and mitogenic effects of the c-ets-2 proto-oncogene.

Methods Employed:

The procedures for restriction enzyme digestions, gel electrophoresis, isolation of DNA insert from gels by electroelution, ligation of insert to vector DNA, bacterial transformation, screening of clones by in situ colony hybridization, DNA probes by nick-translation, small-scale DNA preparation and analysis of the DNA by gel electrophoresis have been published.

The DNA transfection of NIH 3T3 cells by the calcium phosphate precipitation procedure, single cell cloning, growth in soft agar, growth in low-serum and serum-free medium, nude mice assay, high molecular DNA extraction, RNA extraction, Southern blots and Northern blots, DNA and RNA blot hybridization, were according to the published procedures.

Protein labeling with [^{35}S]-methionine, protein extraction, immunoprecipitation, and Western blotting with antibodies and [^{125}I] protein have been described previously.

Major Findings:

1. Construction of the human and the mouse ets-2 expression vectors. We have constructed the ets-2 expression vectors by inserting the cDNAs corresponding to the human and mouse ets-2 in a vector carrying the mouse metallathionein promoter.

2. Generation of the human and mouse ets-2 cell lines. NIH 3T3 cells were transfected with the human and the mouse ets-2 expression vectors. The cell lines containing the multiple copies of human or mouse ets-2 gene were selected and further examined for the ets-2 expression and morphological alterations.

3. Mitogenic effects of the c-ets-2 proto-oncogene. The NIH 3T3 cells transfected with the mouse c-ets-2 construct showed foci of densely-growing, morphologically altered cells when grown either in low-serum (0.05%) or serum-free medium. In contrast, the control NIH 3T3 cells transfected with vector alone without the c-ets-2 insert did not grow in serum-free medium, and in the presence of low serum did not show characteristic foci observed with ets-2-transfected cells. Similar studies are in progress with the human c-ets-2 construct.

4. Anchorage-independent growth. NIH 3T3 cells transformed with the mouse ets-2 proto-oncogene gave rise to colonies when seeded in suspension in soft agar.

5. Tumorigenicity assay. The NIH 3T3 transformed with the mouse ets-2 vector induced tumors in nude mice in less than 3 weeks. The analysis of ets-2 RNA and protein expression in the tumors is in progress.

6. Expression of the ets-2 protein. In order to test whether the growth alterations observed with c-ets-2-transfected cells are mediated by the expression of the ets-2 gene. We have cloned several foci and the analysis of the ets-2 mRNA and protein expression is in progress.

Publications:

Fujiwara S, Fisher RJ, Seth A, Bhat NK, Papas TS. Human ets-1 and ets-2 proteins: identification and intracellular localization. In: Voellmy RW, Ahmad F, Black S, Burgess DR, Rotundo R, Scott WA, Whelan WJ, eds. Advances in gene technology: the molecular biology of development. Cambridge: Cambridge University Press, 1987;77.

Fujiwara S, Fisher RJ, Seth A, Bhat NK, Papas TS. The products of the human homologs of the v-ets oncogene: ets-1, a cytoplasmic 51 Kd protein and ets-2, a nuclear 56 Kd protein. Oncogene 1988;2:99-103.

Papas TS, Bhat NK, Chen TT, DuBois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human disease. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences 1987;171-91.

Samuel KP, Seth A, Konopka A, Lautenberger JA, Papas TS. The 3'-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Lett 1987;218:81-6.

Samuel KP, Seth A, Zweig M, Showalter SD, Papas TS. Bacterial expression and characterization of nine polypeptide-encoded segments of the envelope gene of human immunodeficiency virus. Gene 1988;64:121-34.

Seth A, Blair DG, Dunn KJ, Fisher RJ, Vande Woude GF. High-level expression of human growth hormone mos ($p58^{hGH\text{-}mos}$) product in C-127 cells. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;112.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05567-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Gene Products of the erg (ets-related gene) Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. S. Papas	Chief	LMO	NCI
Others:	A. De Klein	Visiting Fellow	LMO	NCI
	R. Fisher	Expert	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid and Protein Synthesis Laboratory, Program Resources Inc., Frederick, MD, (M. Zweig and S. D. Showalter)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The presence of putative erg proteins encoded by the erg (ets-related gene) locus has been examined in specific human cell lines. To probe the erg proteins, polyclonal antisera have been prepared against synthetic polypeptides representing amino acid sequences deduced from the DNA sequence of the erg gene locus. Furthermore, we have to facilitate further analysis of the putative erg proteins; we have purified a bacterially expressed erg protein and are using this highly purified protein to raise polyclonal erg antiserum. In immunoprecipitates from the nuclear fractions of 35S-methionine-labeled COLO 320 cells we detect two proteins of 56 and 63 Kd which reacted specifically with the specific erg-R1 antiserum. Whether these proteins represent the cellular erg gene products is currently under investigation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
A. De Klein	Visiting Fellow	LMO	NCI
R. J. Fisher	Expert	LMO	NCI

Objectives:

The objective of the proposed research is to identify and characterize the protein products of the human erg (ets-related gene) locus. The properties of these erg proteins will be compared with the closely related viral-ets oncogene protein, the cellular ets-1 and ets-2 proto-oncogene proteins and a bacterially expressed erg protein.

Methods Employed:

Two human cell lines, maintained by in vitro altering, were used in these studies: a colon carcinoma cell line, COLO 320, and a T-cell leukemia cell line, CCRF-CEM. The cells are routinely labeled with ^{35}S-methionine and subfractionated by differential centrifugation. To immunoprecipitate the erg proteins from the different subcellular components, we used site-specific antibodies raised against putative erg amino acid sequences deduced from different regions of the erg gene. The specificity of these site-specific anti-erg antibodies was tested on bacterially expressed erg and ets proteins using immunoblots and immunoprecipitation.

Prokaryotic expression vectors containing the carboxyl-terminal part of the erg gene were utilized for the synthesis of erg proteins in E. coli. The bacterially expressed erg proteins were purified using the detergent chaotrope procedure (Knippl et al., Proc Natl Acad Sci USA 1984;81:6988-92) and reverse phase high pressure liquid chromatography (HPLC).

Major Findings:

Recently, a novel gene named erg, closely related to the v-ets oncogene, was identified in COLO 320, a human colon carcinoma cell line (Reddy et al., Proc Natl Acad Sci USA 1987;84:6131-5). Northern blot analysis using poly A-isolated mRNA from COLO 320 cells and Molt-4 (a human T-cell leukemia cell line) showed different erg mRNA species of 5.0 and 3.2-3.6 Kb. These mRNA species differ both in the 5′ coding regions as well in the 3′ untranslated region. This would suggest that translation of the 5.0 Kb and 3.2 Kb erg mRNA transcripts results in two erg polypeptides of 363 and 462 amino acids, respectively, which differ in their amino- terminal region (Rao et al., Science 1987;237:629-35). In the presented study, we try to identify and characterize the erg gene products in human cell lines using three different site-specific erg antibodies (R1; R2; R3) and a pan-ets antibody (ZC50), all raised against synthetic polypeptides. The pan-ets antibody and erg antiserum R_2 detect both the ets-2 and the erg bacterially expressed proteins. However, using the same bacterially expressed proteins, we showed that the erg R1 antiserum reacted

only with the expressed erg gene product. The same site-specific anti-sera (R_1 and ZC50) were used in immunoprecipitations using ^{35}S-methionine-labeled COLO 320 cells. In these COLO 320 cells, we have identified proteins of 56 and 63 Kd which specifically reacted with the R1 and ZC50 antisera and, thus, might represent the cellular erg proteins. Subcellular fractionation suggests that these proteins, like the ets-2 proteins, are present in the cell nucleus. However, two-dimensional gel electrophoresis and tryptic mapping of these putative erg proteins, and comparison with tryptic maps of in vitro translated and/or bacterial expressed erg genes will be necessary to establish unequivocally whether these proteins are, indeed, the cellular erg gene products.

Publications:

Sacchi N, De Klein A, Showalter SD, Dagna Bricarelli F, Papas TS. Lack of evidence for association of meiotic nondisjunction with particular DNA hapotypes on chromosome 21. Proc Natl Acad Sci USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05568-01 LMO

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Molecular Mechanism of Transactivation by the HIV-1 tat Gene Product

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. W. Schweinfest	Staff Fellow	LMO NCI
Others:	C. L. Jorcyk	Biologist	LMO NCI
	J. A. Lautenberger	Research Chemist	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The transactivator of the transcription gene product (tat) of human immunodeficiency virus-1 (HIV-1) may modulate cellular gene expression. Infection of H9 cells with HIV-1 is demonstrated to produce changes in the level of expression of some serum-inducible genes, although oncogene expression appears to be unaffected. Currently, uncharacterized genes (cDNA clones) whose expression may be repressed have been isolated by differential hybridization of infected vs. uninfected H9 cell cDNA libraries. A construct in which functional tat can be expressed under control of the heat shock promoter is being used to construct permanent cell lines.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. W. Schweinfest	Staff Fellow	LMO NCI
C. L. Jorcyk	Biologist	LMO NCI
J. A. Lautenberger	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

The HIV-1 tat gene product has been demonstrated previously to activate transcription from the HIV-1 long terminal repeat (LTR) in trans. However, evidence of direct binding of tat to the LTR or the transcription process has so far proved elusive. Furthermore, the cell line in which transactivation is measured appears to affect the level of transcription. These observations have led to the hypothesis that cellular genes play a role in modulating the course of viral infection and that viral gene products, such as tat, may act indirectly on the viral LTR by first acting directly on cellular genes. In these studies, the effect of tat on cellular genes and gene products will be investigated.

Methods Employed:

1. A heat shock-inducible vector system has been constructed which utilizes the highly inducible Drosophila hsp70 promoter. Expression of tat will be done in both transiently and stably transfected cell lines. The human T-cell line, H9, is the host cell of choice because it permits HIV growth and is similar to that of the natural host T-cells. DEAE dextran and electroporation are used to construct transient and stable lines, respectively.

2. Two-dimensional gel analysis of labeled proteins from tat^+ and tat^- stable transfectants will be used to assay for modulation in protein levels that may be attributable to tat.

3. Differential hybridization to cDNA libraries from tat^+ and tat^- stable trans-fectants will allow for the isolation of both induced and repressed clones. Presently, differential hybridization to HIV-infected and uninfected H9 cells has been done.

4. Northern blot hybridization is used to analyze modulation in the level of expression of oncogenes and serum-inducible genes in established HIV-infected (H9) and tat-expressing (HeLa) cell lines.

Major Findings:

1. Functional HIV-1 tat gene product is expressed in transiently transfected H9 cells under control of the Drosophila heat shock promoter. By co-transfection of the pHS tat plasmid with a plasmid containing the bacterial chloramphenicol acetyl transferase (CAT) gene linked to the HIV-1 LTR,

transactivation (CAT activity) was demonstrated to occur only when cells were heat shocked for 2 hrs at 42°C.

2. A permanent cell line in H9 cells has been constructed (by electroporation) in which tat-lacking constructs are integrated into H9 genomic DNA. This line will serve as a negative control relative to a permanent tat-expressing cell line.

3. Six cDNA clones isolated by differential hybridization from an H9 library may be repressed relative upon infection by HIV-1. The clones are not yet characterized.

4. A survey of oncogene expression (including c-myc, ets-1, ets-2, v-raf, n-myc, v-Ki-ras, v-sar, r-abl, erbB, erbA, v-myb, v-sis) in infected vs. uninfected H9 cells failed to reveal any modulation in gene expression. However, some serum-inducible genes are repressed and/or have altered polyadenylation patterns upon HIV infection of H9 cells.

5. All genes modulated upon HIV infection are candidates for modulation by tat and are to be tested in tat-expressing permanent cell lines when available.

Publications:

Schweinfest CW, Jorcyk CL, Fujiwara S, Papas TS. A heat shock inducible eukaryotic expression vector. Gene (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05569-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of c-myc on Cellular Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. W. Schweinfest	Staff Fellow	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI

COOPERATING UNITS *(if any)*

Northwestern University Medical School, Chicago, IL (L. F. Lau)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A key role for the c-myc oncogene in cellular proliferation has long been postulated. C-myc may act by modulating the expression of other cellular genes whose products directly control proliferation. Permanent cell lines (in which the endogenous c-myc gene is tightly regulated by growth factors and cell/cell contact) have been constructed in which expression of an exogenously transfected c-myc gene is controlled by the Drosophila heat shock 70 promoter. Transcription and subsequent translation of the exogenous c-myc gene is specifically induced by mild heat shock. The endogenous c-myc gene is not expressed under these conditions. Compared to heat-shocked cell lines which contain constructs lacking c-myc, several changes in cellular gene expression are observed: (1) two-dimensional analysis of the proteins from c-myc-containing cell lines shows the induction of eight protein species and the repression of five protein species relative to cell lines lacking c-myc; (2) the transcripts of two genes (3CH77 and 3CH92) previously identified as serum inducible are induced when c-myc is expressed; (3) the endogenous heat shock 70 gene may be specifically induced in response to c-myc. Therefore, c-myc expression alters the expression of other cellular genes, including the induction of some known to be expressed only in proliferating cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. W. Schweinfest	Staff Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI
S. Fujiwara	Visiting Associate	LMO NCI

Objectives:

A large body of circumstantial evidence has implicated c-myc as having a role in cellular proliferation in both normal and neoplastic cells. Like most oncogenes, a specific biological function for c-myc has yet to be defined. Since c-myc is one of a class of nuclear oncoproteins and has been demonstrated to have DNA binding capability (albeit nonspecific), c-myc's biological function may be to regulate the expression of other genes. Therefore, the objective of this work is to study the effect of c-myc on the regulation of other genes and, by understanding the function of these target genes, be able to define the role of c-myc.

Methods Employed:

1. A set of cell lines has been constructed in which the human c-myc gene (exons 2 and 3 only and exons 1, 2 and 3) can be expressed under control of the Drosophila heat shock 70 promoter. Permanent cell lines were constructed in Balb/c 3T3 cells by co-transfection of the myc-containing plasmids along with the selectable G418-resistance plasmid, pSV2neo, followed by selection in 400 μg/ml G418. Resistant clones are screened for heat shock-inducible expression of c-myc. A control cell line was transfected with a construct lacking the c-myc gene. Under conditions of serum arrest, the endogenous c-myc gene is not expressed.

2. Protein from c-myc-expressing cell lines are analyzed by two-dimensional gel electrophoresis. Non-expressing cell lines are similarly analyzed.

3. The expression of G_0/G_1 transition genes, as well as an S-phase specific gene is assayed as a function or specific c-myc induction by Northern blot hybridization analysis.

4. New genes, whose expression may be modulated by c-myc, are screened for by differential hybridization of cDNA libraries constructed from heat shock-induced, myc-expressing cell lines.

Major Findings:

1. Exogenously transfected human c-myc can be expressed under control of the Drosophila heat shock 70 promoter.

2. Two-dimensional gel electrophoresis, which compares proteins from induced vs. uninduced cells (as well as heat shock-induced negative control cells) reveals the induction of eight protein species and the repression of five protein species.

3. Two genes (3CH77 and 3CH92), previously identified as transcriptionally induced during proliferation, are induced in response to c-myc expression. In response to growth factors, these genes are expressed prior to myc (which also responds to growth factors). However, c-myc expression in the absence of added growth factors precedes expression of 3CH77 and 3CH92. Expression of an S-phase gene upon c-myc induction is not observed.

4. The endogenous heat shock gene itself may be expressed at even more elevated levels in heat shock cells expressing c-myc than in those lacking c-myc. A cDNA clone, #32, which is found to be induced by c-myc-expressing cells, hybridizes with a known hsp70 probe.

5. The cDNA library from c-myc-expressing cells has also been used to find a completely coded mouse ets-2 cDNA (see Project Z01CP05238-07 LMO).

Publications:

Schweinfest CW, Fujiwara S, Papas TS. C-myc protein expression can induce the expression of G_0/G_1 transition genes. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington, DC: IRL Press, 1988;113.

Schweinfest CW, Fujiwara S, Lau LF, Papas TS. C-myc can induce the expression of G_0/G_1 transition genes. Mol Cell Biol (In Press)

Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest CW, Papas TS. Human and mouse ets-2 genes encode for highly conserved proteins. Proc Natl Acad Sci USA (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05570-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Expression of HIV-1 and HTLV-I Proteins in Prokaryotic Vectors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Takis S. Papas	Chief	LMO	NCI
Others:	J. A. Lautenberger	Research Chemist	LMO	NCI
	F. Wong-Staal	Biologist	LTCB	NCI
	R. Ascione	Research Chemist	LMO	NCI
	D. Hodge	Special Volunteer	LMO	NCI
	C. Jorcyk	Chemist	LMO	NCI
	C. W. Schweinfest	Staff Fellow	LMO	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (K.P. Samuel); Bionetics Research, Inc., (G. Pavlakis and B. Felber)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	1.1	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Construction of prokaryotic expression vectors for the production of high levels of human immunodeficiency virus-1 (HIV-1)- and human T-cell leukemia virus-I (HTLV-I)-specific proteins has been accomplished. Using these systems, eight HIV-envelope-specific polypeptides have been expressed at high levels and have been purified to near homogeneity. Using these purified proteins as antigens, it has been possible to elicit specific polyclonal and monoclonal antibodies against these specific retroviral proteins that are able to react against the authentic cellular proteins in vivo. One clone, 566, expresses a protein which is highly immuno-reactive after a 100,000-fold dilution of acquired immunodeficiency syndrome (AIDS)-positive human sera; further, this antigen is able to recognize all HIV-positive sera by enzyme-linked immunosorbent assay (ELISA) testing and is as sensitive in these assays as the natural gp41 viral env-encoded protein. We have also expressed a portion of the HIV-genome encoding the non-structural proteins 3′-orf and sor in our prokaryotic vector systems. We have been able to obtain specific antibodies, using these reagent-antigens, that recognize the native viral product in HIV-infected cellular systems. Our monoclonal antibody against the HIV-1 3′-orf also recognizes the bacterially-expressed HIV-2 3′-orf polypeptide and preliminary studies using the expressed 3′-orf for biochemical analysis shows that the HIV-2 3′-orf is capable of binding guanosine triphosphate (GTP). Our bacterially expressed sor protein has been used to screen a panel of sera from homosexual men that showed no reactivity to commercial HIV-antigens; five out of nine individuals tested prior to seroconversion were positive in a Western immunoblot assay using the bacterially-expressed sor product. These results would suggest that sor may be a valuable reagent to employ for the immunodiagnosis of HIV-reactivity during preclinical stages of infection.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

T. S. Papas	Chief	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
F. Wong-Staal	Biologist	LTCB	NCI
R. Ascione	Research Chemist	LMO	NCI
D. Hodge	Special Volunteer	LMO	NCI
C. Jorcyk	Chemist	LMO	NCI
C. W. Schweinfest	Staff Fellow	LMO	NCI

Objectives:

The human retrovirus, human T-cell leukemia virus (HTLV-I), has been shown to be associated with the disease, adult T-cell leukemia (ATL). Recently, another human retrovirus, the human immunodeficiency virus (HIV), has been shown to cause acquired immunodeficiency syndrome (AIDS). Thus, it is possible that other human diseases are caused by, as yet, unidentified human retroviruses. Therefore, it is of interest to investigate these human retroviruses and their causally-related diseases, both of which have the inherent hazards of working with live virus, or infected patient sera. To circumvent these dangers, we have expressed a number of retroviral gene products of HTLV-I and HIV in prokaryotic vector systems and are preparing large amounts of specific human retrovirus proteins. These selected proteins can be purified to near homogeneity and analyzed for their seroreactivity with antibodies from patients having diseases that may be caused by the retroviruses, HIV-1 and HTLV-I. Such expressed retroviral products can be used for both diagnostic and reagent purposes.

Methods Employed:

1. Construction of Plasmids. DNA fragments were produced by digesting plasmid DNA with restriction enzymes and were resolved on agarose or polyacrylamide gels. Fragments were isolated from agarose gels by dissolving the gel in NaI and absorbing the DNA to glass beads. After washing the glass beads, the fragment was eluted with low-salt buffer and reisolated from acrylamide gels electroelution. Fragments are joined by the action of DNA ligase and the resultant constructs were introduced into E. coli cells that had been made competent by CaCl treatment.

2. Protein Expression. Escherichia coli WPS18 cells containing the vector plasmid, pJL6, or its modified derivatives, were induced at 42° for 1 hr, and proteins were extracted as described by Shoner et al. (Biotechnology 1985;3:151-4). Western blot analysis was performed as described (Science 1984;226:1094-7) on each of the fractions using antibody raised against a peptide coded for by the retroviral gene or infected patient sera, where applicable. The major bands of reactivity that corresponded to proteins in question were further purified by isolation and successive washing of the bacterial inclusion bodies; the fractions solubilized in 8M urea or 7M Guanidine-HCl. Often, these proteins are visible on stained sodium dodecyl sulfate (SDS)-gels and these particulate inclusion fractions are approximately 50% pure without any further chromatographic procedures.

3. The cells used were from the HTLV-I-infected T-cell line, C10/MJ, and the uninfected T-cell line, H9, or the acutely HIV-infected H-9 cells. All bacterial cells were grown at the permissive temperature in LB or NZYDT broth. Proviral HIV clones BH-8 and BH-10 were derived from unintegrated linear DNA obtained from acutely (HIV)-infected H9 cells.

4. Computer Analysis of DNA and Protein Sequences. A wide variety of sequence analyses were performed in connection with the studies described in Major Findings. Recently, extensive use was made of the University of Wisconsin software package implemented on the VAX computer of the Advanced Scientific Computer Laboratory (ASCL) for such applications as data entry, restriction mapping, protein sequence prediction, and alignment of multiple protein sequences. Other analyses required the data base search procedure, SEQFT, run on the Cray-XMP supercomputer of the ASCL. Some specialized applications required the development of new programs. These include a reverse translation program for the design of deoxyinosine oligonucleotide probes, and a program for the creation of hard-copy graphic display of multiple alignments on laser printers that emulate Tektronics 4014 terminals.

Major Findings:

Eight bacterially produced envelope gene-encoded protein fragments from different subregions of human immunodeficiency virus (HIV) glycoprotein molecule (gp120/gp41) have been characterized by Western immunoblot analysis against well-characterized HIV-positive human antisera and polyclonal antisera prepared against highly purified gp120 and gp41 glycoproteins. Each of the recombinant proteins, which account for ~96% of the entire gp160 envelope glycoprotein of HIV, have been shown to be immunogenic, antigenic, and are summarized below:

Bacterial Clone	Region of Env Gene Expressed	Reactivity	
		Immunogenic	Antigenic
486	N-terminus of gp120	-	+
318	Middle of gp120	++	++
569	Middle of gp120	++	++
347	C-terminus of gp120	++	++
719	C-terminus gp120 + N-terminus gp41	++	Not tested*
1016	Middle gp120 + N-terminus gp41	++	Not tested*
566	N-terminus gp41	++	++
503	C-terminus gp41	++	++

*(Though not tested, these polypeptides are believed to be antigenic since they are contained in the region encompassed by the antigenic gp120/gp41).

Polyclonal rabbit antisera were prepared against proteins of clones 318 and 566. Mouse monoclonal antibodies are currently being developed against each protein. Scale-up production and purification of each protein is also being performed for use in diagnostic screening of AIDS and pre-AIDS sera. The identification of conserved or variable immunogenic and antigenic determinants on HIV envelope gp120/gp41 molecules could be determined by the type of analyses.

Partial characterization of eight recombinant envelope polypeptides of HIV gp120 and gp41 by Western blot analysis against HIV-positive human reference serum and polyclonal gp120 and gp41 sera has been achieved. Antibodies have thus far been raised against: clones (1) 318, ~17 kDa polypeptide (polyclonal); (2) 347, ~15 kDa polypeptide (polyclonal); (3) 569, ~23 kDa polypeptide (polyclonal); (4) 566, ~21 kDa polypeptide (monoclonal and polyclonal); and (5) 503, ~17 kDa polypeptide (polyclonal). Each recombinant env polypeptide was screened against a panel of human sera to determine the pattern and spectrum of antibodies in patient sera specific for each region of the gp120 envelope. We have purified to homogeneity the 17 kDa polypeptide of clone 318 and the 22 kDa polypeptide of clone 566, using gel filtration through Sephacryl S100 and reversed phase high pressure liquid chromatography (HPLC). We have evaluated the immunoreactivity of the purified polypeptides of clones 318 and 566 against a panel of HIV-positive and negative sera in ELISA assays. The results show that the 22 kDa polypeptide of clone 566 is highly immunoreactive after 100,000-fold dilution of positive human sera, or at a 20,000-fold dilution of the 566 antigen. This antigen identifies all HIV-positive sera by ELISAs.

We have produced large quantities of a portion of the 3'-orf protein (p27 3'-orf) of HIV, and prepared rabbit antibodies against this protein which can be used for the identification of this protein in the cytoplasmic fractions of H9-infected T-cells. Current efforts are designed to identify p27 3'-orf as either a protein kinase activity or as substrate for cellular protein kinase. A high-titered rabbit anti-p27 3'-orf protein and several mouse monoclonal antibodies have been developed. A monoclonal anti-p27 3'-orf antibody has been characterized and is currently being used for affinity purification of the protein. A structural homology study of the 3'-orf protein was recently conducted. We have shown a similarity in structure with nucleotide binding proteins. Current biochemical analysis of H9/HIV-infected and uninfected cytoplasmic fractions reveal distinct guanosine triphosphate (GTP)-binding activities that comigrate with the 3'-orf and p24 gag proteins. We have analyzed these extracts further by two-dimensional gel analysis, GTP-binding and immunological probing assays to determine whether the HIV-proteins bind GTP. Immunoaffinity-purified 3'-orf and p24 gag proteins from H9 cells infected with HIV-1 (IIIB) are currently being used to determine whether these viral proteins bind GTP/guanosine diphosphate (GDP). Preliminary results suggest that both proteins bind GTP/GDP. We are continuing the GTP/GDP-binding studies and assaying for GTPase and autophos- phorylation activities in the purified p27 3'-orf and p24 gag proteins from H9/HIV-IIIB and other infected T-cell lines. We have expressed the entire HIV-2-3'-orf protein in bacteria and are currently purifying a large quantity of the protein for biochemical analysis. Preliminary binding studies with a partially-purified extract of the HIV-2-3'-orf protein show that it binds GTP. In addition, our HIV-1-3'-orf rabbit antisera and a few monoclonal antibodies also recognize the bacterially produced HIV-2-3'orf protein. Several mouse monoclonals developed against our bacterially expressed HIV-1 (BH8 clone) 3'-orf protein segment identify

epitopes in 3'-orf of HIV-1, but not in different strains of HIV-1 (PH, HXB2, or RF).

Purification of bacterially-expressed HIV-1 sor gene product (b-sor) and preparation of polyclonal antibodies against this protein have been successfully achieved. Characterization of the authentic HIV-1 sor gene product by making use of these reagents has been performed in collaboration with other investigators in the Division of Cancer Etiology. We are now beginning investigations into the usefulness of b-sor in immunodiagnostic assays of HIV-1 infection.

Presently, we are injecting mice with the bacterially expressed sor protein in order to prepare monoclonal antibodies against this protein. Such antibodies will be useful in studying the kinetics of the production of the sor gene product throughout the infection cycle. These studies should provide a more sensitive assay for this protein than the present polyclonal rabbit sera raised against the bacterially expressed protein. Bacterially expressed sor protein produced in our Laboratory has been used by Ranki et al. (Lancet 1987;2:589-93) to survey stored serum samples from homosexual men that had been collected prior to seroconversion (as defined by commercial ELISA assay). In five out of nine such individuals, pre-seroconversion sera reacted with the b-sor in an immunoblot assay. This indicates that the b-sor has potential usefulness for diagnosis of latent HIV-1 infection.

The HIV tat gene has been shown to regulate transcription initiated at the viral LTR and to possibly regulate translation of viral proteins. Since tat has not been shown to bind HIV DNA, this protein may induce the expression of cellular genes. We plan to investigate this possibility by studying the influence of the HIV tat gene on the expression of cellular genes. This gene has been inserted into the retroviral vector, pGVI, under the transcriptional control of the HIV LTR. The recombinant plasmid was transfected into psi2 cells so as to produce an ecotropic viral stock. This virus was used to infect psiAM cells so as to induce G418-resistant colonies. At present, these colonies are being purified and tested for the production of amphotropic helper-free retroviruses. These viruses will be used to infect human lymphoid cells so as to establish cells constitutively expressing the tat gene. Such lines will be useful for the identification of cellular genes whose expression is controlled by the tat gene. Recombinant plasmids containing an inserted fragment with HIV LTR and tat sequences in each orientation have been constructed (plasmids pGVlLTRtat-s and pGVlLTRtat-a). The recombinant plasmids are being used to introduce the HIV tat gene into lymphoid cells under HIV LTR control. It has been shown that the recombinant plasmids can induce psi2 cells to produce an ecotropic virus that can generate G418-resistant colonies in psiAM cells. Resistant colonies will be tested for virus production in HeLa. Clones that produce high viral titer will be used to make virus for infection of human lymphoid cells so as to establish cell lines constitutively expressing the tat gene. Such lines will be used for the identification of cellular genes whose expression is controlled by this gene.

Publications:

DuBois GC, Samuel KP, Showalter SD, Papas TS. Expression and purification of proteins encoded by the envelope and 3'-orf genes of human immunodeficiency virus. In: Brew K, Ahamd F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J,

Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production Oxford/Washington DC: IRL Press, 1988;178.

Samuel KP, DuBois GC, Showalter S, Zweig M, Papas TS. Expression of fused and unfused proteins encoded by the envelope and 3'-orf genes of human immunodeficiency virus in Escherichia coli. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;182.

Samuel KP, Seth A, Konopka A, Lautenberger JA, Papas TS. The 3'-orf protein of human immunodeficiency virus shows structural homology with the phosphorylation domain of human interleukin-2 receptor and the ATP-binding site of the protein kinase family. FEBS Lett 1987;218:81-6.

Samuel KP, Seth A, Zweig M, Showalter SD, Papas TS. Bacterial expression and characterization of nine polypeptides encoded by segments of the envelope gene of human immunodeficiency virus. Gene 1988;64:121-34.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05571-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies of E26 Avian v-ets and its Cellular Homologue in Mouse Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Blair	Supv. Research Chemist	LMO	NCI
Others:	Q. Yuan	Visiting Fellow	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. Watson	Research Microbiologist	LMO	NCI
	K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid and Protein Synthesis Laboratory, PRI, Frederick, MD
(M. Zweig, S. D. Showalter)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.9	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A replication-defective murine retrovirus, ME26, was constructed by inserting the avian gag-myb-ets sequences derived from the cloned avian acute leukemia virus, E26, into an Abelson murine leukemia virus (MuLV)-derived retroviral vector. Both ME26 DNA transfected nonproducer cells and ME26-infected cells expressed a 135 Kd gag-myb-ets fusion protein, termed p135m. p135m is localized primarily in the nucleus and can easily be washed with a low-salt buffer containing detergents. NIH 3T3 cells infected with ME26 exhibit morphological alterations and increased proliferations, and also form small colonies in soft agar. ME26 induces an increased incidence of leukemia, primarily erythroid and myeloid, with long latencies when injected into newborn mice. Analysis of frameshift and deletion mutants is consistent with the v-ets sequences being necessary to mitogenically stimulate NIH 3T3 proliferation in culture.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. Blair	Supv. Research Chemist	LMO	NCI
Q. Yuan	Visiting Fellow	LMO	NCI
A. Seth	Visiting Scientist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
D. Watson	Research Microbiologist	LMO	NCI
K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI

Objectives:

To study the mechanism and cooperative role of the myb and ets oncogenes of the avian erythroleukemia virus, E26, in oncogenesis.

To determine the biological function of v-ets and its cellular homologue in altering cell growth and hematopoietic development, and to develop biological assays to characterize these functions.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA trans- fection, measurement of tumorigenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect specific cellular proteins.

Major Findings:

1. ME26 virus, expressing the gag-myb-ets oncogene fusion protein of the avian E26 leukemia virus, induces myeloid and erythroid leukemias in newborn mice. We have previously shown that amphotropic pseudotypes of the ME26 viral construct induced an increased incidence of leukemias with long latencies. Histological analysis of diseased organs indicated that the majority of these (54%) were erythroid and myeloid neoplasms, while the remainder were lymphoblastic leukemias similar to that observed with helper virus alone. Leukemic tissue from early incidence of disease contained multiple copies of integrated ME26 provirus and the restriction patterns suggested at least some of the tumors were clonal in origin. The amphotropic helper virus alone failed to induce erythroid leukemias, with 88% showing lymphoblastic leukemia only, with a latency approximately 100 days longer than observed in ME26 infected animals.

2. ME26 infected or transfected NIH 3T3 cells form foci of mitogenically stimulated, morphologically altered cells in defined media at low serum levels, and can grow in serum-free media in the absence of added growth factors. We have established a reproducible tissue culture assay to detect the biological activity of ME26. ME26-infected NIH 3T3 cells have a reduced serum dependence and discrete foci could be readily recognized in cells maintained in a defined

media (QBSF) containing 0.03-0.1% calf serum. Thymidine incorporation experiments have also indicated that 0.03-0.1% serum is the optional condition for the proliferation of ME26-infected NIH 3T3 cells. Virus replication was not required for mitogenic stimulation, since stocks of helper-free ME26 prepared in the packaging cell line, PA317, also induced foci on 3T3 cells. Foci could not be detected in serum-free media supplemented with epidermal growth factor, although this was sufficient to maintain the growth of both infected and uninfected NIH 3T3 cells.

3. Analysis of ME26 deletion and frameshift mutants indicates that the ets portion of the gag-myb-ets polyprotein is required for the in vitro growth stimulation. We constructed deletion and insertion mutants to study the oncogenic potential of the v-ets oncogene. Four mutants were constructed and their protein products were detected by immunoprecipitation of NIH 3T3 cells co-transfected with construct DNAs and pSV2neo. Viruses were harvested from MuLV superinfected, pooled G418-resistant colonies. Biological activity was observed by both reseeding pooled G418-resistant colonies and virus infecting NIH 3T3 cells in QBSF media with 0.03% and 0.1% calf serum. Deletions of gag and the 5′ end of myb did not affect the focus-inducing properties of the constructs, but a frameshift inserted in the 5′end of the ets coding region abolished activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05572-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Isolation of Potential Oncogenes from Teleost Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	R. J. Van Beneden	Guest Researcher	LMO	NCI
	K. W. Henderson	Special Volunteer	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

U.S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD (W. Van der Schalie, H. Gardner)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies have suggested that fish have oncogene sequences homologous to those found in mammalian and avian species. We were the first to confirm the presence of fish oncogenes by isolating and sequencing the c-myc gene from rainbow trout (Van Beneden et al., 1986). Other fish genes homologous to known mammalian oncogenes were identified by Southern blot hybridization. In order to examine the role of fish oncogenes, Southern blots were prepared using DNA digests from tumor and normal tissue. Hybridization to known oncogene sequences did not detect rearrangements or gene amplifications. In order to detect other oncogenes, we developed a transfection system in which fish DNA in the presence of calcium phosphate was transfected into NIH 3T3 cells. The transforming ability of fish tumor DNA was examined by standard focus assay, nude mouse assay, and colony selection assay. DNA from diethylnitrosamine-induced mesothelioma in medaka was the most efficient in transformation of NIH 3T3 cells. Secondary transfectants caused formation of tumors in nude mice of >20mm in 1-1/2 weeks following injection. Southern blot analysis of these transfectant DNAs hybridized to medaka genomic DNA probe showed bands present in tumor-induced transfectants. No bands were present in DNA from NIH 3T3 controls and cells transfected with non-tumorigenic medaka DNA. This suggests the presence of specific fish sequences in transformants. These sequences do not appear to be homologous to K-ras, H-ras, N-ras, c-myc, m-met or v-erbB. This study will continue to identify, clone, and sequence the transforming gene from fish in order to examine its role in tumor formation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO	NCI
R. J. Van Beneden	Guest Researcher	LMO	NCI
K. W. Henderson	Special Volunteer	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The purpose of this investigation is to examine DNA isolated from teleost tumors for the presence of transforming genes. These gene(s), isolated by transfection assay, will be cloned and characterized. This will allow us to determine whether they are cellular homologs of known oncogenes or if they represent a new oncogene sequence. Transforming genes isolated from chemically induced lesions will enable us to examine the mechanism of chemical carcinogenesis.

Methods Employed:

1. Tumors and normal tissue were isolated from both feral and laboratory-raised fish. Tissue was either used immediately for DNA isolation or frozen in liquid nitrogen and stored at -70° until used. White perch (Morone americana) were caught by trawling in the Back River (Chesapeake Bay). Tumors were characterized by histopathological analysis of a section of the liver. Northern pike (Esox lucius) were trapped at Ostego Lake, Michigan. Sections from the lymphosarcoma (external lesion) and internal organs were preserved for histopathological examination. Tumors (primarily hepatocellular carcinoma) were induced in laboratory-reared Japanese medaka (Oryzias latipes) by treatment with either (1) diethylnitrosamine (DEN), 200 mg/l, 48 hr exposure to 14-day old post hatch (2) methylazoxymethanol acetate (MAM-Ac).

2. High molecular weight DNA was prepared by the quick-dounce homogenization method of C. Cooper. Individual tissues were homogenized in buffer containing sodium dodecyl sulfate (SDS) and proteinase K followed by phenol-chloroform extractions. When very cartilagenous tissue was used, it was pulverized in liquid nitrogen prior to homogenization. DNA was then precipitated in ethanol, resolubilized and incubated sequentially with RNAse A and proteinase K. Following phenol-chloroform extraction and resolubilization, DNA concentration was estimated by absorbance at 260 nm.

3. DNA isolated from both normal and tumor tissue was digested with restriction enzymes, electrophoresed on agarose gels, transferred to nitrocellulose, and the resulting Southern blots (Southern, 1975) were hybridized to nick-translated oncogene sequences. Fragments used as hybridization probes included v-erbB, H-ras, v-myb, v-abl, v-erbA, v-sis, v-src (all from Oncor Science), c-myc (rainbow trout) and v-ets (fragment E1.2B, Watson et al., 1985).

4. In order to identify transforming sequences, high molecular weight DNA isolated from fish tumors and from normal tissues (control) was examined by transfection assay. A stock of NIH 3T3 cells (490 N3T) was obtained from Dr. Donald Blair (See Project #Z01CP05295-07). Cells were maintained at levels below confluency in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum. To each plate of 3×10^5 NIH 3T3 cells, 25 μg of fish genomic DNA (prepared as above) was co-transfected with 2 μg of a neomycin-resistant plasmid (pSV2neo) in the presence of calcium phosphate (Pellicer et al., 1980). A total of four plates (100 μg fish DNA) of each sample was tested, which was expected to provide one genomic equivalent of DNA. Cells were grown in the presence of geneticin (G418) for two weeks. Drug-resistant colonies were selected, harvested by trypsinization, and replated (in the absence of G418) in a standard focus assay, in a colony selection assay, and/or injected into athymic mice.

5. Standard focus assay. G418-selected cells were replated in media containing no G418 and grown to confluency. Plates were examined for foci. Foci were counted, picked and expanded. DNA was isolated by the Hirt extraction procedure.

6. Colony-selection assay. Cells from the same pool as above were replated in minimal media (QBSF or QBSF supplemented with 0.1% serum). Cells that formed colonies were counted and DNA isolated as above.

7. Nude-mouse assay. The remaining G418-selected cells were injected into athymic mice (1.5×10^6 cells/mouse). Mice were examined for tumors at the site of injection (positive results usually occurred $\leq$ 6-8 weeks). Tumors were excised and portions frozen for DNA extraction (see 2.) and/or diced and placed in media for growth of tumor explants.

8. Southern blots (see 3.) were prepared using DNA isolated from transfected cells, nude mouse tumors and tumor explants. These were hybridized to either known oncogene probes (under low stringency conditions) in order to identify homologous oncogenes or to high molecular weight fish probes to identify fish-specific sequences.

9. In order to examine cells for contact inhibition, cells from foci were examined for their ability to grow in soft agar (0.23%).

10. Genomic libraries were prepared from white perch, northern pike and medaka DNA. High molecular weight genomic DNA isolated from fish testes was partially digested with Sau 3AI. Fragments of 12-20 kb were isolated from a 5-25% NaCl gradient (Glover, 1985). Fragments were ligated into the cloning vector EMBL 3 and packaged, following Stratagene's recommended procedure. Recombinants were selected by plating library on E. coli strain P2392.

11. Clones containing presumptive repetitive fish sequences were isolated from each of the three libraries by screening under high stringency conditions with genomic DNA from the homologous fish species.

12. Fish genomic DNA was enriched for repetitive sequences by shearing DNA to ~300-800 bp, denaturing at 97°, and allowing to reanneal at 60° to a Cot of 40.

Double-stranded DNA was isolated from a hydroxyapatite column maintained at 60° in 0.12 M phosphate buffer.

Major Findings:

1. Southern blots of restriction digests of DNA from normal fish tissue, compared to tumor tissue, showed no detectable differences in hybridization pattern or band intensity when hybridized to known oncogene sequences (v-erbB, H-ras, v-myb, v-abl, v-erbA, v-sis, v-src, c-myc, K-ras and v-ets). There appeared to be no tissue-specific polymorphisms among tissues of a single individual nor among individuals of the same species. These data indicate that for those oncogenes and tissues examined, there appear to be no gross rearrangements nor amplification of oncogenes in tumor tissue relative to control samples.

2. DNA isolated from a diethylnitrosamine-induced mesothelioma in medaka very rapidly transforms NIH 3T3 cells as determined by standard focus assay, colony selection in minimal media (QBSF), and the nude mouse assay. Tumors in nude mice grow to 20 mm after only 1-1/2 weeks. Cells isolated from foci demonstrate anchorage-independence by growth in soft agar.

3. DNA isolated from northern pike lymphosarcoma and white perch cholangioma is also able to transform NIH 3T3 cells, but is not as aggressive as the DNA isolated from medaka mesothelioma.

4. DNA isolated from a MAM-Ac-induced rhabdomyosarcoma had only a marginal ability to transform NIH 3T3 cells, although this is a very aggressive lesion. The findings suggest that perhaps a "transforming gene," if present, does not function well in this system.

5. Hybridization of Southern blots of EcoRI and BamHI digests of DNA isolated from two secondary transfectants (induced by medaka mesothelioma DNA) to medaka genomic sequences at high stringency reveal specific bands. These bands are not present in DNA isolated from NIH 3T3 cells alone or in DNA isolated from cells transfected by nontumorigenic medaka DNA. This suggests that the transformation of the NIH 3T3 cells is due to specific fish sequences. These sequences have not yet been identified, but don't appear to be homologous to H-ras, N-ras, K-ras, v-erbB, myc, or m-met, as determined by Southern blot hybridization.

Publications:

Van Beneden RJ, Power DA. Glucosephosphate isomerase allozymes from the teleost Fundulus heteroclitus. J Mol Biol Evol (In Press)

Van Beneden RJ, Watson DK, Chen TT, Lautenberger JA, Papas TS. Teleost oncogenes: evolutionary comparison to other vertebrate oncogenes and possible roles in teleost neoplasms. Marine Environ Res 1988;24:339-43.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05573-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of Oncogene Expression in Transformed Cells on Topoisomerase Functions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Priel	Visiting Scientist	LMO NCI
Others:	D. Blair	Supv. Research Chemist	LMO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Accumulating data suggest that due to their influence on the topological state of the DNA, topoisomerases I and II (topo I, II) may control various biological processes. Because of their involvement in the control of such fundamental biological activities, it is tempting to speculate that the deranged expression of these enzymes may play an important role in neoplastic transformation. Therefore, in order to elucidate this possibility, we have searched for the possible in vivo interrelationships between these enzymes and activated oncogenes. The enzymatic activities of the topo II in oncogene-transfected cells differed from those observed in untransfected cells, mainly on the degree of dependence on adenosine triphosphate (ATP), spermidine and Mg+2. Normal rat kidney (NRK) cells infected with a temperature-sensitive mutant (ts110) of Moloney murine sarcoma virus (Mo-MuSV) had a very slight topo I activity at the permissive temperature (34°), while at the non-permissive temperature (39°) they exhibit a normal topo I activity. These cells produced p85gag-mos proteins at 34°C, but not at 39°C. Moreover, these cells were resistant to topo I inhibitor effects at 34°C, but were sensitive at 39°C. Further experiments are needed to determine whether this absence inhibition of topo I activity is a direct effect of the p85 gag-mos protein.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

E. Priel	Visiting Scientist	LMO	NCI
D. Blair	Supv. Research Chemist	LMO	NCI

Objectives:

To evaluate the relationship between topoisomerase distribution and function, and the expression of various oncogenes.

To determine the mechanism by which oncogenes alter topoisomerase functions, and the relationship of these alterations to the initiation and maintenance of the transformed phenotype.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells; immunoprecipitation and polyacrylamide gel analysis of labeled cell extracts to detect specific cell proteins. Topoisomerase II was assayed by measuring the ATP-dependent relaxation, linearization and catenation, and the ATP-independent knotting of purified, supercoiled plasmids on agarose gels following incubation with cytoplasmic and nuclear extracts of transformed and normal cells. Topoisomerase I was assayed by measuring the ATP-independent relaxation using the method described above.

Major Findings:

1. Oncogene transformed NIH 3T3 and Balb/c A31 cells transformed by cloned mos, ras and src oncogenes show alterations in topoisomerase II function. We initiated a comparative study of the characteristics of topoisomerase enzyme in both normal and malignant cells transformed by a variety of defined oncogenes. All the examined oncogene and proto-oncogene transfected cell lines possessed both cytoplasmic and nuclear topo II activity. In general, the cytoplasmic activities differed from the nuclear activities. The enzymatic characteristics of both nuclear and cytoplasmic topo II activities in oncogene-transfected cells differed from those observed in untransfected cells in the degree of dependence on ATP spermidine and Mg^{+2}. The most significant effect on topo II activity was observed in mos-transfected cells.

2. NRK cells transformed with ts110, a temperature-sensitive isolate of Moloney murine sarcoma virus, exhibits temperature-dependent alterations of topoisomerase. We investigated the effect of mos on topoisomerase activity in NRK cells infected with a temperature-sensitive mutant (ts110) of Mo-MuSV. These cells have transformed properties when maintained at 34°C, but revert to a normal phenotype at 39°C. We examined topo I and topo II activities in these cells at both 34°C and 39°C. We could not detect a significant topo I activity in these cells at 34°C, but a topo I activity was detected in NRK ts110 cells maintained at 39°C for 4-6 days. These cells were reverted to a normal phenotype. Cells maintained at 39°C for only one day didn't revert to normal phenotype and had a

low level of topo I activity. Topo I activity was not detected in ts110 cells maintained at 37°C for six days. To further substantiate these findings we analyzed the effect of topo I specific inhibitor-camptothecine (CPT) on ts110 cell growth and on RNA, DNA and protein synthesis. CPT is a cytotoxic alkaloid which inhibits RNA and DNA synthesis and causes rapid and reversible fragmentation of DNA in mammalian cells. Growth of the wild-type NRK cells was completely inhibited by 0.2 μm CPT, while the growth of NRK-ts110 cells maintained at 34°C was not affected. Moreover, the growth of the NRK-ts110 cells maintained at 39°C was significantly inhibited by 0.2 μm CPT and these cells, at 39°C, were even more sensitive to the drug than the normal cells. CPT severely inhibited RNA and DNA synthesis in NRK cells and had no effect on protein synthesis. In the NRK-ts110 cells, at 34°C, CPT caused only slight inhibition of RNA and DNA, but these cells, at 39°C, showed a significant inhibition of DNA and RNA synthesis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05574-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Drosophila melanogaster -- ets and ets-like Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	L. J. Pribyl	Biologist	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Department of Biochemistry, Georgetown University, Washington, DC (J. Chirikjian)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Organisms from humans to Drosophila have been found to contain cellular sequences and transcripts that are homologous to viral-onc genes. The normal function of the genes encoded for by these cellular sequences is unknown. Only by understanding the normal function of these cellular genes (c-onc) will we have a possible means to understand how they can become transforming upon transduction by the virus. With this goal in mind, this study using Drosophila was begun.

Cellular sequences homologous to the ets region of the chicken retrovirus, E-26, have been found in Drosophila in this laboratory. The characterized portion of this gene corresponds to the last two exons of the chicken c-ets-1 gene, and has over 90% homology at the predicted amino acid level. It is designated D-ets-2 and is localized on chromosome 3R at position 58 A/B, and it produces a single transcript of 4.7 Kb in all developmental stages. Low stringency hybridization of Drosophila genomic DNA shows several other bands that also hybridize with a viral ets probe, E1.28. Hybridization of a cDNA library under these conditions led to the isolation of a cDNA clone which shows considerable homology to v-ets but is not D-ets-2. This gene, called D-elg for Drosophila ets-like gene, has ~60% homology with D-ets-2 and is located on chromosome 3R at 97D. It produces two transcripts of 2.3 and 2.0 Kb in embryo, pupae, and adult stages. Drosophila appears to have conserved the 3' region of the ets gene very well in at least two different genes, and now it is hoped that Drosophila will provide a system to determine the function of these genes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO NCI
L. J. Pribyl	Biologist	LMO NCI
R. Ascione	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To determine if there are sequences present in the Drosophila genome that are homologous to the v-ets sequences of the avian retrovirus E26. If such sequences do exist, it will then be necessary to characterize this gene or gene family which will provide data necessary to understand the structural evolution of these genes. This would then allow one to develop a model for the domains along the ets protein which may have functional implications and may provide a functional assay.

Methods Employed:

1. Preparation of high molecular weight DNA from Drosophila developmental stages and cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.

2. Digestion of genomic and clonal DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction from low melting agarose and were used either to prepare ets-specific DNA probes by nick-translation using E. coli DNA polymerase and DNase I, or in the preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot partial technique and hybridization of ets probes, or in the construction of a partial recombinant phage library by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

3. Isolation of phage from the libraries containing virus-related sequences by hybridization of ets-specific probes to nitrocellulose filters containing phage DNA prepared from plaques by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA from colonies lifted from plates by the method of N. Brown (personal communication).

4. Subcloning of isolated Drosophila DNA fragments into appropriate plasmid vectors, as required.

5. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560).

6. Total cellular RNA from cultured cells or developmental stages was prepared by the urea method (Biochemistry 1973;12:2330-8). Separation of polyA+ and

polyA- RNA by one cycle of purification through oligo(dT) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

7. Chromosomal in situ hybridization of Drosophila third instar larvae, using the method of Pardue and Gall (Methods Cell Biol 1975;10:1-16).

8. Total protein extractions from Schneider cells and developmental stage were done, with the resultant proteins electrophorized on polyacrylamide gel and then transferred by electroblotting to nitrocellulose paper.

9. Western analysis using antibodies created against viral peptides were applied. Peroxidase conjugated antibody technique was used to detect reactivity.

Major Findings:

1. In Drosophila there are three genes that are related to the v-onc gene ets. These have been designated D-ets-2, D-elg, and a possible D-ets-1 gene. The D-ets-2 is a gene whose 3′ end has been characterized from a single genomic clone. This region is highly homologous to the last two exons of the chicken c-ets-1 gene. It is also 94% homologous to the human ets-2 gene in this region. The 5′ region of D-ets-2 is now being isolated. A cDNA clone of 1.5 Kb from the D-elg gene has 50% homology to the D-ets-2 gene. The D-ets-1 genomic clone is only partially characterized, but appears to be related to the 5′ region of the ets gene.

2. D-ets-2 is expressed as a 4.7 Kb transcript in embryo, larvae, pupae and adult stages. In contrast, Schneider cells, an embryonic cell line with a single point mutation in the D-ets-2 coding region, encodes a 3.8 Kb transcript. D-elg is found to have two transcripts of 2.8 Kb and 2.0 Kb in embryo, pupae and adult stages with no expression in the larvae. D-ets-1 is expressed only in the pupae and adult stages by a 2.6 Kb message.

3. All three genes have been localized to different chromosomes. D-ets-2 is located on chromosome 2R at position 58A/B. D-elg is on the right arm of the third chromosome, at 97D, while D-ets-1 is on the left arm of the third chromosome, at position 66A.

4. The 3′ end of the ets gene characterized to date is very highly conserved. This extensive homology persists in Drosophila, perhaps suggesting a conservation of function for a region with such great structural conservation.

Publications:

Chen ZQ, Kan NC, Pribyl LJ, Lautenberger JA, Moudrianakis E, Papas TS. Molecular cloning of the ets proto-oncogene of the sea urchin and analysis of its developmental expression. Dev Biol 1988;125:432-40.

Chen ZQ, Kan NC, Pribyl LJ, Lautenberger JA, Moudrianakis E, Papas TS. Proto-oncogene c-ets of sea urchins: transcripts and sequence homology with v-ets and human c-ets genes. In: Voellmy RW, Ahmad F, Black S, Burgess DR, Rotundo R,

Scott WA , Whelan WJ, eds. Advances in gene technology: the molecular biology of development. Cambridge: Cambridge University Press, 1987;14.

Papas TS, Bhat NK, Chen TT, Dubois G, Fisher RJ, Fujiwara S, Pribyl LJ, Sacchi N, Seth A, Showalter SD, Watson DK, Zweig M, Ascione R. The ets genes in cells and viruses: implications for leukemias and other human diseases. In: Peschle C, ed. Normal and neoplastic blood cells: from genes to therapy. New York: New York Academy of Sciences, 1987;171-91.

Pribyl LJ, Watson DK, McWilliams MJ, Ascione R, Papas TS. Characterization of the ets-2 gene in Drosophila melanogaster. In: Voellmy RW, Ahmad F, Black S, Burgess DR, Rotundo R, Scott WA, Whelan WJ, eds. Advances in gene technology: the molecular biology of development. Cambridge: Cambridge University Press, 1987;93.

Pribyl LJ, Watson DK, McWilliams MJ, Ascione R, Papas TS. The Drosophila ets-2 gene: molecular structure, chromosomal localization, and developmental expression. Dev Biol 1988;127:45-53.

Pribyl LJ, Watson DK, McWilliams MJ, Ascione R, Papas TS. The ets-like gene (elg) of Drosophila melanogaster. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;111.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05575-01 LMO

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

ets Oncogene Expression in Bacteria and Yeast

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. Lautenberger	Research Chemist	LMO	NCI
Others:	C. Samalekos	Visiting Fellow	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc. Frederick, MD (S. Showalter); Bionetics Research, Inc., Frederick, MD, (D. Court)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The c-ets oncogene was originally discovered through its viral equivalent v-ets gene of the avian acute leukemia virus, E26. This oncogene codes for nuclear proteins against which a number of monoclonal antibodies have been raised. These antibodies were used to check for ets protein expression in bacterial (E. coli w3110) and yeast (S288C haploid) cells. The antibody directed against the 56 Kd ets-2 protein was found to give a signal in yeast cells, as well as in the COLO 320 double-minute (DM) cells used as a positive control, but failed to do so in the bacterial cells. In addition, a new monoclonal antibody tested was found to give a positive signal in both the yeast and the human COLO 320 DM cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. Lautenberger	Research Chemist	LMO	NCI
C. Samalekos	Visiting Fellow	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

Ever since the ras oncogene protein was shown to be closely homologous to a protein in yeast cells, a lot of attention has focused on discovering more such yeast genes that would be homologous to mammalian oncogenes. In this project, emphasis has been given in discovering the yeast homologue of the ets oncogene. If this gene is found in yeast cells, we intend to utilize the well-known genetic model of yeast to investigate the function of this gene in this organism. Such conclusions might be applicable to understanding the function of ets in mammalian systems. It would be interesting to investigate whether the yeast ets-homologous gene is a part of the Cdc genes that control the cell cycle and division of the organism. Also, the sequence and organization of the yeast gene will be investigated in order to identify the regions of close homology of this gene and the other ets genes already identified. This would give an insight as to the evolution of this gene.

Methods Employed:

Initially, the search for the ets gene in yeast and bacteria was concentrated at the protein level. Bacterial and yeast cells were lysed and their proteins were fractionated into nuclear and cytoplasmic fractions. The proteins were then separated by electrophoresis on 12.5% polyacrylamide gels and were then transferred onto nitrocellulose filters by Western blotting. In parallel, cells, both bacterial and yeast, were grown in minimal medium to which 18 amino acids were added (except all 20 common amino acids, methionine and cysteine). The cells were labelled by the addition of ^{35}S-methionine and ^{35}S-cysteine to the medium. Proteins were isolated by lysis with radio-immune precipitation assay (RIPA) buffer.

In both cases, the proteins were investigated using monoclonal antibodies raised against the P35 bacterially expressed ets protein (ZC50) and monoclonal antibodies raised against a synthetic peptide corresponding to a 5 amino acid sequence of the P35 ets protein. Also, ZC50 polyclonal antibodies were used. Around 20 of these monoclonals were screened for the first time.

The filters obtained by Western blotting were incubated with the above antibodies and filters were then exposed. Also, proteins from the labelled cells were immunoprecipitated using the same antibodies in the presence of protein A-sepharose. After boiling, samples were electrophoresed in 12.5% polyacrylamide gels. The gels were then dried and exposed.

Recently, my work has also concentrated on detecting the ets oncogene, primarily in yeast, by Southern hybridization, using 18 to 24 mer synthetic oligonucleotides

as probes. Also, a 360 bp DNA fragment from the sea urchin ets gene is used as a probe. The oligonucleotide probes were selected by use of a computer program that identified regions of minimum degeneracy in the codons predicted from a consensus of metazoan ets amino acid sequences (i.e., human, mouse, chicken, Drosophila, and sea urchin). Deoxyriboinosine was incorporated into ambiguous positions.

Major Findings:

From the immunoprecipitation method a number of proteins of different sizes gave signal in yeast using three monoclonal antibodies that have previously been used to detect the ets protein in human cells. Also, the polyclonal antibody (ZC50) and one of the 20 new monoclonal antibodies that were screened gave signal of ets protein in yeast cells. The same antibodies also reacted with the COLO 320 DM cells used as positive controls. We are currently trying to confirm these findings by employing competition between the purified P35 protein and the synthetic peptide.

In bacteria, too, when grown in minimal medium, a protein of aroung 43 Kd gave a signal when using the ZC50 polyclonal antibody in immunoprecipitation. This protein will be separated and recovered by isoelectric focusing and 2 dimensional electrophoresis, and its sequence will be determined.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) uses viruses as tools for probing cellular regulatory mechanisms; (3) develops and applies biological, biochemical and immunological procedures to obtain evidence for the mechanism by which antigens are recognized by the immune system; (4) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (5) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells.

The Virus Tumor Biology Section (1) characterizes the nucleotide sequence from regions of viral and cellular DNA thought to be involved in gene expression; (2) investigates the properties of cellular and viral transforming genes and their protein products; (3) evaluates the mechanisms by which viral and cellular proteins affect the level of gene expression; and (4) develops eukaryotic viral vectors to study gene expression.

The Cell Physiology Section (1) investigates the molecular elements essential for cellular transformation; (2) studies the properties of cell surface molecules in expression of the cellular phenotype; (3) uses recombinant DNA techniques and molecular genetics to study the elements involved in gene regulation; and (4) employs prokaryotic host-vector systems to examine sequences involved in efficient gene expression and protein production.

The elucidation of signals associated with gene expression is among the primary objectives of the Laboratory of Molecular Virology. In particular, our interest has been directed toward regulatory events which take place at the level of transcription and processing of RNA. We have been involved in the elucidation and analysis of novel genetic elements, enhancer sequences, which appear to be responsible for controlling the rate at which particular genes are transcribed. We have demonstrated the existence of these enhancer sequences not only in the genomes of DNA viruses such as simian virus 40, JC virus and BK virus, but also in the long terminal repeats (LTRs) of retroviruses. Using a combination of in vivo and in vitro assays, we have demonstrated that enhancer sequences often show host-cell specificity, and thus may be among the elements involved in controlling the host range of certain viruses as well as the tissue-specific expression of certain eukaryotic genes. A number of laboratories have now shown that enhancers are critical elements in determining the activity of eukaryotic genes and that they function in a tissue- or organ-specific fashion. A major effort in our laboratory will be directed at determining whether or not enhancer sequences play a role in the developmental and tissue-specific regulation of gene expression. In vivo and in vitro experiments have been designed in an attempt to examine the mechanism by which

the activator/enhancer sequences function. Currently, we have embarked on a number of experiments to define and characterize the biological macromolecules which interact with these regulatory elements.

JC virus is a human papovavirus that has been associated with the demyelinating disease, progressive multifocal leukoencephalopathy. Virus production in tissue culture is restricted to human fetal glial cells and is regulated at the level of transcription and DNA replication. Oligonucleotides have been synthesized which span the 98 base-pair repeated region of the JC virus enhancer. Gel retardation and ultraviolet cross-linking experiments using these oligonucleotides have identified three proteins from human fetal glial cell extracts which are associated with this region. Two proteins of 82 kilodaltons (kd) and 80 kd recognize the 5′ and 3′ regions of the 98 base-pair region, respectively. The molecular weights of these proteins are similar to those of proteins similarly identified from human HeLa cell extracts. One protein specifically binds to the central region of the JC virus repeat, but was found to have a molecular weight of 45 kd in human fetal glial cell extracts and 85 kd in human HeLa cell extracts. Studies are in progress to determine the role of each of these proteins in the tissue specific expression of JCV.

A particularly important avenue of investigation has involved the development of transgenic mice. Using JC virus, we have been able to establish lines of mice with specific neurologic defects including a demyelination syndrome and tumors of the adrenal medulla. Animals have also been established which carry "foreign" class I histocompatibility antigens (see below). These will be important in studying a number of basic principles associated with immunosurveillance and the distinction between foreign and self. We have also derived mice which carry and express the human T-lymphotropic virus type I (HTLV-I) tat gene under the control of its own regulatory element. These mice develop neurofibromatosis (NF) which resembles human von Recklinghausen's disease. NF is particularly prevalent in the southern part of Japan, which also happens to be endemic for HTLV-I infection. Transgenic mice carrying the human immunodeficiency virus (HIV) tat-3 gene give rise to Kaposi's sarcomas.

Another major focus of the laboratory has been the transcriptional regulatory role of the 3′ long open reading frame of the HTLV-1 which encodes a 40 kd protein ($p40^{tax}$). This protein positively regulates transcription directed by the HTLV-1 LTR in a phenomenon known as *trans*-activation. We have been unable to attribute any sequence-specific DNA binding properties to $p40^{tax}$, suggesting that the protein activates the HTLV-1 promoter in an indirect fashion using cellular transcription factors. Our objective is to understand the biochemical mechanism of *trans*-activation by the $p40^{tax}$ protein and the involvement of cellular transcription factors in this process. Important findings include: (1) $p40^{tax}$ appears to *trans*-activate responsive LTR elements through the induction of a 180 kd cellular protein; (2) mutational analyses correlate the *trans*-activation of the HTLV-1 LTR by $p40^{tax}$ with the presence of a cAMP responsive octonucleotide; (3) two signal transduction agents, cAMP and TPA, are both potent activators of the HTLV-LTR. Relevant to the last observation, we have defined both the cAMP-responsive and the TPA-responsive sequence elements with the HTLV-1 LTR.

An understanding of the structure and function of the class I histocompatibility antigens (the classical transplantation antigens) and in

particular, the roles of these cell-surface antigens in relation to the neoplastic state has been a subject of considerable interest. These studies are of singular importance because the ability of the immune system to identify and destroy tumor cells depends upon their presentation by the class I antigens to the cytotoxic T-lymphocytes. A major goal is to obtain an understanding of the factors which govern immune recognition of foreign cells. Attempts are directed at in vivo and in vitro immune modulation which, hopefully, will enhance the ability of the host to recognize tumor cells as "foreign" and to eliminate them by immunologic means.

Molecular cloning and identification of class I loci has led to the finding of a gene that encodes a soluble or secreted class I-related antigen. Because of variations in the level of expression of this gene in different inbred mouse strains and an unusual tissue-restriction in its expression, it was suggested that this soluble histocompatibility antigen represented a serum protein, perhaps a tolerogenic form of the class I antigens, which could act as a blocking factor to regulate the function of cytotoxic T-cells in the process of immune surveillance. Studies are in progress to test this hypothesis by using the secreted class I antigen to modulate T-lymphocyte recognition of tumor cells.

A considerable effort has been directed toward the study of mammalian oncogenes and their counterparts in yeast. It is hoped that this information will contribute to an understanding of the process of cell transformation and tumorigenesis. We have examined cell lines transformed by the ras oncogene, and by ras and E1A, and have observed a striking difference in their metastatic potential as assayed in nude mice. The ras alone transformants are highly metastatic, while the two gene transformants show a very low metastatic potential. Transfection of the serotype 2 E1A gene, but not the serotype 12 E1A gene, into rat transformants results in a substantial reduction in the metastatic potential of these cell lines. Studies in progress are designed to investigate the role of oncogenes in inducing cell transformation, as well as the cellular factors which contribute to metastases in animal models.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05101-10 LMV

PERIOD COVERED: October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Studies on the Molecular Mechanisms for Malignant Transformation of Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Rudy Pozzatti	Senior Staff Fellow	LMV	NCI
	Jonathan Vogel	Senior Staff Fellow	LMV	NCI
	Laura Napolitano	Howard Hughes Scholar	LMV	NCI

COOPERATING UNITS *(if any)*: Department of Human Pathology, University of California at Davis (Steven Hinrichs)

LAB/BRANCH: Laboratory of Molecular Virology

SECTION: Cell Physiology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have examined the ability of the tat (trans-activation of transcription) genes from both human T-lymphotropic virus type-1 (HTLV-1) and human immunodeficiency virus type-1 (HIV-1) to transform early passage rat embryo cells. Transfection of either the HTLV tat-1 or the HIV tat-3 genes alone showed no effect on these cells. However, co-transfection of either the tat-1 or tat-3 genes with the activated ras oncogene resulted in the formation of foci of morphologically transformed cells. Focus formation was substantially higher for co-transfection of ras and tat-1 versus ras and tat-3. Five out of five ras plus tat-1-transformed cell lines formed tumors after subcutaneous injection into athymic nude mice. At least two out of five ras plus tat-3 transformed cell lines were also tumorigenic in nude mice. These results demonstrate that the trans-activating genes tat-1 and tat-3 from human retroviruses are capable of contributing to the transformation of rodent cells, and suggest that they may be involved in oncogenesis in humans infected with either HTLV-1 or HIV-1.

Based upon the above observations, we have introduced separately the HTLV tat-1 and the HIV tat-3 genes into the germ line of mice to test their potential to induce tumors in vivo. Transgenic mice carrying the tat-1 gene from HTLV-1 developed von Recklinghausen's neurofibromas and those carrying the tat-3 gene from HIV-1 gave rise to Kaposi's sarcomas. These results provide direct evidence that a single gene from either HTLV-1 or HIV-1 can induce tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Rudy Pozzatti	Senior Staff Fellow	LMV	NCI
Jonathan Vogel	Senior Staff Fellow	LMV	NCI
Norman Lassam	Visiting Associate	LMV	NCI

Objectives:

To understand the mechanism of transformation by different human tumor viruses.

Methods Employed:

Introduction of cloned genes into fertilized mouse eggs by microinjection and analysis of proteins by immunoprecipitation using either conventional or monoclonal antibodies. Transfection of plasmid DNA into early passage rodent cells, extraction of RNA and protein from cell lines, Northern and Southern blotting analysis of RNA and DNA, immunoprecipitation of proteins, Western blot analysis, and tumorigenicity assays in nude mice.

Major Findings:

1. Transfection of the HTLV tat-1 or HIV tat-3 genes alone into primary rat embryo cells showed no effect either on the morphology of the cells or in delaying the onset of senescence.

2. Co-transfection of either the tat-1 or tat-3 genes with the activated ras oncogene gave rise to foci of morphologically transformed cells. Focus formation was at least tenfold higher in frequency with ras and tat-1 versus ras and tat-3.

3. Cell lines have been established from individual foci transformed by ras and tat-1 or ras and tat-3. Five out of five ras plus tat-1 lines formed tumors after subcutaneous injection into nude mice. Two out of five ras plus tat-3 lines also formed tumors after injection into nude mice. Taken together, these data raise the possibility that the trans-activating genes of HTLV-1 and HIV-1 may play a role in the development of cancers that occur in individuals infected with these viruses.

4. Transgenic mice, which carry and express the HTLV-1 tat-1 gene under the control of its own regulatory element, developed neurofibromas (NF) which resemble human von Recklinghausen's disease. This study provides direct evidence that a single gene from HTLV-1 can cause cancer in mice. Because NF is particularly prevalent in Southern Japan, which also happens to be endemic for HTLV-1 infections, we suggest that HTLV-1 may have a neurotropic target and may induce neurologic malignancies in humans.

5. Transgenic mice which carry and express the HIV-1 tat-3 gene under the control of its own regulatory element developed Kaposi's sarcoma (KS) which is detected in some 60 percent of acquired immunodeficiency syndrome (AIDS) patients at autopsy. This study offers direct proof that a single gene from HIV-1 can induce a form of malignancy in mice which resembles what has been detected in AIDS patients.

Publications:

Green J, Vogel J, Jay G. HTLV-1 transgenic mice: a model for studying viral pathogenesis. In: Roman GC, Vernant J-C, eds. HTLV-1 and the nervous system. New York: AR Liss, Inc. (In Press).

Hinrichs SH, Nerenberg M, Reynolds KR, Jay G. A transgenic mouse model for human neurofibromatosis. In: Rivera DS, ed. 1988 Year Book of Cancer. Houston: Year Book Medical Publishers (In Press).

Hinrichs SH, Nerenberg M, Reynolds KR, Khoury G, Jay G. A transgenic mouse model for human neurofibromatosis. Science 1987;237:1340-3.

Hinrichs SH, Vogel J, Jay G. Use of transgenic animals to study human retroviruses. In: Fidler IJ, Poste G, eds. Cancer and metastasis reviews. Boston: Martinus Nijhoff Publishing (In Press).

Nerenberg M, Hinrichs SH, Reynolds K, Khoury G, and Jay G: The tat gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science 1987;237:1324-9.

Nerenberg M, Hinrichs SH, Reynolds KR, Vogel J, Jay G. A transgenic mouse model for the study of human T-lymphotropic virus type 1. In: Liu DT, Marshak DR, Schechter AN, eds. Therapeutic peptides and proteins: assessing the new technology. New York: Cold Spring Harbor, 1987;201-3.

Nerenberg M, Jay G: Expression of HTLV in transgenic mice. In: Notkins AL, Oldstone MBA, eds. Concepts in viral pathogenesis, vol. II. New York: Springer-Verlag (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05216-08 LMV

PERIOD COVERED
October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ras Oncogene Regulation in Yeast

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ravi Dhar	Visiting Scientist	LMV	NCI
Others:	Taduru Sreenath	Visiting Fellow	LMV	NCI
	Nadera Ahmed	Microbiologist	LMV	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Expression of the ras1 and ras2 genes of Saccharomyces cerevisiae has been examined at the transcriptional and translational levels. The ras2 mRNA level was high and nearly constant until late in the exponential phase and decreased considerably as cells entered the stationary phase. These data suggest that translational control is important in regulating ras2 gene expression in cells grown on glucose. Nutrient starvation, leading to G1-arrest and sporulation in diploids, had little effect on the rate of ras2 protein synthesis, but led to decreased amounts of ras2 mRNA. This decrease was accomplished in part by selective repression of ras2 transcripts with particular 5′ ends. Our data also suggest that nutrient starvation is another condition in which translational control is prominent in regulation of ras2 expression. The fact that a large decrease in the amount of ras2 mRNA occurs in the stationary phase and starvation conditions, but is associated with little effect on ras2 protein synthesis, suggests that ras2 transcriptional control in these conditions is designed primarily to offset changes in translational efficiency.

We have reported a mutation in a strain of S. cerevisiae which results in the accumulation of previously unidentified intermediates in the processing of ras2 protein. We have identified phosphorylation on the ras2 protein and have shown phosphorylation of ras2 protein to be regulated in S. cerevesiae.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ravi Dhar	Visiting Scientist	LMV NCI
Taduru Sreenath	Visiting Fellow	LMV NCI
Nadera Ahmed	Microbiologist	LMV NCI

Objectives:

Our major objectives are:

1. To study the transcriptional and translational regulation of the two *ras* genes in yeast and their extragenic suppressors.

2. To study the phenotype of *ras* mutants and correlate gene regulation with biological function.

Methods Employed:

Recombinant DNA technology, Southern and Northern blot analysis, immunoprecipitation, and Western blots.

Major Findings:

We have shown that the two *ras* genes, c-*ras*1 and c-*ras*2, are differentially regulated.

1. In the presence of glucose, yeast cells express a large amount of *ras*1 mRNA and *ras* protein early in their growth phase.

2. Low levels of *ras*1 mRNA are made very early when cells are grown in nonfermentable carbon sources like ethanol or acetate. Such regulation provides an explanation for the inability of *ras*2$^-$ cells to grow on nonfermentable carbon sources.

3. The inability of *ras*2$^-$ cells to grow on nonfermentable carbon sources can be reversed by an extragenic suppressor, sra 6-15.

4. *Ras*1 cannot substitute for *ras*2 protein in sporulation, even when *ras*1 protein is overproduced.

5. C-*ras*2 is regulated at the level of transcription resulting in three major RNA 5′ ends.

6. *Ras*2 protein synthesis is under translational control.

7. Transcriptional control is primarily designed to offset changes in translational efficiency.

8. Sporulation of yeast cells occurs in the absence of the ras2 protein in the media. However, in the presence of the ras2 gene product, its activity is regulated during conditions of sporulation.

9. G_1 arrest has little effect on the rate of ras2 protein synthesis, but leads to decreased amounts of ras2 mRNA.

10. We proposed the following sequence of events for ras2 protein processing: p41> p40-1> p40, where p40-1 represents a ras2 intermediate required for the targeting of the protein to the plasma membrane.

11. We have identified phosphorylation on the ras2 protein in S. cerevisiae.

12. Phosphorylation maps at the C terminal half of the ras2 protein.

13. Phosphorylation of the ras2 protein is regulated by an upstream gene product, cdc25.

Publications:

Breviario D, Hinnebusch A, Dhar R. Multiple regulatory mechanisms control the expression of ras1 and ras2 genes of Saccharomyces cerevisiae. EMBO 1988;7:1805-13.

Breviario D, Maurizio B, Dhar R. Accumulation of processing of the ras2 protein in strain 12 of Saccharomyces cerevisiae. Biochem Biophys Res Comm 1988; 15:1346-51.

Nieto A, Qasba PK, Nakhasi HL, Dhar R. Differential regulation of c-Ha-ras and c-Ki-ras gene expression in rat mammary gland. Carcinogenesis 1987;8:1955-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05217-08 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on the Regulation of SV40 Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Mary Loeken	Guest Researcher	LMV	NCI
	Janet Duvall	Bio Lab Tech	LMV	NCI

COOPERATING UNITS *(if any)*

Dana Farber Cancer Institute, Boston, MA (Dr. David Livingston)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The simian virus 40 (SV40) early region contains coding sequences for three proteins, large T-antigen, small t-antigen and simian virus 40 early leader protein (SELP). We have previously demonstrated that the SV40 T-antigen acts as a trans-acting transcriptional activator protein to increase expression of viral and cellular genes. In addition, we have demonstrated that carboxy terminal mutants of the SV40 T-antigen severely affect transcription and translation of SV40 late mRNA. Analysis of SV40 T- and t-antigens suggested both proteins have domains which are similar to regions of the adenovirus E1A protein that are important for transformation, transcriptional activation and transcriptional repression. In view of these homologies, we have analyzed the ability of the SV40 t-antigen to act as a trans-acting regulatory protein. Using transient transfection assays, we have demonstrated that t-antigen acts as a trans-acting transcriptional activator protein, capable of inducing transcription from promoters which are responsive to E1A. Co-transfection of plasmids containing the cDNA sequence for t-antigen with the adenovirus E2A promoter increases expression from the promoter greater than tenfold, similar to the activity observed with E1A. Transcriptional activation by t-antigen is dependent upon the presence of E2A enhancer sequences. Analysis of t-antigen mutants demonstrates that amino acids 112-173, which are unique to t-antigen, are required for activation of the E2A promoter. Using S1 nuclease analysis, we have also demonstrated that t-antigen activates transcription from polymerase III promoters.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Mary Loeken	Guest Researcher	LMV	NCI
Janet Duvall	Bio Lab Technician	LMV	NCI

Objectives:

To study the function of the SV40 gene products

Methods Employed:

Tissue culture; DNA transfection; radiolabeling of DNA, RNA and protein; DNA cloning; polyacrylamide gel electrophoresis; Southern blotting; Northern blotting and hybridization; detection and analysis of protein by immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Major Findings:

SV40 provides a unique model system for the study of gene regulation. We have used it to make the following observations:

1. SV40 t-antigen acts as a *trans*-acting regulatory protein to increase expression of both polymerase II and polymerase III genes.

2. Carboxy terminal mutants of SV40 T-antigen severely affect transcription and translation of SV40 late mRNA.

Publications:

Brady J, Buckler CE, Salzman NP. Simian virus 40 genetic map. In: O'Brien, SJ, ed. Genetic maps. New York: Cold Spring Harbor, 1987;64-7.

Green J, Brady J, Khoury G. 72 bp Element contains a critical control region for SV_{40} late gene expression in *Xenopus laevis* oocytes. Virology 1987;159:339-49.

Khalili K, Brady J, Khoury G. Translational regulation of SV40 early mRNA defines a new viral protein. Cell 1987;48:639-45.

Khalili K, Brady J, Pipas JM, Spence S, Sadofsky M, Khoury G. COOH-terminal mutants of SV40 T-antigen: a role for the early protein late in the lytic cycle. Proc Natl Acad Sci USA 1988;85:354-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05220-08 LMV

PERIOD COVERED
October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the Structure and Function of Cell Surface Antigens

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Takayuki Yoshioka	Visiting Fellow	LMV	NCI
	Lian-Sheng Chen	Visiting Fellow	LMV	NCI

COOPERATING UNITS *(if any)*
Department of Biology, Yale University (Charles Bieberich)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Cell Physiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The regulated expression of major histocompatibility complex (MHC) class I antigens is essential for assuring proper cellular immune responses. To study class I gene regulation, we have transferred a foreign class I gene into inbred mice. We demonstrated that these mice expressed the transgenic class I molecule on the cell surface. Skin grafts from transgenic mice were rapidly rejected by mice of the background strain, indicating that the transgenic antigen was expressed in an immunologically functional form. As with endogenous class I genes, the class I transgene was inducible by interferon treatment and suppressible by human adenovirus 12 (Ad12) transformation. Linkage analysis indicated that the transgene was not closely linked to endogenous class I loci, suggesting that trans-regulation can occur for class I genes located outside the MHC. The function of the transgenic D-d class I molecule in the induction of immunologic tolerance and in directing MHC restriction were also investigated. All of the transgenic mouse strains were found to be tolerant for the D-d antigen. Spleen cells from transgenic mice were immunocompetent but consistently failed to generate an anti-D-d cytotoxic T lymphocyte response in vitro. These data suggest that the D-d antigen was recognized as a self molecule. In addition, the transgenic D-d mice generated antigen-specific D-d-restricted cytotoxic T lymphocytes, indicating that the D-d antigen also functioned as a restriction element for antigen recognition. The observations demonstrate the usefulness of the transgenic mouse system for studying class I antigen expression and function.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Takayuki Yoshioka	Visiting Fellow	LMV	NCI
Lian-Sheng Chen	Visiting Fellow	LMV	NCI

Objectives:

To study the organization and expression of the genes which encode the class I histocompatibility antigens by molecular cloning techniques.

Methods Employed:

Recombinant DNA techniques, nucleic acid hybridization, gel electrophoresis, electron microscopy, protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Since regulated expression of class I antigens is indispensable for proper function of the immune system, it is important to understand the mechanisms which govern their expression. Germ line transformation of mice provides a powerful system to study gene regulation. Transgenic B6 mice carrying the D^d gene cloned from a BALB/c mouse can express the D^d antigen in a tissue-dependent manner. We have examined the level of cell surface expression of the transgenic class I antigen and of the endogenous K^b and L^b antigens on the same cells. While the level of D^d antigen on spleen cells from the D8 transgenic line was similar to that seen on BALB/c spleen cells, the level on cells from the D24 line was twofold higher. In both D8 and D24 lines, the level of expression of the endogenous K^b antigens on spleen cells was the same as in B6 control cells. These data strongly suggest that the efficient expression of a transgenic class I antigen does not alter the level of expression of the endogenous class I genes. The rapid rejection of skin grafts from D^d transgenic mice on the background B6 mice indicates that the D^d product expressed in tail skin can be recognized as foreign and can stimulate a vigorous immune response.

Our results demonstrated that induction of MHC class I gene expression by treatment with interferon (IFN)-gamma and its suppression by transformation with Ad12 occurred for class I genes located outside of the MHC in primary cells from transgenic mice. Furthermore, the induction was similar to that seen for endogenous class I antigens. These observations argue strongly for mechanisms of class I gene regulation that involve *trans*-acting factors. Given that class I antigen expression is essential for the immune presentation of aberrant cells, it will be of utmost importance to define the nature of these factors and their target sequences.

Although the D^d antigen expressed on the surface of spleen cells from D8 mice was recognized by D^d-stimulated B6 effector cells, and must then be immunologically functional, D8 spleen cells consistently failed to generate an anti-D^d response. These observations suggest that D8 mice were tolerant for the transgenic antigen. The failure of D8 cells to respond to D^d-antigenic determinants could not be overcome by in vivo priming or by the addition of helper factors in vitro. The results of limiting dilution assays strongly suggest that the observed tolerance for the D^d antigen in D8 mice is due to a lack of anti-D^d CTL precursors. In addition to being seen as a self molecule in transgenic D^d mice, the D^d antigen can also serve as a functional restriction element. Spleen cells from these mice were able to generate a D^d-restricted anti-I-AED response when presented with D^d-expressing I-AED-modified stimulating cells.

The results of these experiments demonstrate the usefulness of applying the transgenic mouse technology to the study of tolerance and MHC restriction.

Publications:

Bieberich C, Yoshioka T, Tanaka K, Jay G, Scangos G. Functional expression of a heterologous major histocompatibility complex class I gene in transgenic mice. Mol Cell Biol 1987;7:4003-9.

Rubin LA, Hoekzema GS, Nelson DL, Greene WC, Jay G. Reconstitution of a functional interleukin-2 receptor in a non-lymphoid cell. J Immunol 1987;139:2355-60.

Tanaka K, Yoshioka T, Bieberich C, Jay G. Role of the MHC class I antigens in tumor growth and metastasis. Annu Rev Immunol 1988;6:359-80.

Yoshioka T, Bieberich C, Scangos G, Jay G. A transgenic MHC class I antigen is recognized as self and acts as a restriction element. J Immunol 1987;139:3861-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05254-07 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Kuan-Teh Jeang	Senior Staff Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

Dana Farber Cancer Institute, Boston, MA (David Livingston)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

We are interested in determining whether enhancer-dependent gene regulation occurs through a "looping" or a "sliding" model. We have approached this issue by bracketing the SV40 enhancer element with the lac operator sequences and studying the biology of this modified enhancer element in the presence of lac repressor molecules. Recently, we have extended this approach to the study of TPA and cAMP-inducible sequence elements. Experiments to determine if these elements can be similarly down-regulated are in progress.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Kuan-Teh Jeang	Senior Staff Fellow	LMV	NCI

Objectives:

The goal of this study is to understand the regulation of genes by differentiating between a "looping" or "sliding" mechanism for gene expression.

Methods Employed:

Transient assay systems, recombinant DNA technology, RNA analysis by hybridization and nuclease protection.

Major Findings:

1. Lac repressor protein is functional in eukaryotic cells.

2. Bracketing the SV40 enhancer with lac repressor protein can negatively modulate gene expression.

Publications:

Brown M, Figge J, Hansen U, Wright C, Jeang, K-T, Khoury G, Livingston DM, Roberts TM. lac Repressor can regulate transcription from a hybrid SV40 early promoter containing a lac operator in animal cells. Cell 1987;49:603-12.

Jeang K-T, Khoury G. The role of enhancer elements in the molecular mechanism of eukaryotic transcription. Bioessays (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05354-06 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Studies on the Activated Form of the Human Proto-oncogene, c-Ha-ras

PRINCIPAL INVESTIGATOR *(List other professionel personnel below the Principal Investigator.) (Nama, title, laboratory, and institute affiliation)*

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Rudy Pozzatti	Senior Staff Fellow	LMV	NCI
	Mary McCormick	Senior Staff Fellow	LMV	NCI
	Lance Liotta	Chief	LMV	NCI
	Mary Ann Thompson	Guest Researcher	LMV	NCI

COOPERATING UNITS *(if eny)*

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have transfected various viral and cellular oncogenes into primary cultures of rat embryo cells and have obtained lines of morphologically transformed cells. Transformation with the ras oncogene alone was observed; however, a tenfold increase in the transformation frequency was obtained when ras was cotransfected with the adenovirus E1A gene. We have examined cell lines transformed by the ras oncogene alone and by ras plus E1A, and have observed a striking difference in their metastatic potential as assayed in nude mice. Specifically, the ras alone transformants are highly metastatic, while the two gene transformants show a very low metastatic potential. Transfection of the serotype 2 E1A gene, but not the serotype 12 E1A gene, into the ras alone transformants results in a substantial reduction (at least tenfold) in the metastatic potential of these cell lines. Experiments are in progress to investigate the mechanism by which the adenovirus type 2 E1A gene reduces the metastatic potential of the ras alone transformants.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Rudy Pozzatti	Guest Researcher	LMV	NCI
Mary McCormick	Senior Staff Fellow	LMV	NCI
Lance Liotta	Chief	LP	NCI
Mary Ann Thompson	Guest Researcher	LP	NCI

Objectives:

We have observed that primary rat embryo cells transformed by the ras oncogene are highly metastatic when assayed in nude mice. This study is designed to determine how the adenovirus type 2 E1A gene is able to suppress the metastatic phenotype associated with the ras-transformed rat embryo cells.

Methods Employed:

Isolation and cloning of specific fragments, construction of recombinant vector molecules, extraction of mRNA, Northern and Southern blotting analysis of RNA and DNA, DNA transfection, tumorigenesis assays in nude mice, construction of cDNA libraries, and screening libraries by colony hybridization with cDNA probes.

Major Findings:

1. We have transfected oncogenes into second passage rat embryo cells and have examined the frequency of morphological transformation that results from using a single gene (ras) versus two "cooperating" genes (ras and the adenovirus E1A gene). Transfection of the ras gene alone resulted in low frequency of transformation (one cell in 10^5). Transfection of two-gene transformants resulted in a tenfold increase in the transformation frequency (one cell in 10^4). Cell lines were established from the one- and two-gene transformants in order to examine their phenotype properties.

2. The most striking phenotypic difference between the two classes of transformants was observed when tumorigenicity assays were performed. Both one- and two-gene transformants formed rapidly growing tumors in nude mice when injected subcutaneously. However, seven of the eight ras alone transformants formed metastases in the lungs of animals bearing subcutaneous tumors. None of six two-gene transformants formed metastases after subcutaneous injection. When the metastatic potential of transformed cell lines was analyzed by intravenous injection of cells, all eight ras alone transformants formed large numbers (>200) of metastatic nodules in the lungs of IV-injected animals. In contrast, the two-gene transformants showed very low numbers of lung nodules (<5) after intravenous injection.

3. We have tested the hypothesis that the adenovirus type 2 E1A gene may be able to suppress the metastatic phenotype of ras alone transformed cells by

transfecting E1A into five independent ras alone transformed cell lines. Four of the five cell lines showed a substantial reduction (at least tenfold) in metastatic potential as a result of expression of the type 2 E1A gene. We have also observed that the adenovirus serotype 12 E1A gene is not capable of suppressing the metastic potential of ras alone transformed cells.

4. Through a collaboration with Dr. Lance Liotta, DCT, NCI, we have observed a difference between the metastatic and non-metastatic cell lines in the levels of secretion of an enzyme that specifically degrades type IV collagen. Secretion of collagenase IV activity is a property often found associated with metastatic cell lines. We observed that the metastatic ras alone transformed cell lines all secrete high levels of collagenase IV activity, while the ras and Ad2 E1A transfected cell lines were all low in secreted collagenase IV activity, comparable to normal untransfected rat embryo cells.

5. Dr. Gregory Goldberg from Washington University in St. Louis has provided us with a cDNA clone of the human collagenase IV gene. This clone will allow us to determine the levels of expression of the collagenase IV gene in our ras and ras plus Ela-transformed cell lines at the transcriptional level. If the collagenase IV gene is regulated by the Ad2 Ela gene at the transcriptional level, we will characterize the 5′ regulatory sequences of the collagenase IV gene and identify those sequences that are responsive to the Ela gene.

Publications:

Garbisa S, Negro A, Kalebic T, Pozzatti R, Muschel R, Saffiotti U, Liotta LA. Type IV collagenolytic activity: linkage with the metastatic phenotype induced by ras transfection. In: Hellman K, Liotta LA, Prodi G, eds. International congress on cancer metastasis, biological and biochemical mechanisms and clinical aspects. New York: Plenum Corps (In Press).

Pozzatti R, McCormick M, Thompson MA, Garbisa S, Liotta LA, Khoury G. Regulation of the metastatic phenotype by the Ela gene of adenovirus-2. In: Hellman K, Liotta LA, Prodi G, eds. International congress on cancer metastasis, biological and biochemical mechanisms and clinical aspects. New York: Plenum Corps (In Press).

Pozzatti, R, McCormick M, Thompson MA, Khoury G. The Ela gene of adenovirus-2 reduces the metastatic potential of ras transformed rat embryo cells. Mol Cell Biol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05355-06 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Immune Surveillance Against Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Roberta Reynolds	Research Microbiologist	LMV	NCI
	Kazuhiko Koike	Visiting Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

Department of Medicine, Harvard Medical School (Kurt J. Isselbacher)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

There is increasing evidence for the importance of events that govern and influence the interaction between the transformed cell and its host being ultimately responsible for the establishment of the cancer phenotype. To derive an animal model that will allow us to define some of these phenomena at the molecular level, we have chosen to induce the expression of a viral oncogene in all tissue types, with the hope of identifying sites that are more susceptible to malignant transformation. When the gene for simian virus 40 (SV40) large tumor antigen (T antigen) was placed under the control of a major histocompatibility complex class I gene enhancer, the resulting transgenic mice not only developed choroid plexus papillomas, as seen with wild-type SV40, but also lymphoid hyperplasia and multiple endocrine neoplasias. The development of lymphoid hyperplasia was preceded by an elevated level of expression of T antigen in these tissues at an early age. Surprisingly, the striking thymic hyperplasia has not been observed to progress toward malignancy. The multiple endocrine neoplasias developed later in life and involved the pancreas, pituitary, thyroid, adrenals, and testes. While not preceded by an elevated level of expression of T antigen, once endocrine tumors appeared they quickly progressed toward malignant growth. Although other tissues also exhibited a basal level of expression of the viral oncogene similar to that detected in endocrine tissues, they rarely developed tumors. This transgenic mouse model seems particularly suitable for a molecular understanding of events responsible for certain tissue types being so much more susceptible to neoplastic conversion, with others being so refractory.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Roberta Reynolds	Research Microbiologist	LMV	NCI
Kazuhiko Koike	Visiting Fellow	LMV	NCI

Objectives:

To study the mechanisms involved in regulating immune surveillance.

Methods Employed:

Recombinant DNA techniques, nucleic acid hybridization, gel electrophoresis, electron microscopy, protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

Over the past two decades, the major effort to understand the molecular biology of the cancer cell has focused primarily on defining the mechanism of transformation, a process whereby a normal cell is converted in culture to a state of uncontrolled proliferation. A large number and variety of oncogenes and proto-oncogenes have been defined, and while the study of their activities is central to our understanding of the etiology of cancer at the molecular level, the phenomena that govern and influence the interaction between the transformed cell and its host are ultimately responsible for the establishment of the cancer phenotype.

We have chosen to deregulate the expression of a viral oncogene in transgenic mice with the hope of identifying sites that are either more privileged or more susceptible to tumor growth. The availability of such a transgenic model should allow us to dissect the molecular events underlying the basis for tumor growth in a setting where cell-cell interactions remain intact.

Introduction of the SV40 large T oncogene under the control of a major histocompatibility complex class I gene enhancer gave rise to transgenic mice with a distinct predisposition to multiple endocrine neoplasia. Every mouse that was allowed to grow to maturity came down with malignant tumors in the pancreas, pituitary gland, thyroid gland, adrenal glands, and testes. In addition, these same mice also had extensive lymphoid hyperplasia with the involvement of the thymus, spleen, and lymph nodes.

In this study, we have derived a transgenic mouse model that seems appropriate for a molecular definition of events responsible for tumor growth. One can now begin to ask relevant questions based on our observations. Why are endocrine tissues more susceptible to neoplastic changes in this mouse model? We have noted that most of the endocrine tumors, as well as the choroid plexus tumors, were highly vascular. It is possible that endocrine tumor cells are more

capable of secreting growth factors that can induce angiogenesis. Endocrine tissues have also been known to be targets for autoimmunity, such as diabetes mellitus and autoimmune thyroiditis. If such disorders are the result of heightened immune recognition of otherwise immunologically "privileged" sites, then transformed cells derived from such tissues would be more capable of escaping immune detection. Why, despite extensive hyperplastic growth, do the lymphoid tissues not undergo malignant conversion? Unlike endocrine cells, which are terminally differentiated and cannot "tolerate" cell division, lymphoid cells are inherently able to divide and are perhaps more permissive to sustained proliferation without triggering a neoplastic state of growth.

Publications:

Reynolds RK, Hoekzema GS, Vogel J, Hinrichs SH, Jay G. Multiple endocrine neoplasia induced by the promiscuous expression of a viral oncogene. Proc Natl Acad Sci USA 1988;85:3135-9.

Tanaka K, Jay G, Isselbacher KJ. Expression of heat-shock and glucose-regulated genes: differential effects of glucose starvation and hypertonicity. Biochim Biophys Acta (In Press).

Vogel J, Tanaka, K, Hoekzema GS, Jay G. Experimental strategies for modification of histocompatibility antigens in tumor cells. In: Fidler IJ, Poste G, eds. Cancer metastasis reviews, vol 6. Boston: M Nijhoff Publishing, 1988;677-83.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05390-05 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

How Do Tumor Cells Escape Immune Surveillance?

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Norman Lassam	Senior Staff Fellow	LMV	NCI
	Steven A Rosenberg	Surgeon	SB	NCI

COOPERATING UNITS *(if any)*

Surgery Branch, National Cancer Institute (Steven A. Rosenberg)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cancer may be thought of as an immunological disorder that arises because certain "transformed" cells, endowed with the propensity to divide, have learned to evade detection by the immune system. The prospect of intervention by "immunotherapy" depends very much on our ability to either render cancer cells more recognizable to the immune system or potentiate the immune system towards a more effective recognition of cancer cells. There is now direct evidence that suppression of the major histocompatibilty complex class I antigens, a family of cell-surface glycoproteins required for the presentation of cancer cells to the immune system, is directly responsible for the ability of tumor cells to escape immune surveillance. It has been shown that cancer cells can be made immunogenic either by the expression of an exogenous class I gene introduced by DNA-mediated gene transfer, or by the derepression of endogenous class I genes with interferon; these cells are efficiently rejected by the immune system. Even more interesting is the finding that the immune system can be potentiated to reject tumors by immunization with homologous tumor cells that have been manipulated to express normal levels of class I antigens; these findings suggest a direct route to immunotherapy that involves debulking of the tumor mass, raising the level of class I antigens in a small number of explanted tumor cells and re-immunizing the host.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV NCI
Norman Lassam	Senior Staff Fellow	LMV NCI

Objectives:

Molecular analysis of the escape by tumor cells from immune surveillance.

Methods Employed:

Recombinant DNA techniques, nucleic acid hybridization, gel electrophoresis, electron microscopy, and protein analysis by immunological methods in combination with polyacrylamide gel electrophoresis.

Major Findings:

1. The immune system is involved not only in defense against infections but also against "spontaneously derived" aberrant cells. This latter immune function appears to be essential for the removal of autonomous cell variants that presumably arise frequently in all multicellular organisms. The development of malignant tumors, therefore, represents not only neoplastic transformation, but the failure of host resistance to eliminate certain abnormal cells. Transformation of a cell is insufficient to ensure its escape from immune surveillance. Cells transformed in culture almost invariably do not induce tumors when transplanted back into immunocompetent syngeneic hosts. It is those properties of certain tumor cells allowing them to resist immune recognition which are ultimately responsible for their tumorigenicity.

2. The major histocompatibility complex class I (H-2) antigens (designated K, D and L in mice) are indispensable for the presentation of cells bearing "foreign" antigens to the cytotoxic T-lymphocytes. The finding that certain malignant tumors, including teratocarcinomas, eccrine porocarcinomas and cervical carcinomas, have markedly reduced levels or nondetectable levels of cell-surface class I antigens (in contrast to their normal cellular counterparts) suggests a possible mechanism for their escape from immune surveillance. In support of this hypothesis is the recent finding that cells transformed by the highly oncogenic strains of human adenovirus (Ad12), in contrast to the nononcogenic strains (Ad5), also express reduced levels of class I antigens on their surfaces. This observation with Ad12 provides an experimental system for demonstrating that the absent or reduced expression of class I antigens is directly responsible for oncogenicity.

3. We have shown that transfection of a functional class I gene into a highly tumorigenic Ad12-transformed cell line that expresses no detectable class I surface antigens resulted in its complete loss of oncogenicity. Since

interferon can induce the expression of class I genes, treatment of mice bearing Ad12 tumors with interferon led to total suppression of tumorigenicity. These findings indicate one possible mechanism for the escape of certain tumors from immune surveillance and suggests future therapeutic approaches for the reversal of certain malignancies.

Publications:

Hayashi H, Tanaka K, Jay F, Khoury G, Jay G. Modulation of the tumorigenicity of human adenovirus 12-transformed cells by interferon. In: Rivera DS, ed. 1987 Year Book of Cancer. Houston: Year Book Medical Publishers, 1987;534-5.

Lassam N, Vogel J, Jay G. MHC class I antigens and tumorigenesis. In: Compans R, Helenius A, Oldstone, MBA, eds. Cell biology of virus entry, replication and pathogenesis. New York: AR Liss (In Press).

Tanaka K, Gorelik E, Watanabe M, Hozumi N, Jay G. Experimental strategies for the abrogation of the tumorigenicity of B16 melanoma. Mol Cell Biol 1988;8:1857-61.

Weber JS, Jay G. MHC class I gene expression by tumors: immunotherapeutic implications. Curr Top Microbiol Immunol (In Press).

Weber JS, Jay G, Tanaka K, Rosenberg SA. Immunotherapy of a murine tumor with interleukin-2: increased sensitivity after MHC class I gene transfection. J Exp Med. 1987;166:1716-33.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05391-05 LMV

PERIOD COVERED
October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Analysis of the JC Virus Enhancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	James Remenick	Visiting Scientist	LMV	NCI
	Taduru Sreenath	Visiting Fellow	LMV	NCI

COOPERATING UNITS *(if any)*
National Institute of Neurological and Communicative Disorders and Stroke, NIH (Eugene Major)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

JC virus is a human papovavirus that has been associated with the demyelinating disease, progressive multifocal leukoencephalopathy. Virus production in tissue culture is restricted to human fetal glial cells and is regulated at the level of transcription and DNA replication. Oligonucleotides have been synthesized which span the 98 base-pair repeated region of the JC virus enhancer. Gel retardation and ultraviolet cross-linking experiments using these oligonucleotides have identified three proteins from human fetal glial cell extracts which associate with this region. Two proteins of 82 kilodaltons (kd) and 80 kd recognize the 5′ and 3′ regions of the 98 base-pair region, respectively. The molecular weights of these proteins are similar to those of proteins similarly identified from human HeLa cell extracts. One protein specifically binds to the central region of the JC virus repeat, but was found to have a molecular weight of 45 kd in human fetal glial cell extracts and 85 kd in human HeLa cell extracts. Single base pair and deletion mutations are being created in the JC virus enhancer and tested for their ability to support JC virus transcription in vivo and in vitro. Proteins which recognize the essential regions of the enhancer will be purified and tested for functional activity in the in vitro transcription system. Ultimately, the proteins responsible for tissue-specific expression of the JC virus early region will be cloned.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
James Remenick	Visiting Scientist	LMV	NCI
Taduru Sreenath	Visiting Fellow	LMV	NCI

Objectives:

To identify the transcriptional sequences and the proteins which they bind in the regulation of RNA polymerase II transcription.

Methods

Recombinant DNA techniques, CAT and Luciferase analyses, in vitro transcription, nucleic acid hybridization, construction of single base-pair and deletion mutations, and DNA transfection.

Major Findings:

1. The 98 base-pair repeat of JC virus binds to at least three proteins that have been identified in human fetal glial and HeLa cell extracts.

2. The JC virus promoter is active in vitro in reactions containing either glial cell or HeLa cell extract; however, only glial cell extracts will produce a properly initiated transcript.

3. Early transcription from the JC virus promoter is restricted to certain glial and neuroblastoma type cells that may represent a specific stage of neural development.

Publications:

Feigenbaum L, Khalili K, Major E, Khoury G. Regulation of the host range of human JCV. Proc Natl Acad Sci USA 1987;84:3695-8.

Khalili K, Feigenbaum L, Khoury G. Evidence for a shift in 5′-termini of early RNA during the lytic cycle of JC virus. Virology 1987;158:469-72.

Khalili K, Rappaport J, Khoury G. Nuclear factors in human brain cells bind specifically to the JCV regulatory region. EMBO J 1988;7:1205-10.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05392-05 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Regulation of Transcription by Large T-Antigen

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John Brady	Acting Chief	LMV	NCI
Others:	Mary Loeken	Guest Researcher	LMV	NCI
	Janet Duvall	BioLab Tech	LMV	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The simian virus 40 (SV40) late promoter can be trans-activated by SV40 T/t antigen in the absence of DNA replication. Transfection experiments suggest that T/t-antigen trans-activation may involve either direct promoter binding or induction of one or more cellular transcription factors. Using an oligo-nucleotide probe containing SV40 late promoter sequences between 287-309, we have utilized the gel shift and ultraviolet (UV)-cross-linking assays to identify a protein of 80-90Kd that interacts specifically with the transcriptional control sequence. The addition of partially purified protein to in vitro transcription assays decreases the level of SV40 late transcription. We are presently analyzing whether SV40 T/t-antigen modifies the binding or transcriptional activity of this transcription factor.

To gain further understanding of the mechanisms by which trans-acting factors regulate gene expression, we have examined the regulation of transcription factors which bind to the adenovirus E2A enhancer. Adenovirus E2A upstream sequences which contain the binding sites for ATF and EIIF are sufficient to act as an enhancer in response to both SV40 T/t-antigen and adenovirus E1A. Specific mutation of the ATF and EIIF binding sites demonstrates that both act as positive regulators. Using gel-shift analysis, we demonstrated that the binding activity of EIIF is increased following adenovirus infection. In contrast, the binding activity of ATF is decreased by adenovirus infection.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Mary Loeken	Guest Researcher	LMV	NCI
Janet Duvall	BioLab Tech	LMV	NCI

Objectives:

Identify mechanisms by which eukaryotic genes, transcribed by RNA polymerase II, are regulated at the transcriptional level.

Methods Employed:

Recombinant DNA techniques, in vitro transcription, eukaryotic cell transfection nucleic acid hybridization, electrophoretic immunoblot analysis of protein, gel electrophoresis, construction of deletion and point mutants, and in vivo competition assays.

Major Findings:

1. SV40 large T-antigen stimulates transcription from the SV40 late promoter. Analysis of promoter mutants and in vivo competition experiments indicate that binding of T-antigen to site II and the 72-bp repeats are important for efficient induction of late transcription. The spacing between the sequences is critical, suggesting a cooperative interaction.

2. Using gel-shift and UV-cross-linking assays, we have identified a protein of 80-90 Kd that interacts specifically with the SV40 major late upstream control region. Addition of partially purified protein to in vitro transcription assays decreases the level of SV40 late transcription.

3. Adenovirus E2A regulatory sequences which contain the binding sites for two DNA binding proteins, ATF and EIIF, are sufficient to act as an inducible enhancer in reponse to SV40 T/t-antigen and E1A. Adenovirus infection results in the modulation of both ATF and EIIF binding activity.

Publications:

Brady J, Loeken M, Thompson MA, Khoury G. Regulation of viral transcription units by SV40 T-antigen. In: Aloni Y, ed. Molecular aspects of papovavirus. Boston: Martinus Nijhoff Publishing, 1987;119-35.

Khoury G, Khalili K, Duvall J, Brady J. The role of *cis*- and *trans*-acting functions in SV40 gene regulation. In: Crooke ST, Poste G, eds. New frontiers in the study of gene functions. New York: Plenum Press, 1987;1-19.

Razzaque A, Jahan N, Jariwalla R, Jones C, Brady J, Rosenthal LJ. Localization and sequence analysis of the transforming domain mtrII of human cytomegalovirus. Proc Natl Acad Sci USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05393-05 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effects of JC Virus Early Region in Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Others:	Lionel Feigenbaum	Microbiologist	LMV	NCI
	Jeffrey Green	Biotechnology Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

Molecular Therapeutics, Inc., West Haven, CT (George Scangos)

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Cell Physiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

JC virus (JCV) is a ubiquitous human papovavirus that has been strongly associated with the demyelinating disease, progressive multifocal leukoencephalopathy (PML). PML occurs in patients who are immunosuppressed by illness, immuonsuppressive therapy or genetic disorders. JCV exhibits a highly specific host range and tissue specificity. In immunosuppressed humans, viral particles are detected in brain cells of glial origin, specifically oligodendrocytes and astrocytes. It is the intent of this study to determine if introduction of JCV into transgenic mice would provide an animal model to study these human diseases. Transgenic mice have been produced containing JC virus early region genes under the control of the JCV promoter/enhancer element. Five mice were obtained containing JCV sequences. Three female founder mice succumbed to tumors, resulting from metastases of adrenal medullary neuroblastomas. Two of five mice produced offspring which developed a neurological disorder related to myelin deficiency. Neuropathological analysis indicated a myelin deficiency in the central nervous system apparently correlated with the expression of JCV T-antigen in brain tissue.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gilbert Jay	Chief, Cell Physiology Section	LMV	NCI
Lionel Feigenbaum	Microbiologist	LMV	NCI
Jeffrey Green	Biotechnology Fellow	LMV	NCI

Objectives:

To study the biology of JC virus (JCV) using transgenic mice as an animal model.

Methods Employed:

Mouse embryo injection, embryo transfer to pseudopregnant female mice, Southern and Northern hybridization, immunofluorescence, immunoprecipitation, and in situ hybridizations.

Major Findings:

1. JC virus is capable of expression when inserted into the mouse genome and is apparently regulated in a tissue-specific manner.

2. Neurological disease results from expression of JC virus in the brain of transgenic mice, specifically in the oligodendroglia or the myelin-producing cells. This causes a dysmyelination in the central nervous system.

3. Tumors are induced in the transgenic mice and are highly specific to the adrenal medulla. It is possible that pregnancy-induced immunosuppression was the basis for development of tumors in the female transgenic mice.

Publications:

Feigenbaum, L, Khalili K, Major E, Khoury G. Regulation of the host range of human papovavirus JCV. Proc. Natl. Acad. Sci. USA 1987;84:3695-8.

Khalili K, Feigenbaum L, Khoury G. Evidence for a shift in 5′-termini of early viral RNA during the lytic cycle of JC virus. Virology 1987;158:469-72.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05394-05 LMV

PERIOD COVERED

October 1, 1987 through September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Enhancer Elements in B-Lymphocytes and T-Lymphocytes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Imre Boros	Visiting Fellow	LMV	NCI
	Kuan-Teh Jeang	Senior Staff Fellow	LMV	NCI
	Michael Radonovich	Technician	LMV	NCI
	Susan Marriott	IRTA Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A focus of this project has been the role of the 3' long open reading frame of the human T-cell leukemia virus type-I (HTLV-1) which encodes a 40-Kd protein (p40tax). This protein positively regulates transcription directed by the HTLV-1 long terminal repeat (LTR) in a phenomenon known as trans-activation. We have been unable to attribute any sequence-specific DNA binding properties to p40tax, suggesting that the protein activates the HTLV-1 promoter in an indirect fashion using cellular transcription factors. Our objective is to understand the biochemical mechanism of trans-activation by the p40tax protein and the involvement of cellular transcription factors in this process.

We have extended our observations on the trans-activation properties of the HTLV-1 p40tax protein. Salient findings include: 1) p40tax appears to trans-activate responsive LTR elements through the induction of a 180 kd cellular protein; 2) mutational analyses correlate the trans-activation of the HTLV-1 LTR by p40tax with the presence of a cAMP responsive octonucleotide; 3) two signal transduction agents, cAMP and TPA, are both potent activators of the HTLV-1 LTR. Relevant to the last observation, we have defined both the cAMP-responsive and the TPA-responsive sequence elements with the HTLV-1 LTR.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Imre Boros	Visiting Fellow	LMV	NCI
Kuan-Teh Jeang	Senior Staff Fellow	LMV	NCI
Michael Radonovich	Technician	LMV	NCI

Objectives:

This project is focused on understanding the mechanism of action of the HTLV-1 $p40^{tax}$ protein.

1. Purification of cellular proteins that interact with the HTLV-1 LTR.

2. Definition of cellular pathways for the activation of the HTLV-1 LTR.

3. Mutagenesis of the HTLV-1 $p40^{tax}$-responsive elements.

Methods Employed:

Recombinant DNA techniques, bacterial expression vectors, somatic cell fusion techniques, protein purification, gene expression using transient and permanent assays, and RNA and protein analysis.

Major Findings:

1. We have defined two signal transduction pathways, cAMP and TPA, that activate the expression of the HTLV-1 LTR. The cAMP responsive element is contained within three repeated octomeric sequences found in the HTLV-1 LTR. The TPA responsive sequence is contained within two 51 bp elements.

2. We have correlated the binding activity of a cellular 180 kd protein with the biological trans-activation properties of $p40^{tax}$.

3. We have extensively mutagenized the HTLV-1 21 bp $p40^{tax}$-responsive element to define the minimally essential core nucleotides necessary for biological response.

Publications:

Brady J, Jeang K-T, Boros I, Giam C-Z, Duvall J, Radonovich M, Khoury G. Transcriptional regulatory sequences in the HTLV-1 LTR. In: Harlow E, ed. Nuclear oncogenes. New York: Cold Spring Harbor Laboratory, 1987;120-5.

Brady J, Jeang K-T, Duvall J, Khoury G. Identification of $p40^{tax}$ responsive regulatory sequences within the HTLV-1 LTR. J. Virol. 1987;61:2175-81.

Jeang K-T, Boros I, Radonovich M, Duvall J, Khoury G, Brady J. Cellular proteins and DNA sequences involved in trans-activation of the HTLV-1 LTR by $p40^{tax}$. In: Cullen B, Franza R, Wong-Staal F. eds. HIV and HTLV-1 gene expression, Banbury Conference. New York: Cold Spring Harbor Laboratory (In Press).

Jeang, K-T, Brady J, Radonovich M, Duvall J, Khoury G. $p40^{tax}$ trans-activation of the HTLV-1 LTR promoter. In: Mechanisms of control of gene expression. UCLA Symposium Proceedings, New Series, vol. 67.1988;181-9.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR CELL BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 to September 30, 1988

The objectives of the Laboratory of Tumor Cell Biology (LTCB) are to develop, implement, and analyze data obtained from studies of cellular proliferation, cell differentiation, and biochemical growth characteristics of normal and malignant mammalian cells both in vivo and in vitro. Particular attention is given to leukemogenesis and immune deficiency. Because of unusual access to human blood cells and because of the interest of this group in retroviruses and human herpes virus (HHV-6), there is special focus on human leukemias and lymphomas, and acquired immunodeficiency syndrome (AIDS). It is anticipated that an enhanced understanding of cell regulatory mechanisms will permit the optimal use of anti-tumor agents in the therapy of cancer and AIDS.

The Laboratory of Tumor Cell Biology is concerned with several biological and biochemical problems: (1) Studies on the cellular and molecular origin and pathogenesis of human leukemia. Biochemical control mechanisms involved in cell differentiation and neoplastic transformation are examined. Tumor viruses of animals are used both as tools as well as for help in understanding mechanisms of naturally occurring animal leukemias and AIDS. Also, studies designed to determine the distribution of human T lymphotropic retroviruses in T-cell leukemia patients, patients with AIDS, and normals in different parts of the world are being carried out; (2) Studies on the biochemical events preceding mitosis. An understanding of these events appears essential to the control of proliferation, and information derived from such studies may lead to more effective inhibitors of neoplastic cell growth. Events leading to mitosis are also of interest since many of the effective anti-tumor agents are useful only when cells are in DNA replication or in mitosis; (3) Attempts to develop new approaches to cancer chemotherapy and antiviral agents for treatment of AIDS using information gained from basic cellular studies. In addition, several approaches are being explored to obtain a vaccine against the AIDS virus; (4) Studies on the development of biochemical and immunological markers for malignant cells are carried out. Biochemical and immunological studies are also conducted in individuals with disorders associated with an increased incidence of neoplasia; (5) Controls regulating cellular growth and differentiation, and the process of malignant transformation in hematopoietic cells; and (6) Growth factors (and their receptors) that control the growth and differentiation of blood cells have been isolated and are under intensive study.

Progress made in the past year in various phases of this research effort is summarized below:

Kaposi's Sarcoma

Long-term cell cultures were established from lung tissue and pleural effusion obtained from AIDS patients with disseminated Kaposi's sarcoma (KS). These cells were initiated in culture with the aid of a recently described endothelial cell

growth factor(s). Morphological, biological, immunological, and cytochemical studies demonstrated that these KS-derived cells were similar to the characteristic endothelial-like (KSEL) spindle cells, prominent in KS lesions and that they were possibly of nonvascular origin. These cells were found to produce factors with diverse biological activities including those supporting their own growth (autocrine) and that of normal endothelial and fibroblastic cells (paracrine). Condition media from KSEL cells also induced chemotaxis and supported chemoinvasion by other KSEL and normal vascular endothelial cells. These cells also contained and expressed a potent angiogenic activity, as detected by chorioallantoic membrane (CAM) and nude mouse assays. Furthermore, when inoculated subcutaneously into nude mice, these KSEL cells induced the development of lesions composed of murine cells with histological features similar to KS lesions. These observations suggest that AIDS-associated and possibly other types of KS may be initiated by cells with properties similar to the KSEL cells described here. The autocrine and paracrine activities of such cells may then progressively lead to the development of the characteristic KS lesions in a multifocal fashion. The cell culture systems described here should provide in vitro and in vivo models for the study of the pathogenesis of KS.

Human B Lymphotropic Virus (HBLV, Human Herpesvirus 6, HHV6)

The isolation of the novel human herpesvirus, HBLV, from patients with a variety of hematological disorders was first reported by us in 1986. Of the six original isolates, two were from human immunodeficiency virus-1 (HIV-1)-positive individuals, one with AIDS-KS and one with dermatopathic lymphadenopathy. Subsequently, other groups reported the isolation of a virus genetically identical to HBLV from African patients with AIDS. Their association, possibly coincidental with HIV-1-infected donors who develop AIDS, was extended to a larger population by seroepidemiological studies which included symptomatic and asymptomatic HIV-1-infected individuals. Approximately 70% of these donors, compared to an incidence of 24% in random control samples, were seropositive for HBLV. Similar results were obtained in a smaller study involving HIV-1 seropositive and seronegative homosexual males.

While the concomitant infection by HIV-1 and HBLV clearly could be coincidental, other observations suggest a possible synergistic role for HBLV in AIDS. For example, the incidence of HBLV seropositivity is similar between symptomatic and asymptomatic donors, although the titer of antibodies to HBLV is consistently higher in patients developing AIDS. In addition, while the initial report of HBLV described its presence in B-cells from infected patients, recent in vitro studies have shown that HBLV can infect a number of other cell types. For example, we reported the infection of fresh peripheral blood lymphocytes with predominant T-cell markers, including CD-4 surface antigen. This infection resulted in a dramatic cytopathic effect on these cells. Furthermore, a number of cell lines consisting of T- and B-lymphocytes as well as cells of other origin, e.g., megakaryocytes, could also be infected by HBLV. Many of these same cell lines are also susceptible to infection by HIV-1.

Detailed evaluation of fresh leukocytes and some established cell lines have further demonstrated that individual CD-4 positive T-cells can be infected in vitro by both HIV-1 and HBLV. As a consequence these cells appear to be killed at an accelerated rate compared to those infected by either of the viruses independently. This dual or co-infection of cells was also observed with some established cell lines. For example, the dual infection of a susceptible cell

line by HIV-1 and HBLV results in an accelerated loss of viable cells. While these observations are consistent with a possible synergistic activity, the acute mechanisms involved are unknown. Since HBLV is cytopathic for CD-4 T-cells, infection by HBLV could contribute to the development of immune suppression by directly causing the death of CD-4 T-cells. Alternatively, it could contribute indirectly by activating or stimulating HIV-1 expression. It was observed that other human herpesviruses contain regulatory elements capable of interacting with HIV-1 LTR affecting virus expression. Several observations suggest the possibility of HBLV involvement in immune suppressive events associated with infection by HIV-1. Further serological and virus isolation prospective studies should help clarify the possible role of HBLV as a co-factor in the pathogenesis of AIDS.

Isolation of Human T-Lymphotropic Virus-II (HTLV-II) from Patients with a Variety of Neoplastic Diseases

In contrast to HTLV-I there are only a few isolates of HTLV-II, and there is very little information about its incidence or disease association. One reason for the paucity of information on HTLV-II is the difficulty in distinguishing it from HTLV-I by routine serological tests. In a survey of sera from patients with various lymphoproliferative disorders, 20 patients with diseases not routinely ascribed for HTLV-I and HIV-1 were seropositive for HTLV-I/HTLV-II antibodies. Both HTLV-II and HIV-1 were subsequently isolated by passage onto selective target cells. For example, HTLV-II infected selected B-cells as well as T-cells, whereas HIV-1 did not infect the B-cells used. HTLV-II was also isolated from one patient with prolymphocytic T-cell leukemia and from three patients with hairy cell leukemia. Comparison of the restriction enzyme maps of three of these isolates with that of the prototype virus HTLV-II_{Mo} revealed some heterogeneity among the HTLV-II genomes. The findings indicate that HTLV-II is more widely disseminated than previously appreciated; its association with malignancy is not limited to hairy cell leukemia of the T-lymphocyte type; it is B-lymphotropic as well as T-lymphotropic; and it exhibits greater heterogeneity than HTLV-I but less than HIV-1.

Persistence and Modulation of HIV-1 Expression in Cells of Mononuclear Phagocyte Lineage

In our previous studies we have shown that the monocyte/macrophages are highly susceptible and permissive host-cells for HIV-1. This cell system can be successfully applied for virus isolation in situations when the T-cell system fails. Using peripheral blood (PB)-derived monocyte/macrophages as targets for HIV-1, the virus was recovered from brain, peripheral blood, bone marrow, lung and skin specimens taken from HIV-1-infected individuals at various stages of disease development. Over 30 HIV-1 isolates have been obtained and most of them characterized by nucleic acid hybridization analysis. It has been successfully demonstrated that in some cases of early infection with the virus (antibody negative, acute infection) or in clinically asymptomatic antibody-positive individuals, HIV-1 could be recovered only from monocyte/macrophages (e.g., HTLV-III_B infection of a laboratory worker). In addition, the monocyte/macrophage system enabled us to isolate and study the so-called "low" replicative types of HIV-1. Three such isolates have been obtained: one from esophageal tissue of a patient with acute HIV-1 infection (HTLV-III_{RH-eu}), one from brain (HTLV-III_{CC-br}) and one from thymic tissue (HTLV-III_{CC-thy}) of a pediatric case with full-blown AIDS.

Studies have been undertaken to define the level of HIV-1 expression in monocyte/macrophages infected with these "low" replicative isolates as compared to "high" replicative HIV-1 isolates which were also recovered from and propagated only in monocyte/macrophages (e.g., HTLV-III_{Ba-L}, HTLV-III_{WR-SK}). HIV-1 expression was followed by in situ hybridization and by reverse transcriptase (RT) assay. Using the same multiplicity of HIV-1 infection, it was shown that there was at least a 10-fold difference in virus production between "low" and "high" replicative isolates. To delineate which factors can modulate HIV-1 expression, PB-derived monocyte/macrophages infected with low replicative isolates were exposed to several cytokines. Unlike the case of the model system of HIV-1-infected U-937 neoplastic cells, culture fluids harvested from phytohemagglutinin (PHA)-stimulated T-cells and MLR (mixed lymphocyte reaction) had no effect on HIV-1 expression in monocyte/macrophages infected with "low" replicative HIV-1 isolates. These results suggest that observations obtained from the model system of HIV-1 neoplastic T- or monocytic cell lines may have limited application for in vivo situations of HIV-1-natural host cell interaction. In contrast, using colony stimulating factors (CSF), both the purified CSF as well as the recombinant CSF accelerated the virus expression in monocyte/macrophages which were infected with "low" replicative HIV-1 isolates and cultured in the presence of these lymphokines. In addition, CSF produced by giant cell tumor (GCT) not only accelerated, but actually increased HIV-1 production in monocyte/macrophages infected with these "low" replicative isolates. However, a single and most effective factor for significant increase of HIV-1 replication in a given monocyte/macrophages infected with "low" replicative HIV-1 isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative HTLV-III_{CG-br} isolate into a "high" replicative one occurred within four in vitro passages of the propagated viral progeny in monocyte/macrophages. This increased production of the HTLV-III_{CG-br} isolate was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in PB-derived monocyte/macrophages enables us to study important parameters of virus-host cell interactions essential for our understanding of HIV-1 pathogenesis, namely heterogeneity of HIV-1 isolates and the dynamics (conversion from "low" to "high" production) with respect to cytopathic effect(s) exhibited on host cells.

Identification of DNA Sequences Within the HIV-1 Genome Responsible for Efficient Infection and Replication of HIV-1 in PB-Derived Monocyte/Macrophages

In our previous studies we have characterized several HIV-1 isolates quantitatively for their capacity to infect T-cells and monocyte/macrophages. There were significant differences in ED_{50} between different HIV-1 isolates. For instance, HTLV-III_{Ba-L} recovered from and propagated only in monocyte/macrophages productively infected these cells at multiplicities of infection (MOI) of 0.5 to 1 X 10^2 cpm/ml of reverse transcriptase (RT) activity. In contrast, the prototype HTLV-III_B, which readily infects T-cells at these low MOIs, requires an MOI of 5 X 10^5 cpm RT activity/ml to PB-derived monocyte/macrophages. These 1000 to 10000-fold differences between HTLV-III_{Ba-L} and HTLV-III_B in capacity to infect monocyte/macrophages are sufficient to permit definitive testing of recombinants generated from these two isolates. In collaboration with Dr. M. Reitz, Jr. (Z01CP005538-02 LTCB), a 4.2 Kb Hind-III fragment of HTLV-III_{Ba-L} containing *tat*, *trs*, *env* and a portion of 3'*orf* has been cloned and sequenced. Nucleic acid sequence analyses have suggested that the 4.2 Kb fragment of the HTLV-III_{Ba-L} (monocyte/macrophage "tropic") isolate does not

contain a characteristic portion within the viral genome which would be specifically responsible for efficient infection and replication of the HTLV-$III_{B_{a-L}}$ isolate in monocyte/macrophages. Preliminary data from infection of T-cells and monocyte/macrophages with hybrid viruses generated from the HTLV-IIIB and HTLV-$III_{B_{a-L}}$ isolates, along with data from sequencing of the vDNA (Hind-III fragment) suggest that minor nucleic acid sequences distributed throughout the whole HIV-1 genome most likely are involved in allowing the efficient replication of an HIV-1 isolate in monocyte/ macrophages. This approach, generation of biologically active hybrids composed of two biologically distinct viral isolates, provides a unique opportunity to identify critical sequences within the HIV-1 genome responsible for significant biological changes.

Development of Highly Specific Immunological Reagents (Monoclonal Antibodies) for Rapid Detection and Discrimination of Human (HIV-1, HIV-2) and Simian (SIV) Immunodeficiency Viruses

Four mouse monoclonal antibodies (MoAbs) were developed following immunization with one HIV-2 isolate and tested for reactivity with different HIV-1, HIV-2 and SIV isolates in an immunofluorescence assay and by Western blot analysis. One of them, an anti-p24 antibody called R1C7, reacted with all HIV-1, HIV-2 and SIV isolates, thus identifying an epitope shared by all human and simian immunodeficiency viruses. Another anti-p24 antibody named A4F6 reacted with three HIV-2 isolates (HIV-2/NIH_{z1}, $LAV\text{-}2_{Rod}$ and LKO01ST9), some SIV isolates ($STLV\text{-}III_{AGM}$, SIV-251 and SIV-309) and no HIV-1 isolates. Two anti-p16 antibodies named R5C4 and R5F6 reacted strongly only with the HIV-2 isolates. The use of these MoAbs can be effectively utilized for rapid discrimination and identification of AIDS-related retroviruses.

Inhibitors of HIV-1 Replication

A number of drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA synthesis chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC).

D-penicillamine (DPA), a thiol amino acid, which presumably interacts with sulfhydryl group-containing proteins, blocks HIV-1 replication in cell cultures. In clinical trials on 30 asymptomatic patients with generalized lymphadenopathy syndrome (LAS), and ARC, suppression of virus expression was seen in 60% of patients treated with this drug. A large-scale study involving 60 patients is currently being carried out. The exact mechanism of action of this compound is not clear but it appears that it interacts with the HIV-1 *tat* protein which is a zinc-containing sulfhydryl protein or other sulfhydryl group-containing HIV-1 proteins such as the nucleic acid-binding protein and the *env* glycoproteins.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, is a member of the polyene macrolide group of antifungal antibiotics, which interacts with sterols and irreversibly binds to them. It is known to be active against a variety of lipid-enveloped RNA and DNA viruses, several oncogenic retroviruses and different strains of herpesviruses. Both amphotericin B and AME have been found to be potent inhibitors of HIV-1 replication in cell culture without any toxic side effects. Results with amphotericin B treatment of a few LAS patients indicate suppression of HIV-1 in the peripheral blood of these

patients. Clinical studies with AME are likely to begin shortly. A combination of AME with several other drugs including foscarnet, 3'-azido-3'-deoxythymidine (AZT), 2'-3'-dideoxycytidine (DDC), and dextran sulfate showed both additive and synergistic effects and it appears that combination therapy may prove to be more effective in the treatment of AIDS patients.

The inhibition of HIV-1 replication by the use of antisense oligonucleotides (synthetic oligonucleotides), which inhibit virus replication by competition hybridization has been utilized. Since the complete nucleotide sequence of the HIV-1 genome is known, we picked several regions for the preparation of antisense oligomers. These include regions adjacent to the primer binding site and tat-3 gene splice acceptor and donor sites. Oligonucleotides of chain length 20 were found to be most active in inhibiting virus replication. The greatest inhibition of virus replication was observed by oligonucleotides specific for the tat-3 gene splice acceptor and donor sites. Approximately 8% of the oligonucleotides added to cell culture are taken up by the cells, thus giving a high enough concentration inside the cell to be effective. Oligomer derivatives such as thiophosphates, morpholidates and methylphosphonates were also found to be very effective inhibitors of HIV-1 replication. In addition, a combination of several of these oligomers showed both an additive as well as synergistic effect. Safety and toxicity studies in mice show that these compounds are completely safe and nontoxic. Whether the antisense oligomer approach will prove to be useful in the treatment of HIV-1-infected patients remains to be determined, and limited clinical trials are likely to be initiated in the near future.

Use of Synthetic Peptides as Vaccines for AIDS

With an estimated 60,000 cases of AIDS already reported in the United States and with more than 33,000 known deaths, it is extremely urgent and important to look for ways to protect the at-risk population by immunization with an effective vaccine. Several approaches to the development of an AIDS vaccine are being explored. They include the use of (a) infectious HIV-1, (b) inactivated HIV-1, (c) subunits of HIV-1, (d) molecularly cloned subunit proteins, (e) synthetic peptides both to the envelope proteins (gp120) of the virus and the gag protein (p17), (f) infectious recombinant virus with vectors such as vaccinia virus, and (g) the use of anti-idiotypes as a vaccine.

We have explored the potential usefulness of synthetic peptides as candidate AIDS vaccines, especially the peptide generated from the p17 sequence of HIV-1. The p17 protein was recently found, by the immunogold-labeling techniques by electron microscopy, by Gelderblom (Max Planck Institute, Berlin, FRG) et al. (unpublished results) to be associated with the envelope glycoproteins of HIV-1. Similar conclusions have been drawn by computer modeling techniques. This observation suggests that the p17 epitope may be exposed on the surface in the virions. Hence, gag gene products are important in any considerations of a potential vaccine for AIDS. In addition, because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as 20% divergence in the amino acid sequence of gp120, it is important to look for other approaches to the development of a vaccine for AIDS.

A 30 amino acid peptide analog of HIV-1 p17 (termed HGP30) has been obtained, and antibodies prepared against this peptide inhibit HIV-1 replication in cell culture of three different strains of HIV-1 and appear to be group-specific rather than type-specific. These studies indicate that HIV-1 p17 may be

important in the cell-mediated immunity and that these antibodies may be protective against HIV-1 infection. A number of synthetic peptides covering the HIV-1 p17 sequence have recently been made and are being evaluated for immune response in animals. These antibodies will be examined in HIV-1 virus neutralization assays to identify the epitope responsible for maximal virus neutralization for potential usefulness in vaccine trials.

Tropical Spastic Paraparesis (TSP) and Neurological Dysfunction

Adult T-cell leukemia (ATL) and TSP are diseases that are prevalent in Jamaica and the Caribbean and these areas are endemic for HTLV-I. HTLV-I antibodies have been identified in a large number of patients with TSP. TSP is a neurological syndrome in which patients progressively develop difficulty in walking, stiffness of legs and back pain. A similar disease called HTLV-I-associated myelopathy (HAM) has recently been described in Japan. We have recently isolated HTLV-I from the CSF of a TSP patient from Jamaica. This virus isolate shows subtle differences from the prototype HTLV-I isolate from ATL patients, by restriction enzyme mapping, suggesting the possibility that the TSP isolate is similar to but not identical to the prototype HTLV-I isolate. Further gene cloning and sequencing of the HTLV-I isolate from TSP patients will establish the similarity and differences between this isolate and HTLV-I. We are also utilizing the polymerase chain reaction (PCR) technique to evaluate various tissues from TSP patients for the presence of retrovirus infection.

Immune Response to HIV and Vaccine Development

Results of a 6-year prospective study of HIV-1 seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods during which individuals remain disease free. This observation confirms and extends our earlier findings which suggested that in both adults and pediatric patients these antibodies are associated with better clinical outcome. This study, therefore, supports continued efforts to generate vaccines aimed at eliciting high-titer, broadly reactive neutralizing antibody but does not preclude an equally important role of cell-mediated immune responses in preventing infection or retarding disease progression.

In 1986, we reported the in vitro generation of an HIV-1 variant, obtained by culturing an infectious molecularly cloned virus in the presence of a neutralizing antibody positive serum. Following cloning and subsequent sequencing of the variant envelope, a single amino acid substitution at position 582 in the transmembrane protein was localized. That this point mutation was, in fact, responsible for the altered neutralizing ability, was confirmed by making this change in the parental virus clone and illustrating that the altered parental virus possessed the same neutralization resistance as the original variant. These experiments indicate that although sera from HIV-1-infected humans in general are broadly group-specific in their neutralizing antibody response, they possess type specificities which may influence the propagation of mutant viruses arising during the course of infection. Moreover, our data show that the viral transmembrane protein possesses determinants important for the neutralizing antibody response. Whether the amino acid substitution observed marks a distinct neutralization epitope, or whether it is part of a non-contiguous conformational epitope is not yet resolved.

A major type-specific neutralization epitope was mapped to 24 amino acids within the exterior viral envelope protein (gp120) using a mouse monoclonal antibody specific for gp120. The monoclonal antibody used in these studies can inhibit HIV-1 infection by fusion with virus-infected cells as well as by cell-free virus, indicating that vaccines directed toward neutralization epitopes may be broadly protective. A further important finding with regard to future vaccine development was the observation that gp160, produced in the insect bacculovirus system, is immunogenic and can elicit high titer neutralizing antibody in inoculated goats. This material provides a possible source of vaccine material. Recently, a double retroviral infection of HTLV-I and HIV-2 in a healthy African woman from Guinea Bissau was observed. The study illustrates that in areas where several retroviruses may be endemic, as Africa, serologic profiles may be difficult to interpret because of immunologic cross reactivities. Thus, more specific diagnostic tests may need to be developed. Moreover, an extensive seroepidemiologic survey demonstrated that Portugal is not a viral endemic area for HTLV-I.

Modulation of T- and B-Cell Function by Viral Proteins

Reconstruction experiments with partially purified viral proteins, recombinant-derived viral proteins, and proteins derived from viral mutants with altered function have succeeded in producing in vitro effects resembling the in vivo pathology of human AIDS. Viral gp120 produces an immunosuppressive effect on normal T-cells and a sequence localized within the junction region between gp41 and 3'orf causes a form of B-cell suppression in which the production of immunoglobulin (IgG) is polyclonally stimulated and the B-cells lose the ability to produce specific antigen. These effects suggest that immunosuppressive properties of the viral proteins may contribute, at least in part, to the pathology of HIV-1 infection.

Distribution of HBLV in the Normal Population

Serologic tests for detection of anti-HBLV antibodies have been applied to normal blood donor populations from Minneapolis, Minnesota and Kansas. The tests suggest relatively wide-spread distribution of HBLV in the areas tested (>80%). Quantitative and qualitative parameters of immunological response are under investigation. These studies have so far shown that although low antibody titers are widespread, unusually high titer antibodies to Burkitt's lymphoma, acute lymphocytic leukemia (ALL), Hodgkin's lymphoma and chronic fatigue syndrome have so far only been found in certain West African countries.

Transfection/Mutagenesis

The availability of full-length molecular clones of HIV which, upon introduction into human T-cells, give rise to replicating cytolytic virus, has provided a model system in which to systematically examine the functions of different components with the viral genome. In addition to the gag, pol, and env genes (which encode structural components of the retrovirus), the HIV genome contains at least five other genes: vip, tat, rev, vpr and nef (previously named sor, tat, trs, R and 3'orf). Studies of the transactivator gene of HIV (tat) have shown that this gene is an absolute requirement for virus replication. Mutants in which the coding region of this gene was removed or the splice acceptor (used to generate nature tat mRNA) deleted were unable to generate virus or produced only low levels of virus. These defects could be corrected by providing a functional

tat in trans or the tat protein itself. The functional domain of tat has been localized by site-directed mutagenesis, to a central core region that is rich in cysteine residues. This core sequence resembles the metal binding finger proteins which are known to bind nucleic acids in regulating gene expression. To further define the functions of the tat and rev genes, translational stop codons were independently generated in the 5' portion of each gene. We have focused on the first coding exon of both genes since this exon has been shown to be necessary and sufficient for tat function. A chain-terminating mutation early in the rev gene resulted in an increase in transcription of viral mRNA measured by nuclear run-on experiments, but only one major species of viral mRNA (1.8kd) was detected. As a result, little or no viral structural proteins were made. Thus, the rev gene product may have a negative trans-regulatory role in transcription when overexpressed but is essential for expression of viral proteins by promoting accumulation of unspliced mRNA. Recent data suggest that rev may selectively stabilize unspliced mRNA as well as transport it into the cytoplasm.
The role of the nef gene in virus production was investigated, and it appears that a major coding region of the nef gene could be removed without adversely affecting the replicative capacities of the virus or its ability to kill human lymphocytes. In fact, the replicative potential of nef mutants appeared higher than that of the wild type. Analysis of the mutant (10-1) in which deletion in the nef extended into the carboxyl terminus of gp41 revealed a mutant genome which replicates efficiently but produced virus which was severely compromised in its ability to kill lymphocytes.

Studies directed at defining the role of the vip gene which lies between the pol and tat genes of HIV and encodes a 23kd protein have shown that this gene is crucial in producing infectious virus capable of cell-free transmission; mutants deprived of vip could be transmitted, rather inefficiently, by coculture with $T4^+$ target cells but failed to establish stable infection when transmitted in a "cell-free" fashion. Since cells transfected with vip mutant genomes produce normal levels of viral RNA, structural proteins, tat and HIV particles, we currently suspect that the vip gene exerts its effect on virus replication at a post-translational level (perhaps by enhancing assembly of infectious particles).

DNA/Protein Interactions

A new assay has been developed for microscale analysis of DNA binding proteins. The assay involves biotinylation of sequences known to have specific biological importance. The derivitized oligomers are then added to labeled nuclear extracts pretreated with poly GC competitor (to reduce background); the protein-DNA complex is then bound to streptavidin agarose beads which are centrifuged and washed. The proteins are then visualized after sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS/PAGE) and autoradiography. Using this technique, we have found that a protein of 86kd specifically binds to the enhancer of HIV. This protein is constitutively expressed in H9 cells and can be induced by phytohemagglutinin (PHA) stimulation of Jurkat cells. The protein is a candidate for the transcription factor activity as reported by Nabel and Baltimore (Nature 326: 711-13, 1987). Attempts to localize the mitogen response elements within the HIV long-terminal repeat (LTR) which may control cellular events responsible for HIV latency, show the presence of sequences upstream of -279 which are responsible for a negative regulatory effect by increased mitogen response. Sequences homologous to other mitogen-inducible genes, such as interleukin-1 (IL-1), may also play a role in the inducibility of the HIV LTR since their deletion diminishes such response.

Type-Specific Neutralization Epitopes on Cloned HIV Viruses

One strategy for immunoprophylaxis against acquired immunodeficiency syndrome is the administration of an immunogen intended to elicit protective virus neutralizing antibodies. Recent observations indicate that individual isolates of HIV-1 are composed of populations of genetically and biologically distinct variants. To determine the implications of such heterogeneity for vaccine development and evaluation, we compared the susceptibility of three viruses molecularly cloned from HTLV-IIIB (HIV-I/NIH/USA/1983/HTLV-IIIB) to the neutralizing effects of a number of antisera. The results show that either patients' sera or hyperimmune sera raised against native gp120 or recombinant peptides derived from gp120 of a specific DNA clone from HTLV-IIIB can show up to two log differences in neutralization titer against the three cloned viruses. Our data confirm that a single amino acid change in the virus envelope may result in profound changes in recognition by neutralizing antisera, and extend the previous concept of type-specificity of neutralization to differing variants of a single isolate. This suggests that the group-specific neutralizing activity (NA) of some human sera could be directed against a large number of type-specific determinants as well as conserved epitopes. Inability to demonstrate protection by candidate vaccines after challenge with homologous virus isolates could represent the selection of minor clonal variants not well recognized by the immunized host.

Comparative Analysis of HIV-2 and SIV Isolates

Complete nucleotide sequences have been obtained for two independent isolates of HIV-2 and one isolate of SIV_{MAC}. Infectious clones of these viruses have also been obtained and inoculated into monkeys. These reagents would be very important for developing an animal model for an HIV vaccine. We have also obtained clear evidence that HIV-2 and SIV contain a novel protein which is virion-associated. This protein (vpx) may be useful as a discriminating reagent for HIV-2 infection.

Mechanisms of Pathogenesis of KS

Cultures of endothelial cells from KS patients have been established and examined for the expression of cytokines which may account for the maintenance and expansion of KS cells in vivo. High level expression of several growth factor genes, which can fully account for both autocrine and paracrine modes of growth of cells comprising a typical KS lesion have been observed.

Molecular Cloning and Restriction Enzyme Mapping of HBLV

Human B-lymphotropic virus (HBLV, HHV-6) DNA has been purified by initially banding the virus on sucrose gradients (1.13 to 1.17 gm/ml) followed by gentle lysis and banding on cesium chloride gradients either with or without ethidium bromide. Yields of viral DNA from 2.5 to 10 liter cultures are now in the range of 20 ug by these procedures. Three plasmid libraries which were prepared from CsCl banded virus DNA are being screened. Over 200 clones have been obtained which are now being characterized by restriction analyses, gene walking and sequencing to map and analyze the entire HBLV genome.

Numerous probes which cross-react among the previously identified herpesviruses do not hybridize to HBLV DNA, except the Marek's disease virus (MDV). DNA fragments of HBLV, which hybridize to specific fragments of MDV have been cloned. Sequencing of the genomic clone, ZVH14, has revealed an open reading frame of 1113 amino acids. This is a candidate open reading frame for the HBLV envelope glycoprotein. Studies of the expression of this open reading frame are being pursued.

Genetic Determinants for Macrophage Tropism

A molecular clone containing the 3' half of a provirus representing a macrophage tropic HIV-1 has been obtained and sequenced. The organization of the viral genome is the same as for T-cell tropic HIV-1 isolates, and the sequence divergence between this isolate and other United States HIV-1 isolates is no different in kind or degree than that seen among different HIV-1 isolates. The genetic differences allowing growth in macrophages must therefore be rather small. We have constructed different chimeric proviruses by inserting parts of the genome from the macrophage tropic virus into the biologically active clone of the T-cell tropic virus, including part or all of the gp120, the amino terminal 250 amino acids of the gp41, and *tat* and *trs*, and the biological properties of these chimeric viruses are under study.

Generation and Characterization of HIV-1 Point Mutants

Based on our comparisons of the primary sequence of different HIV-1 isolates, SIV, and various HIV-2 isolates, the 22 cysteine residues of the HIV-1 envelope are completely conserved in all isolates, suggesting that all are functionally important. We have made 14 mutants, each of which have a different cysteine replaced by another amino acid. Seven of eight such mutants lack the ability to infect H9 target cells, confirming the suspected importance of cysteine residues. Another series of mutations are being made to characterize the envelope polyprotein cleavage site. Changing the lysine at position 510 to a leucine abolishes both envelope cleavage and infectivity. A third kind of mutation is in the coding region for the endonuclease which is important for proviral integration into the host cell genome.

Regulatory Genes and Elements of HIV-1 and HIV-2

New human retroviruses (termed HIV-2) have been isolated from sick and healthy individuals resident in West Africa. Some of these HIV-2 isolates may be less pathogenic in the natural host. Thus, HIVs may comprise a spectrum of viruses with varying degrees of pathogenicity. Our studies explore the premise that the pathogenic potential of these viruses is, at least in part, governed by their genetic structure and that viral gene expression and replication underlies pathogenesis. We have observed that like HIV-1, all of the HIV-2 isolates tested contain a functional *tat* gene and *tat* response elements. We have undertaken a detailed insertion and deletion mutant analysis of the regulatory elements of HIV-2 LTR and find that: i) HIV-2 LTR contains positive regulatory elements upstream of the transcriptional initiation site, ii) the *tat* response element is more complex than similar elements in HIV-1 LTR, and iii) there is some evidence that HIV-2 LTR contains negative regulatory element(s) downstream of the transcriptional initiation site. The significance of this downstream negative regulatory element in controlling viral gene expression, particularly in human $T4^+$ lymphocytes, is under investigation. We are now focusing on the detailed

analysis of the tat and particularly of the 3'orf genes of HIV-2 to determine if the tat gene of HIV-2(ST) is weaker and 3'orf gene of HIV-2 (ST) is stronger than the same genes of HIV-1.

Translational Controls in HIV-1 and HIV-2

Chronically HIV-1-infected cells have been shown to contain transcripts with multiple open reading frames (orf). For example, the sor cDNA clones contain the orf for not only sor but also for tat, trs and 3'orf. This transcript exists in the presence of other transcripts containing one or a fewer number of the same orfs. Recent cDNA cloning of HIV-2 has revealed similar transcripts for HIV-2, and we find that different orfs carried on a given transcript are translated with unequal efficiency. We are now determining the factors which govern the translational efficiency of HIV-1 and HIV-2 transcripts.

Induction of Lymphotoxin Expression by HTLV-I

A number of T-cell lines have been examined for expression of lymphotoxin and tumor necrosis factor (TNF). These cell lines include uninfected cell lines, cell lines infected by HIV-1, and cell lines either established by in vitro HTLV-I infection or directly from ATL patients. All cell lines infected with HTLV-I constitutively express high levels of lymphotoxin RNA and protein. Many also express TNF. In contrast, none of the other cell lines, whether uninfected or infected by HIV-1, expressed detectable levels of either lymphokine. A functional helper T-cell clone expressed no detectable lymphotoxin before infection with HTLV-I. We are currently analyzing the lymphotoxin gene for tissue-specific enhancer activity to identify any factors important in the activation of both virus and lymphotoxin expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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Monocyte/Macrophages and Accessory Cells in Pathogenesis of HIV-1 Infection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	A. Minassian	Guest Researcher	LTCB NCI
	K. Ohashi	Guest Researcher	LTCB NCI
	M. Reitz	Research Chemist	LTCB NCI

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LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cell-system of peripheral blood-derived monocyte/macrophages (M/M) was successfully employed for human immunodeficiency virus type-1 (HIV-1) isolation in situations where conventional virus isolation failed. We demonstrated that HIV-1 can be recovered from M/M but not from T-cells in several cases of "early" HIV-1 infection or in advanced acquired immune deficiency syndrome (AIDS) cases. The high sensitivity of this system made it possible to recover several HIV-1 isolates termed "low" replicative isolates because of their poor replication in natural targets. Three such isolates have been studied with regards to their expression in M/M. We showed that some cytokines (e.g., colony stimulating factor [CSFs]) can accelerate the expression of these "low" replicative isolates in these cells. However, a single and most effective factor for significant increase of HIV-1 replication in M/M infected with these "low" replicative isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative human T-lymphotropic virus type-III (HTLV-III-CG-br) isolate into a "high" one occurred within four in vitro passages of this isolate in M/M. The increased virus production was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in M/M enables us to study important parameters of virus-host cell interactions which are essential for our understanding of HIV-1 pathogenesis. In addition, biologically active hybrids were generated from two biologically distinct HIV-1 isolates previously characterized by cloning and sequencing of their vDNA. This study permits identification of critical sequences within the HIV-1 genome responsible for significant biological properties. Furthermore, a set of monoclonal antibodies were developed against core proteins of HIV-2/NIH-Z which enables rapid discrimination of cells infected with HIV-1, HIV-2 and simian immune deficiency virus (SIV).

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Popovic	Visiting Scientist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
S. Gartner	Senior Staff Fellow	LTCB NCI
A. Minassian	Guest Researcher	LTCB NCI
K. Ohashi	Guest Researcher	LTCB NCI
M. Reitz	Research Chemist	LTCB NCI

Objectives:

Detailed characterization of HIV-1 interactions with natural targets, particularly monocyte/macrophages; understanding of virus-host cell interactions including adsorption, penetration, persistence and expression of HIV-1 in cells of the mononuclear phagocyte lineage is mandatory for the development of effective therapeutic measures and prevention of development of HIV-1 associated diseases.

Identification of nucleic acid sequences within the HIV-1 genome responsible for efficient replication of HIV-1 isolates in monocyte/macrophages.

Development of highly specific immunological reagents (monoclonal antibodies) for rapid detection and discrimination of human (HIV-1, HIV-2) and simian (SIV) immunodeficiency viruses.

Methods Employed:

A variety of virological, immunological, nucleic acid and cell biological techniques are employed. The peripheral blood (PB)-derived monocyte/macrophage culture system, which was developed in our laboratory, is utilized for efficient recovery of HIV-1 from various tissues of virus-infected individuals.

Major Findings:

Persistence and Modulation of HIV-1 Expression in Cells of Mononuclear Phagocyte Lineage

In our previous studies we have shown that the monocyte/macrophages are highly susceptible and permissive host-cells for HIV-1. This cell system can be successfully applied for virus isolation in situations when the T-cell system fails. Using PB-derived monocyte/macrophages as targets for HIV-1, the virus was recovered from brain, peripheral blood, bone marrow, lung and skin specimens taken from HIV-1-infected individuals at various stages of disease development. Over 30 HIV-1 isolates have been obtained and most of them characterized by nucleic acid hybridization analysis. It has been successfully demonstrated that in some cases of early infection with the virus (antibody negative, acute infection) or in clinically asymptomatic antibody-positive individuals, HIV-1 could be recovered only from monocyte/macrophages (e.g., HTLV-III_B infection of a

laboratory worker). In addition, the monocyte/macrophage system enabled us to isolate and study the so called "low" replicative types of HIV-1. Three such isolates have been obtained: one from esophageal tissue of a patient with acute HIV-1 infection (HTLV-III_{RH-eu}), one from brain (HTLV-III_{CG-br}) and one from thymic tissue (HTLV-III_{CG-thy}) of a pediatric case with full-blown AIDS.

Studies have been undertaken to define the level of HIV-1 expression in monocyte/macrophages infected with these "low" replicative isolates as compared to "high" replicative HIV-1 isolates which were also recovered from and propagated only in monocyte/macrophages (e.g., HTLV-III_{BA-L}, HTLV-III_{WR-SR}). HIV-1 expression was followed by in situ hybridization and by reverse transcriptase assay. Using the same multiplicity of HIV-1 infection, it was shown that there was at least a 10-fold difference in virus production between "low" and "high" replicative isolates. To delineate which factors can modulate HIV-1 expression, peripheral blood-derived monocyte/macrophages infected with low replicative isolates were exposed to several cytokines. Unlike the case of the model system of HIV-1-infected U-937 neoplastic cells, culture fluids harvested from phytohemagglutinin (PHA)-stimulated T-cells and MLR (mixed lymphocyte reaction) had no effect on HIV-1 expression in monocyte/macrophages infected with "low" replicative HIV-1 isolates. These results suggest that observations obtained from the model system of HIV-1 neoplastic T- or monocytic cell lines may have limited application for in vivo situations of HIV-1-natural host-cell interaction. In contrast, using colony stimulating factors (CSF), both the purified CSF as well as the recombinant CSF accelerated the virus expression in monocyte/macrophages which were infected with "low" replicative HIV-1 isolates and cultured in the presence of these lymphokines. In addition, CSF produced by giant cell tumor (GCT) not only accelerated, but actually increased HIV-1 production in monocyte/macrophages infected with these "low" replicative isolates. However, a single and most effective factor for significant increase of HIV-1 replication in a given monocyte/macrophages infected with "low" replicative HIV-1 isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative HTLV-III_{CG-br} isolate into a "high" replicative one occurred within four in vitro passages of the propagated viral progeny in monocyte/macrophages. This increased production of the HTLV-III_{CG-br} isolate was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in PB-derived monocyte/macrophages enables us to study important parameters of virus-host cell interactions essential for our understanding of HIV-1 pathogenesis, namely heterogeneity of HIV-1 isolates and the dynamics (conversion from "low" to "high" production) with respect to cytopathic effect(s) exhibited on host cells.

Identification of DNA Sequences Within the HIV-1 Genome Responsible for Efficient Infection and Replication of HIV-1 in PB-Derived Monocyte/Macrophages

In our previous studies we have quantitatively characterized several HIV-1 isolates for their capacity to infect T-cells and monocyte/macrophages. There were significant differences in ED_{50} between different HIV-1 isolates. For instance, HTLV-III_{BA-L} recovered from and propagated only in monocyte/macrophages productively infected these cells at multiplicities of infection (MOI) of 0.5 to 1 X 10^2 cpm/ml of reverse transcriptase (RT) activity. In contrast, the prototype HTLV-III_B, which infects readily T-cells at these low MOIs, requires an

MOI of 5 X 10^5 cpm RT activity/ml to PB-derived monocyte/macrophages. These 1000 to 10000-fold differences between HTLV-III_{Ba-L} and HTLV-III_B in capacity to infect monocyte/macrophages are sufficient to permit definitive testing of recombinants generated from these two isolates. In collaboration with Dr. M. Reitz, Jr. (Z01CP05538-02 LTCB), a 4.2 Kb Hind-III fragment of HTLV-III_{Ba-L} containing tat, trs, env and a portion of 3'orf has been cloned and sequenced. Nucleic acid sequence analyses have suggested that the 4.2 Kb fragment of the HTLV-III_{Ba-L} (monocyte/macrophage "tropic") isolate does not contain a characteristic portion within the viral genome which would be specifically responsible for efficient infection and replication of the HTLV-III_{Ba-L} isolate in monocyte/macrophages. Preliminary data from infection of T-cells and monocyte/macrophages with hybrid viruses generated from the HTLV-IIIB and HTLV-III_{Ba-L} isolates, along with data from sequencing of the vDNA (Hind-III fragment) suggest that minor nucleic acid sequences distributed throughout the whole HIV-1 genome most likely are involved in allowing the efficient replication of an HIV-1 isolate in monocyte/macrophages. This approach, generation of biologically active hybrids composed of two biologically distinct viral isolates, provides a unique opportunity to identify critical sequences within the HIV-1 genome responsible for significant biological changes.

Development of Highly Specific Immunological Reagents (Monoclonal Antibodies) for Rapid Detection and Discrimination of Human (HIV-1, HIV-2) and Simian (SIV) Immunodeficiency Viruses

Four mouse monoclonal antibodies (MoAbs) were developed following immunization with one HIV-2 isolate and tested for reactivity with different HIV-1, HIV-2 and SIV isolates in an immunofluorescence assay and by Western blot analysis. One of them, an anti-p24 antibody called R1C7, reacted with all HIV-1, HIV-2 and SIV isolates, thus identifying an epitope shared by all human and simian immunodeficiency viruses. Another anti-p24 antibody named A4F6 reacted with three HIV-2 isolates (HIV-2/NIH_{z1}, $LAV\text{-}2_{Rod}$ and LK001ST9), some SIV isolates (STLV-III_{AGM}, SIV-251 and SIV-309) and no HIV-1 isolates. Two anti-p16 antibodies named R5C4 and R5F6 reacted strongly only with the HIV-2 isolates. The use of these MoAbs can be effectively utilized for rapid discrimination and identification of AIDS-related retroviruses.

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Gallo RC, Popovic M, Sarngadharan MG. US Patent 4,652,599: Method of Continuous Production of Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS using Permissive Cells, March 24, 1987.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1CP05535-02 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Retrovirus Infection, Treatment, Prevention and Etiology of TSP

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. S. Sarin	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	R. Mukhopadhya	Visiting Fellow	LTCB NCI
	T. Ikeuchi	Guest Researcher	LTCB NCI
	C. C. Gajdusek	Chief	CNSS NINCDS
	C. J. Gibbs	Deputy Chief	CNSS NINCDS
	P. R. Johnson	Visiting Scientist	CNSS NINCDS

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Laboratory of Tumor Cell Biology

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Hematopoietic Cellular Control Mechanisms

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A number of approaches have been examined to identify drugs that can block human immunodeficiency virus type-1 (HIV-1) replication in cell culture. Our studies indicate that a combination of drugs gives a more significant additive and synergistic effect than does single drugs alone, and hence combination chemotherapy may be more useful in the treatment of acquired immunodeficiency syndrome (AIDS). Antisense oligonucleotides have also been found to be effective in blocking HIV-1 replication. A combination of oligonucleotides defining different target sites on the HIV-1 genome appears to be synergistic. Antibodies made against a 30 amino acid HIV-1 p17 synthetic peptide (HGP30) were found to inhibit HIV-1 replication in H9 and Molt-3 cells, and these antibodies appear to be group specific. HGP30 has been found to be safe and nontoxic in several animal species, including rhesus and chimpanzees, and the preparation is ready for vaccine trials in humans. Other synthetic peptides covering the sequence of p17 are being analyzed for immune amino acid response and the development of neutralizing antibodies. An human T-lymphotropic virus type-I (HTLV-I)-like virus has been isolated from the cerebrospinal fluid of a patient with tropical spastic paraparesis (TSP) and this virus isolate appears to be similar to but not identical to HTLV-I from adult T-cell leukemia (ATL). Further characterization is in progress.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

P. S. Sarin	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
R. Mukhopadhya	Visiting Fellow	LTCB NCI
T. Ikeuchi	Guest Researcher	LTCB NCI
D. C. Gajdusek	Chief	CNSS NINCDS
C. J. Gibbs	Deputy Chief	CNSS NINCDS
P. R. Johnson	Visiting Scientist	CNSS NINCDS

Objectives:

To identify drugs that could interfere with HIV-1 infection and replication and study their mechanism of action. The identification of an active and non-toxic drug could be very useful in the treatment of AIDS. Advantages of combination therapy are also being explored.

To explore the potential usefulness of synthetic peptides as a vaccine approach for the prevention of AIDS. An HIV-1 p17 synthetic peptide is being evaluated as a vaccine for AIDS. Immune response to a 30 amino acid synthetic peptide (HGP30) is being evaluated in several animal species to determine the type-specific or group-specific antibodies that are produced in these species.

To identify an animal model for AIDS. Studies in several animal species, including chimpanzees, are being carried out.

To examine whether HTLV-I or a variant of HTLV-I is involved in tropical spastic paraparesis (TSP).

Methods Employed:

Standard virological, biochemical and cell biology techniques are utilized in the processing and culture and cocultivation of human and animal cells from peripheral blood, bone marrow and cerebrospinal fluid (CSF). Retrovirus expression in the cell culture is measured by immunofluorescence with monoclonal antibodies against viral antigens, reverse transcriptase assays and electron microscopy. Virus neutralizing antibody and syncytia inhibition assays, as well as assays to examine inhibition of HIV-1 replication in the presence of drugs, are also utilized.

Major Findings:

Inhibitors of HIV-1 Replication

A number of drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA

synthesis chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC).

D-penicillamine (DPA), a thiol amino acid, which presumably interacts with sulfhydryl group-containing proteins, blocks HIV-1 replication in cell cultures. DPA has been used in the past for the treatment of Wilson's disease, chronic hepatitis and rheumatoid arthritis. In clinical trials on 30 asymptomatic patients with generalized lymphadenopathy syndrome (LAS), and ARC, suppression of virus expression was seen in 60% of patients treated with this drug. A large-scale study involving 60 patients is currently being carried out. The exact mechanism of action of this compound is not clear but it appears that it interacts with the HIV-1 tat protein which is a zinc-containing sulfhydryl protein or other sulfhydryl group-containing HIV-1 proteins such as the nucleic acid-binding protein and the env glycoproteins.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, is a member of the polyene macrolide group of antifungal antibiotics, which interacts with sterols and irreversibly binds to them. It is known to be active against a variety of lipid-enveloped RNA and DNA viruses, several oncogenic retroviruses and different strains of herpesviruses. This antiviral property of AME prompted us to examine its activity against HIV-1, which is also a lipid-enveloped retrovirus. The binding of AME to cholesterol in the membrane of cells causes changes in cell permeability and function, and its binding to sterols of lipid-enveloped viruses causes loss of their infectivity. Both amphotericin B and AME have been found to be potent inhibitors of HIV-1 replication in cell culture without any toxic side effects. Results with amphotericin B treatment of a few LAS patients indicate suppression of HIV-1 in the peripheral blood of these patients. Clinical studies with AME are likely to begin shortly. A combination of AME with several other drugs including foscarnet, 3'-azido-3'-deoxythymidine (AZT), 1'-3'-dideoxycytidine (DDC), and dextran sulfate showed both additive and synergistic effects and it appears that combination therapy may prove to be more effective in the treatment of AIDS patients.

The inhibition of HIV-1 replication by the use of antisense oligonucleotides (synthetic oligonucleotides), which inhibit virus replication by competition hybridization, has been utilized. Since the complete nucleotide sequence of the HIV-1 genome is known, we picked several regions for the preparation of antisense oligomers. These include regions adjacent to the primer binding site and tat-3 gene splice acceptor and donor sites. Oligonucleotides of chain length 20 were found to be most active in inhibiting virus replication. The greatest inhibition of virus replication was observed by oligonucleotides specific for the tat-3 gene splice acceptor and donor sites. Approximately 8% of the oligonucleotides added to cell culture are taken up by the cells, thus giving a high enough concentration inside the cell to be effective. Oligomer derivatives such as thiophosphates, morpholidates and methylphosphonates were also found to be very effective inhibitors of HIV-1 replication. In addition, a combination of several of these oligomers showed both an additive as well as the synergistic effect. Safety and toxicity studies in mice show that these compounds are completely safe and nontoxic. Whether the antisense oligomer approach will prove to be useful in the treatment of HIV-1-infected patients remains to be determined, and limited clinical trials are likely to be initiated in the near future.

Use of Synthetic Peptides as Vaccines for AIDS

With an an estimated 60,000 cases of AIDS already reported in the United States and with more than 33,000 known deaths, it is extremely urgent and important to look for ways to protect the at-risk population by immunization with an effective vaccine. Several approaches to the development of an AIDS vaccine are being explored. They include the use of (a) infectious HIV-1, (b) inactivated HIV-1, (c) subunits of HIV-1, (d) molecularly cloned subunit proteins, (e) synthetic peptides both to the envelope proteins (gp120) of the virus and the gag protein (p17), (f) infectious recombinant virus with vectors such as vaccinia virus, and (g) the use of anti-idiotypes as a vaccine.

We are collaborating with various laboratories in pursuing the envelope glycoprotein, vaccinia vector and anti-idiotypes as sources for an AIDS vaccine. We have also explored the potential usefulness of synthetic peptides as candidate AIDS vaccines, especially the peptide generated from the p17 sequence of HIV-1. The p17 protein was recently found, by the immunogold labeling techniques by electron microscopy, by Gelderblom (Max Planck Institute, Berlin, FRG) et al. (unpublished results) to be associated with the envelope glycoproteins of HIV-1. Similar conclusions have been drawn by computer modeling techniques. This observation suggests that the p17 epitope may be exposed on the surface in the virions. Hence, gag gene products are important in any considerations of a potential vaccine for AIDS. In addition, because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as 20% divergence in the amino acid sequence of gp120, it is important to look for approaches to the development of a vaccine for AIDS, other than focusing on the HIV-1 env gene product.

A 30 amino acid peptide analog of HIV-1 p17 (termed HGP30) has been obtained which cross-reacts in the HIV-1 p17 radioimmunoassays (RIA) and can be identified by HIV-1 p17 monoclonal antibodies by enzyme linked immunosorben assay (ELISA) assays and Western blotting. Antibodies prepared against this peptide inhibit HIV-1 replication in cell culture of three different strains of HIV-1 and appear to be group-specific rather than type-specific. These studies indicate that HIV-1 p17 may be important in the cell-mediated immunity and that these antibodies may be protective against HIV-1 infection. A number of synthetic peptides covering the HIV-1 p17 sequence have recently been made and are being evaluated for immune response in animals. These antibodies will be examined in HIV-1 virus neutralization assays to identify the epitope responsible for maximum virus neutralization for potential usefulness in vaccine trials.

Tropical Spastic Paraparesis (TSP) and Neurological Dysfunction

Adult T-cell leukemia (ATL) and TSP are diseases that are prevalent in Jamaica and the Caribbean and these areas are endemic for HTLV-I. HTLV-I antibodies have been identified in a large number of patients with TSP. TSP is a neurological syndrome in which patients progressively develop difficulty in walking, stiffness of legs and back pain. A similar disease called HTLV-I-associated myelopathy (HAM) has recently been described in Japan. We have recently isolated HTLV-I from the cerebrospinal fluid (CSF) of a TSP patient from Jamaica. This virus isolate shows subtle differences from the prototype HTLV-I isolate from ATL

patients, by restriction enzyme mapping, suggesting the possibility that the TSP isolate is similar to but not identical to the prototype HTLV-I isolate. Further gene cloning and sequencing of the HTLV-I isolate from TSP patients will establish the similarity and differences between this isolate and HTLV-I. We are also utilizing the polymerase chain reaction (PCR) technique to evaluate various tissues from TSP patients for the presence of retrovirus infection.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05536-02 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immune Response to HIV: Neutralizing Antibody and Vaccine Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Robert-Guroff	Research Biologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	M. S. Reitz, Jr.	Research Chemist	LTCB NCI
	J. Goedert	Medical Officer	EEB NCI

COOPERATING UNITS *(if any)* Kumamoto Univ., Kumamoto, Japan (S. Matsushita); NY Hosp., Cornell Med. Ctr., NY, NY (P. Giardina); Univ. Med. Dent., Newark, NJ (J. Oleske); Repligen Corp., Cambridge, MA (S. Putney); Portuguese Inst. Oncologia, Lisbon, Portugal (E. Cardoso)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In studying immune responses to human immunodeficiency virus (HIV) infection, we have focused on neutralizing antibodies which we first detected in 1985. Initial studies in infected children and adults showed that neutralizing antibodies are associated with better clinical outcome. A 6-year prospective study has now found that such antibodies are correlated with long periods during which HIV-infected homosexual men maintain good health. Declining or low antibody titers indicate poor prognosis. Future studies of HIV seroconverters will determine if neutralizing antibodies retard disease onset or prolong survival, important information for vaccine development. The effect of HIV envelope heterogeneity on the elicitation and function of neutralizing antibodies is also being pursued. Investigation of an HIV variant, immune selected in vitro by culture of an infectious viral clone in the presence of neutralizing antibody, has shown that type-specific antibody can influence the propagation of mutant viruses. Molecular analysis of the variant revealed that a single amino acid substitution in the viral transmembrane protein conferred resistance to the selecting serum. Thus, conformational epitopes may contribute to neutralizing antibody development. In a separate study, a neutralizing monoclonal antibody localized a highly immunogenic epitope to 24 amino acids in the major envelope glycoprotein of HIV. Preparation of additional monoclonal antibodies will allow further mapping of important envelope epitopes and preparation of anti-idiotypic antibody as potential vaccine material. The simian immunodeficiency virus will be established in a Macaque model to further investigate both humoral and cellular immune responses following viral infection. The effect of double retroviral infections in human disease continues to be pursued.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Robert-Guroff	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
M. S. Reitz, Jr.	Research Chemist	LTCB NCI
J. Goedert	Medical Officer	EEB NCI

Objectives:

The objectives of this project are to learn what natural immune surveillance mechanisms operate in HIV-1-infected individuals, whether such natural responses can be manipulated to enhance protection against virus infection or disease progression, and how to construct vaccine preparations to result in such protective immune responses. As retroviral neutralizing antibodies are known to result in protection against a viral challenge in animal systems, we have initially focused on these antibodies. The specific goals of the present project are:

1. to determine to what extent HIV-1 neutralizing antibodies are protective or influence disease progression;

2. to determine what influence the known heterogeneity of the viral envelope has on elicitation and function of HIV-1 neutralizing antibodies;

3. to identify regions of the virus envelope important for viral infectivity and neutralization;

4. to determine the efficacy of various envelope preparations, including recombinant constructions, subunit fragments, and anti-idiotypic antibodies, as vaccine materials;

5. to probe immune responses and modes of transmission of other human retroviruses in order to apply knowledge gained to investigations on HIV, while further elucidating mechanisms of pathogenesis and protection in other retroviral systems.

Methods Employed:

The method for assaying sera for HIV-1 neutralizing antibodies has been described by M. Robert-Guroff *et al.* (Nature 316:7274, 1985). Long-term prospective studies of neutralizing antibodies in HIV-1-infected individuals were carried out on study subjects who had undergone detailed immunologic and clinical analyses for determination of disease progression. The principle study population analyzed was a cohort of 34 homosexual men, prospectively followed since 1982.

The ability of sera to neutralize the infectivity of the prototype HIV-1 isolate, HTLV-IIIB, as well as other isolates including HTLV-IIIRF, HTLV-IIIMN, HTLV-IIISC, HTLV-IIIWMJ2, HTLV-IIIRUT, and HTLV-III San Francisco isolate (SF2) was

assessed. An isolate of the simian immunodeficiency virus (SIV-K1W) and HIV-2 (NIH-Z) were also employed. Stable stocks of infectious preparations of these isolates were prepared and titered for use in comparative studies.

Routine serologic assays for antibodies to HIV-1 and other human retroviruses were carried out using the enzyme linked immunosorbent assay (ELISA) technique, Western blotting, radioimmunoprecipitation, and immunofluorescent assay.

In collaboration with Dr. Marvin Reitz, Jr. (ZO1CP05538-02 LTCB), molecular genetic analysis of an HIV-1 variant, immune selected in vitro by culturing in the presence of a neutralizing antibody-positive serum, was carried out. Following cloning of the variant virus, a recombinant containing the variant envelope in the parental virus background was constructed. The variant envelope gene was also sequenced, and site-directed mutagenesis was used to change the amino acid residue at position 582 in the parental virus to the one found in the variant. These molecularly engineered viruses were transfected into COS cells and subsequently used to infect H9 cells in order to obtain permanent virus-producing lines. The viruses produced by these lines were analyzed in the neutralizing antibody assay to determine which ones had altered neutralizing ability.

A monoclonal antibody to HIV-1 gp120 with high titer neutralizing activity was used to identify a major type-specific neutralizing epitope. These studies, in collaboration with Dr. Shuzo Matsushita, Kumamoto University, Japan, and Drs. Scott Putney and James Rusche of Repligen Corporation made use of subfragments of the PB1 region of the viral envelope. This region was already shown to be capable of eliciting neutralizing activity. The ability of the monoclonal to bind these subfragments resulted in identification of the type-specific epitope recognized by the antibody.

Cross-neutralization studies to identify immunologic subtypes of HIV-1 isolates involved analysis of goat sera obtained from animals immunized with native and recombinant envelope proteins and subfragments. The titer and breadth of neutralizing antibody activity of these immune sera were assessed.

Major Findings:

Results of a 6-year prospective study of HIV-1-seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods during which individuals remain disease free. This observation confirms and extends our earlier findings which suggested that in both adults and pediatric patients these antibodies are associated with better clinical outcome. This study, therefore, supports continued efforts to generate vaccines aimed at eliciting high-titer, broadly reactive neutralizing antibody but does not preclude an equally important role of cell-mediated immune responses in preventing infection or retarding disease progression.

The same prospective study indicated that declining or low neutralizing antibody titer signaled poor prognosis. The poor neutralizing antibody response of individuals who progressed to AIDS was not due to a lesser ability to generate

neutralizing antibodies capable of inhibiting widely diverse strains. We speculate that immune complexes may be responsible, in part, for the observed low titers.

In 1986, we reported the in vitro generation of an HIV-1 variant, obtained by culturing an infectious molecularly cloned virus in the presence of a neutralizing antibody-positive serum. Following cloning of the variant we showed that the resistance to neutralization by the selecting serum was due to a change in the envelope gene of the variant. Subsequent sequencing of the variant envelope localized the alteration to a single amino acid substitution at position 582 in the transmembrane protein. That this point mutation was, in fact, responsible for the altered neutralizing ability, was confirmed by making this change in the parental virus clone and illustrating that the altered parental virus possessed the same neutralization resistance as the original variant. These molecular analyses were carried out in collaboration with Dr. Marvin Reitz (ZO1CPO5538-02 LTCB). These experiments indicate that although sera from HIV-1-infected humans in general are broadly group-specific in their neutralizing antibody response, they possess type specificities which may influence the propagation of mutant viruses arising during the course of infection. Moreover, our data show that the viral transmembrane protein possesses determinants important for the neutralizing antibody response. Whether the amino acid substitution observed marks a distinct neutralization epitope, or whether it is part of a non-contiguous conformational epitope is not yet resolved. This system is now being exploited in order to elucidate additional envelope regions important for neutralization and ultimately important for vaccine development.

A major type-specific neutralization epitope was mapped to 24 amino acids within the exterior viral envelope protein (gp120) using a mouse monoclonal antibody specific for gp120. This epitope falls within the PB1 region, previously shown to possess a major immunogenic neutralizing domain. The monoclonal antibody used in these studies can inhibit HIV-1 infection by fusion with virus-infected cells as well as by cell-free virus, indicating that vaccines directed toward neutralization epitopes may be broadly protective. These experiments were carried out in collaboration with Drs. Shuzo Matsushita, Kumamoto University, Japan, and Scott Putney and James Rusche of Repligen Corporation, Cambridge, MA.

A further important finding with regard to future vaccine development was the observation that gp160, produced in the insect bacculovirus system, is immunogenic and can elicit high titer neutralizing antibody in inoculated goats. This material provides a possible source of vaccine material. These experiments were in collaboration with Drs. Scott Putney and James Rusche, Repligen Corporation, Cambridge, MA.

Collaborative studies with Dr. Ermelinda Cardoso, Oncology Institute, Lisbon, Portugal, demonstrated a double retroviral infection of HTLV-I and HIV-2 in an African woman from Guine Bissau, who to date remains healthy. The study illustrated that in areas where several retroviruses may be endemic, as Africa, serologic profiles may be difficult to interpret because of immunologic cross reactivities. Thus, more specific diagnostic tests need to be developed. Moreover, an extensive seroepidemiologic survey demonstrated that Portugal is not

a viral endemic area for HTLV-I. Thus, we could not conclude that Portuguese seamen introduced HTLV-I into Portugal or Japan, as has been speculated, in the 16th century.

A collaborative study with Dr. Brice Weinberg, Duke University, Durham, NC, revealed a cluster of HTLV-I infection in North Carolina. The virus apparently was transmitted as a result of both heterosexual contact and intravenous drug abuse. This result extends our previous findings of HTLV-I and HIV-1 double infections in drug abusers and suggests that this population should be followed closely to reveal possible clinical effects of dual retrovirus infection.

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Patents:

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Robert-Guroff M, Gallo RC. (Pending): A Method for Detecting HTLV-III Neutralizing Antibodies in Sera.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1CP05537-02 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopathogenesis of Human RNA and DNA Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. C. Saxinger	Research Microbiologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	F. Wong-Staal	Chief, Molecular Biology Section	LTCB NCI
	P. Levine	Medical Officer	EEB NCI

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Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Viral pathogenesis: Work on the chimpanzee human immunodeficiency virus (HIV) model has suggested new directions for approaches to intervention. Findings are that infection appears to progress by discrete stages which may be variably immunoregulated and that cofactors or cellular immunity, or target cell selection may be fundamental. Also in vitro tests of B- and T-cell immunosuppression by viral proteins and fragments produced by molecular biological techniques have been successful in the preliminary phase. Detailed characterization of mechanisms of immunosuppression are in progress.

U.S. human T-lymphotropic virus type-I (HTLV-I) prevalence: A retrospective random sampling of the U.S. population (HANES-II) and a retrospective geographic drug-abuser population have been tested for HTLV-I antibody. Analysis in progress will indicate frequency of infection and its rate of change in these populations.

Human B-lymphotropic virus (HBLV) prevalence: Enzyme linked immunosorbent assay (ELISA) tests have been successfully developed. Prevalence studies, geographically and epidemiologically oriented, have shown that immunoglobulin (IgG) reactivity to HBLV in normal adults is common (>80%) and that exposure to the virus takes place frequently within the first year after birth.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

W. C. Saxinger	Research Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
F. Wong-Staal	Chief, Molecular Biology Section	LTCB NCI
P. Levine	Medical Officer	EEB NCI

Objectives:

Immunological and Structural Characterization of HBLV

Development of a screening test for antibodies to HBLV in various human populations for studies of disease association. Identification of immunogenic viral proteins and characterization of human humoral response.

Modulation of T- and B-Cell Function by Viral Proteins

Characterization of stimulatory and suppressive effects on human lymphocytes produced by HTLV proteins. Comparison of various HTLV variants with respect to these characteristics in support of studies of mechanisms important in the pathogenesis and spread of HTLV.

Development of Antigen Test for HBLV

Development of a routine, sensitive procedure for detection and quantitation of viral antigens in serum and tissues in support of studies on the association of disease with HBLV infection.

Identification of New Virus Clusters in Humans and Primates

Identification of the environmental distribution of HTLV or of variant viruses related to HTLV in support of studies of mechanisms important in the pathogenesis and spread of HTLV.

Methods Employed:

A variety of immunochemical, immunological, and microbiological techniques are used. Antigen-antibody reactions are measured by standard and in-house developed ELISA, radioimmunoassay (RIA), Western blot, and a variety of other immunoprecipitation techniques. Purification of cellular and viral proteins is accomplished by a variety of chromatographic (gravity and high performance liquid chromatography [HPLC]), electrophoretic, and centrifugation techniques. Large-scale epidemiologic data analysis is performed using a lab-based personal computer coupled with an IBM mainframe system for demographic data entry and storage of immunological test data. Assays for cellular immunity and modulation are performed by standard in vitro tests for hematopoietic cell function by incorporation of radiolabelled, growth or specific plaque assays. Peptide syntheses are performed by standard (9-fluorenylmethyoxycarbonyl amino group

protection (FMOC) chemical procedures integrated with automation devices developed within LTCB.

Major Findings:

Modulation of T- and B-Cell Function by Viral Proteins

Reconstruction experiments with partially purified viral proteins, recombinant-derived viral proteins, and proteins derived from viral mutants with altered function have succeeded in producing in vitro effects resembling the in vivo pathology of human AIDS. Viral gp120 produces an immunosuppressive effect on normal T-cells and a sequence localized within the junction region between gp41 and 3'orf causes a form of B-cell suppression in which the production of IgG is polyclonally stimulated and the B-cells lose the ability to produce specific antigen. These effects suggest that immunosuppressive properties of the viral proteins may contribute, at least in part, to the pathology of HIV-1 infection.

HTLV-I Seroprevalence in the U.S. and Drug Abuser (DA) Populations

Testing of the HANES-II collection for HTLV-I antibody has been completed and the data are now being analyzed for prevalence in the U.S. This study should provide a baseline for HTLV-I seroprevalence in the U.S. as of 1976. Testing of drug abuser sera from geographically diverse regions in 1972 has been completed and the data are being analyzed for prevalence by age, race, sex, drug abuse habits, health status and geographic distribution. These data will provide a basis for assessing the rate of change of HTLV-I infection in this high-risk population from a period predating introduction of the AIDS virus. Analyses of the drug-abuser groups show that antibody reactivity to HTLV-I has existed at high levels in all geographic quarters of the U.S. since 1972, and is not found only in the Southeast as suggested by earlier studies. Overall 3% of white DAs and 9% of black DAs were positive. Race and age were the major risk factors for HTLV-I antibody positivity. These studies indicate that DAs should be investigated for increased incidence of leukemia and lymphoma related to HTLV-I. Analyses of the correlates of HTLV-I antibody in the HANES-II survey are nearly completed and suggest that the prevalence of HTLV-I antibody reactivity in the U.S. population are far greater than 1/10000 recently found in U.S. blood donors by the Red Cross. The latter number is probably an underestimate because the blood donor population underrepresents minority races and (older) age groups.

Distribution of HBLV in the Normal Population

Serologic tests for detection of anti-HBLV antibodies have been developed and applied to normal blood donor populations from Minneapolis, Minnesota and Kansas. Sensitive, quantitative data have been obtained from ELISA tests using either infected cells or purified virus. Some of the developments mark a major improvement in this type of testing, require very small amounts of infected cell material and will probably find wide application in other fields. The tests suggest relatively wide-spread distribution of the areas tested (>80%). Quantitative and qualitative parameters of immunological response are under investigation. These studies have so far shown that although low antibody titers are widespread, unusually high titer antibodies to Burkitt's lymphoma, acute lymphocytic leukemia (ALL), Hodgkin's lymphoma and chronic fatigue syndrome have

so far only been found in certain West African countries. Attempts to prepare proper immune sera in sheep and monoclonal antibodies against the virus are in progress. Sensitive tests for viral antigen have been constructed and are currently being used to investigate possible disease relationships with HBLV.

Publications:

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Patents:

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Saxinger C, Gallo RC. US Patent (Pending): Detection of Human T-Cell Leukemia Virus Type III.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1CP05538-02 LTCB

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October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure and Function of HIV Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Reitz	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	M. Robert-Guroff	Research Biologist	LTCB NCI
	M. Popovic	Research Biologist	LTCB NCI
	S. Gartner	Senior Staff Fellow	LTCB NCI
	E. Tschachler	Visiting Scientist	LTCB NCI
	H. Buchow	Visiting Scientist	LTCB NCI
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COOPERATING UNITS *(if any)*

None

LAB/BRANCH
Laboratory of Tumor Cell Biology

SECTION
Molecular Genetics of Hematopoietic Cells

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Comparative studies on the genomes of human immunodeficiency virus type-1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV) have been facilitated by obtaining the complete nucleotide sequences of an SIV isolate from a macaque and several HIV-2 isolates from persons with immunodeficiency. These all are related to HIV-1, about 55% by overall nucleotide sequence homology, and to each other to about the same extent as HIV-1 isolates are related to each other (70-85%). Conservation of certain areas of the genomes of the two groups of virus are helping to identify those which are functionally critical. Based in part on these analyses, mutants of HIV-1 have been made and are being studied. These include mutations at the envelope polyprotein cleavage site (which in preliminary results result in loss of infectivity), within the integrase gene (which also have resulted in loss of infectivity), and at various cysteine residues in the envelope proteins (some of which have resulted in a loss of infectivity). Another project has involved the selection in vitro by neutralizing antisera of HIV-1 variants which resist neutralization by the selecting antiserum. DNA sequence analyses, construction of viral chimeras, and site-specific mutagenesis of one such variant showed resistance was due to a single amino acid substitution in the transmembrane protein. This has obvious implications for vaccine design. A third project is to identify the genetic determinants which give some strains of HIV-1 the ability to grow on macrophages as well as T-cells. A DNA clone of a macrophage tropic HIV-1 was obtained and completely sequenced. This was used to generate a series of viral chimeras containing different genomic regions derived from the macrophage tropic parent. These are currently being analyzed for their biologic properties.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Reitz	Research Chemist	LTCB NCI
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M. Popovic	Research Biologist	LTCB NCI
S. Gartner	Senior Staff Fellow	LTCB NCI
E. Tschachler	Visiting Scientist	LTCB NCI
H. Buchow	Visiting Scientist	LTCB NCI
H.-G. Guo	Visiting Scientist	LTCB NCI

Objectives:

This work is an effort to understand how structural aspects of the genes of HIV relate to viral biology, including different steps in replication, cell tropism, and interaction with the host immune system. Much of this work concerns the envelope gene and its relati^R
on to the above properties.

Methods Employed:

Standard techniques of molecular biology and virology are being used to pursue these investigations.

Major Findings:

In Vitro Immunoselection of HIV-1 Variants

One series of studies involved a variant HIV-1 derived from a biologically active molecular clone by transmission and cultivation in the presence of a neutralizing antiserum. The variant was resistant to neutralization by the same antiserum. By a combination of DNA cloning and sequence analysis, construction of chimeric viruses, and site-specific mutagenesis, we were able to show that a single substitution of threonine for alanine at amino acid position 582, in the transmembrane protein, was sufficient to confer the neutralization-resistant phenotype. We have made other mutations in the same region and are currently testing them to try to better define the site recognized by the neutralizing antibody, and are also testing synthetic peptides from this region. This site is of particular interest since it is present in many divergent strains of HIV-1.

Genetic Determinants for Macrophage Tropism

We have obtained a molecular clone containing the 3' half of a provirus representing a macrophage tropic HIV-1 and determined the DNA sequence. The organization of the viral genome is the same as for T-cell tropic HIV-1 isolates, and the sequence divergence between this isolate and other United States T-cell tropic HIV-1 isolates is no different in kind or degree than that seen among different T-cell tropic HIV-1 isolates. The genetic differences allowing growth in macrophages must therefore be rather small. We have constructed different

chimeric proviruses by inserting parts of the genome from the macrophage tropic virus into the biologically active clone of the T-cell tropic virus. These include part or all of the gp120, the amino terminal 250 amino acids of the gp41, and tat and trs. The biological properties of these chimeric viruses are currently under study.

Generation and Characterization of HIV-1 Point Mutants

We have made and started to characterize several kinds of mutants of HIV-1. Based on our comparisons of the primary sequence of different HIV-1 isolates, SIV, and various HIV-2 isolates, the 22 cysteine residues of the HIV-1 envelope are completely conserved in all isolates, suggesting that all are functionally important. We have made 14 mutants, each of which have a different cysteine replaced by another amino acid. Seven of eight such mutants lack the ability to infect H9 target cells, confirming the suspected importance of cysteine residues. The exact steps in infection which are blocked in these mutants are under investigation.

Another series of mutations are being made to characterize the envelope polyprotein cleavage site. Changing the lysine at position 510 to a leucine abolishes both envelope cleavage and infectivity. Other mutations are under construction to define the protease recognition site.

A third kind of mutation which is being made and studied is in the coding region for the endonuclease which is important for proviral integration into the host cell genome. Unintegrated DNA is a hallmark of HIV-1 infection, and it is not clear whether or not integration is necessary for infectivity. One of these mutants has a stop codon introduced midway through the integrase coding region. This mutant produces a virus particle which contains reverse transcriptase, and it apparently synthesizes unintegrated viral DNA in H9 target cells. Infection does not, however, seem to be productive, in that virus expression or spread in the target cells has not been noted.

Publications:

Franchini G, Gurgo C, Gallo RC, Collalti E, Guo HG, Fargnoli KA, Hall LF, Wong-Staal F, Reitz MS Jr. Nucleotide sequence of the simian T-lymphotropic virus type III and its relatedness to the human immunodeficiency viruses (HIV-1 and HIV-2). Nature 1987;328:539-43.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05539-02 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mapping of the Regulatory Genes and Elements of Human Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. K. Arya	Research Biologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	A. Sethi	Microbiologist	LTCB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human immunodeficiency virus type-1 (HIV-1) and HIV-2 may comprise a spectrum of human retroviruses with varying patho-genic potential in vivo. This pathogenic potential may, in part, be governed by the genetic structure of these viruses. This project involves the comparative analysis of the structure and functions of HIV-1 and HIV-2 genomes with focus on their regulatory genes and elements. Like HIV-1, various isolates of HIV-2 contain functional tat gene and tat response elements as determined by molecular cloning of the functional tat genes and long terminal repeats (LTRs), and DNA-mediated transfection analysis. Whether there are subtle and quantitative differences in the trans-activation capacities of HIV-1 and HIV-2 tat genes is being determined. Detailed insertion and deletion mutation analysis has revealed multiple regulatory elements in the HIV-2 LTR. This LTR contains positive regulatory elements upstream of the transcription initiation site and a complex tat response element down-stream of the transcriptional initiation site. There is evidence for the presence of a downstream negative element in this LTR which, if confirmed and when fully analyzed, may prove to be significant for regulation of HIV-2 gene expression. Potentially relevant to virus latency and disease progression, HIV-1 and HIV-2 gene expression can be stimulated by T-cell activation signals or immune activation and by heterologous transactivation such as human T-lymphotropic virus type-I (HTLV-I) and cytomegalovirus (CMV).

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

S. Arya	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
A. Sethi	Microbiologist	LTCB NCI

Objectives:

The major objective of this project is to comparatively analyze the structure and functions of the regulatory genes of HIV-1 and HIV-2, particularly those HIV-2 isolates that are apparently less pathogenic. An additional objective is to investigate the factors governing the translational efficiency of HIV-1 and HIV-2 transcripts.

Methods Employed:

Molecular cloning in expression vectors, DNA sequencing, DNA-mediated transfection analysis, transcriptional and translational analyses and other standard methodologies of gene cloning and recombinant DNA technology.

Major Findings:

Regulatory Genes and Elements of HIV-1 and HIV-2

HIV-1 is etiologically associated with acquired immune deficiency syndrome (AIDS). More recently, new human retroviruses (termed HIV-2) have been isolated from sick and healthy individuals resident in West Africa. Some of these HIV-2 isolates may be less pathogenic in the natural host. Thus, HIVs may comprise a spectrum of viruses with varying degrees of pathogenicity. Our studies explore the premise that the pathogenic potential of these viruses is, at least in part, governed by their genetic structure and that viral gene expression and replication underlies pathogenesis. HIVs contain several regulatory genes which up-modulate (e.g., *tat*), down-modulate (e.g., 3'*orf*, *trs*) or ensure proper (e.g., *trs*) viral gene expression. It is possible that up-modulator genes are weaker and/or down-modulator genes are stronger in less pathogenic HIVs. Thus, we are comparatively analyzing the structure and functional capacities of these genes and of the LTR regulatory elements of HIV-1 and HIV-2, particularly the HIV-2 (strain ST) isolate obtained from a healthy individual and which displays much diminished cytopathic effects in vitro. We have determined that, like HIV-1, all of the HIV-2 isolates tested contain a functional *tat* gene and *tat* response elements. We have undertaken a detailed insertion and deletion mutant analysis of the regulatory elements of HIV-2 LTR and have found that HIV-2 LTR contains positive regulatory elements upstream of the transcriptional initiation site. In addition, we defined the *tat* response element which is more complex than similar elements in HIV-1 LTR, and found that there is some evidence that HIV-2 LTR contains negative regulatory element(s) downstream of the transcriptional initiation site. The significance of this downstream negative regulatory element in controlling viral gene expression, particularly in human $T4^+$ lymphocytes,

is under investigation. We are now focusing on the detailed analysis of the tat and particularly of the 3'orf genes of HIV-2 to determine if the tat gene of HIV-2(ST) is weaker and the 3'orf gene of HIV-2 (ST) is stronger than the same genes of HIV-1. In addition, we are analyzing the response of HIV-1 and HIV-2 LTR regulatory elements to T-cell activators (phytohemagglutinin [PHA] and peanutagglutinin [PNA]) and heterologous transactivators (HTLV-I and CMV). This may have a bearing on viral latency, full expression of pathogenic potential, and progression towards clinical disease.

Translational Controls in HIV-1 and HIV-2

We have previously shown that the chronically HIV-1 infected cells contain transcripts with multiple open reading frames (orf). For example, the sor cDNA clones contain the orf for not only sor but also for tat, trs and 3'orf. This transcript exists in the presence of other transcripts containing one or a fewer number of the same orfs. Recent cDNA cloning of HIV-2 has revealed similar transcripts for HIV-2. Is the existence of the transcripts of varying complexities related to the demands of virus replication, status of virus infection and viral latency, and how the actual translation of a given orf from multiple transcripts is attained? We have shown that different orfs carried on a given transcript are translated with unequal efficiency. We are now determining the factors which govern the translational efficiency of HIV-1 and HIV-2 transcripts, with a particular focus on the structure of the inter-orf untranslated sequence elements.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05560-01 LTCB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Induction of Lymphotoxin Expression by HTLV-I Infection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Reitz	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	E. Tschachler	Visiting Scientist	LTCB NCI

COOPERATING UNITS *(if any)*
None

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adult T cell leukemia, which is caused by human T-lymphotropic virus type-I (HTLV-I), is frequently characterized by severe hypercalcemia and lytic bone lesions. The lymphokine lymphotoxin, synthesized by T-cells, has among its activities the ability to activate osteoclasts. We therefore looked at HTLV-I-infected T-cells for evidence of lymphotoxin expression. High levels of constitutive lymphotoxin expression were noted in all HTLV-I-infected cells, but not in uninfected T-cells or those infected by human immunodeficiency virus (HIV-1).

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Reitz	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
E. Tschachler	Visiting Scientist	LTCB NCI

Objectives:

The major objective of this work is to understand the molecular mechanism of hypercalcemia in adult T-cell leukemia (ATL). We also wish to identify cellular factors important in lymphokine expression which are also important in virus expression.

Methods Employed:

Standard molecular biological recombinant DNA methods are being used in these investigations.

Major Findings:

Induction of Lymphotoxin Expression by HTLV-I

We have looked at a series of T-cell lines for expression of lymphotoxin and tumor necrosis factor (TNF). These cell lines include uninfected cell lines, cell lines infected by HIV-1, and cell lines established either by in vitro HTLV-I infection or directly from ATL patients. All cell lines infected with HTLV-I constitutively expressed high levels of lymphotoxin RNA, protein, and activity. Many also expressed TNF. In contrast, none of the other cell lines, whether uninfected or infected by HIV-1, expressed detectable levels of either lymphokine. A functional helper T-cell clone expressed no detectable lymphotoxin before infection with HTLV-I; after infection, high levels of expression were noted. We are currently analyzing the lymphotoxin gene for tissue-specific enhancer activity to identify any factors important in the activation of both virus and lymphotoxin expression.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP07148-05 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on T-Cell Malignancies, Lymphomas and AIDS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. C. Gallo	Chief	LTCB NCI
Others:	S. Z. Salahuddin	Cancer Expert	LTCB NCI
	S. Nakamura	Visiting Scientist	LTCB NCI
	P. Lusso	Visiting Fellow	LTCB NCI
	S. Sakurada	Guest Researcher	LTCB NCI
	P. Biberfeld	Guest Researcher	LTCB NCI
	W. Blattner	Chief, Viral Epidemiology Section	EEB NCI
	D. Ablashi	Senior Investigator	LCMB NCI

COOPERATING UNITS *(if any)*

Imperial Cancer Research Fund, London, England (Robin Weiss); Duke University, Durham, NC (Bart Haynes); M.D. Anderson Hospital and Tumor Institute, Houston, TX (Kenneth McCredie); Harvard University, Boston, MA (Myron Essex)

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Hematopoietic Cellular Control Mechanisms

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.0	3.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell biology studies have focused on the role of human T-lymphotropic retroviruses (HTLV) in human T-cell malignancies and acquired immunodeficiency syndrome (AIDS), and a human B-lymphotropic DNA virus (HBLV, human herpesvirus, HHV-6). HTLV-I is a transforming virus, whereas human immunodeficiency virus (HIV-1) is cytopathic and kills the cells it infects. HTLV-I, HTLV-II and HIV-1 have specificity for OKT4-positive T helper cells. The involvement of these viruses in neuropathy is being examined. HIV-1 has been shown to be associated with cells of monocyte-macrophage lineage. HIV-1 isolates obtained from different patients show genetic variations in the envelope region. Long-term cell cultures have been obtained from lung tissues and pleural effusions of AIDS patients with Kaposi's sarcoma (KS). These cells are typical endothelial cells which excrete a variety of factors that can promote the growth of different cell types by autocrine and paracrine mechanisms. Isolation of HTLV-II from patients with prolymphocytic leukemia and hairy cell leukemia indicate that HTLV-II can infect both B- and T-cells, and a comparison of the genomes of the new isolates with prototype HTLV-II-MO indicates the presence of some heterogeneity among these isolates. HHV-6 has been shown to infect both B- and T-cells as well as megakaryocytes, and it appears that HHV-6 may be involved in immunosuppressive events associated with HIV-1.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

R. C. Gallo	Chief	LTCB NCI
S. Z. Salahuddin	Cancer Expert	LTCB NCI
S. Nakamura	Visiting Scientist	LTCB NCI
P. Lusso	Visiting Fellow	LTCB NCI
S. Sakurada	Guest Researcher	LTCB NCI
P. Biberfeld	Guest Researcher	LTCB NCI
W. Blattner	Chief, Viral Epidemiology Section	EEB NCI
D. Ablashi	Senior Investigator	LCMB NCI

Objectives:

It is anticipated that a greater understanding of the processes involved in the molecular control of cellular growth, differentiation, and the pathogenesis of human neoplasias, AIDS and Kaposi's sarcoma will lead to the ultimate goal of developing improved approaches to the prevention and therapy of human neoplasia.

Pursuing studies leading to an understanding of the origin of tumor viruses, how they acquire their oncogenic potential, how they interact with cells, and how they are transmitted throughout nature.

Leukocyte differentiation in vitro. Attempts are made to study exogenous and endogenous factors which affect the maturation and proliferation of normal and leukemic human bone marrow cells. The mechanisms involved in the maturation process are under study.

Growth of leukemic myeloblasts in liquid suspension and endothelial cells under the stimulus of factor(s) produced by human embryonic culture cells or HTLV-II-infected cells.

The distribution of human T-lymphotropic virus (HTLV) in human T-cell leukemia patients (HTLV-I, HTLV-II) and human immunodeficiency virus (HIV-1) from patients with acquired immunodeficiency syndrome (AIDS) from various parts of the world is being actively pursued.

A number of techniques are being explored for the production of an effective vaccine against AIDS. These include preparation of large quantities of gp160 and fragments of gp120 for potential use in the study of immune response in different animal species and the testing of these antibodies for their capacity to neutralize HIV-1 replication. Synthetic peptides corresponding to the HIV-1 p17 and gp120 sequences are also being evaluated for potential usefulness as a vaccine.

Methods Employed:

A variety of cellular biological, biochemical and virological techniques are employed in the processing and culture of lymphocytes, endothelial cells and brain cells from patients with leukemias, lymphomas and AIDS. Retrovirus expression in the cell cultures is measured by immunofluorescence using monoclonal antibodies, reverse transcriptase assays and by electron microscopy. Other techniques include the use of enzyme linked immunosorbent assays (ELISA), radioimmunoassays, Western blot analysis, neutralizing antibody assays, syncytia inhibition assays and inhibition of virus replication.

Major Findings:

Kaposi's Sarcoma

Long-term cell cultures were established from lung tissue and pleural effusion obtained from AIDS patients with disseminated Kaposi's sarcoma (KS). These cells were initiated in culture with the aid of a recently described endothelial cell growth factor(s). Morphological, biological, immunological, and cytochemical studies demonstrated that these KS-derived cells were similar to the characteristic endothelial-like (KSEL) spindle cells, prominent in KS lesions, and that they were possibly of nonvascular origin. These cells were found to produce factors with diverse biological activities including those supporting their own growth (autocrine) and that of normal endothelial and fibroblastic cells (paracrine). Condition media from KSEL cells also induced chemotaxis and supported chemoinvasion by other KSEL and normal vascular endothelial cells. These cells also contained and expressed a potent angiogenic activity, as detected by chorioallantoic membrane (CAM) and nude mouse assays. Furthermore, when inoculated subcutaneously into nude mice, these KSEL cells induced the development of lesions composed of murine cells with histological features similar to KS lesions. These observations suggest that AIDS-associated and possibly other types of KS may be initiated by cells with properties similar to the KSEL cells described here. The autocrine and paracrine activities of such cells may then progressively lead to the development of the characteristic KS lesions in a multifocal fashion. The cell culture systems described here should provide in vitro and in vivo models for the study of the pathogenesis of KS.

Human B Lymphotropic Virus (HBLV, Human Herpesvirus 6, HHV6)

The isolation of the novel human herpesvirus HBLV from patients with a variety of hematological disorders was first reported by us in 1986. Of the six original isolates, two were from human immunodeficiency virus-1 (HIV-1)-positive individuals, one with AIDS-KS and one with dermatopathic lymphadenopathy. Subsequently, other groups reported the isolation of a virus genetically identical to HBLV from African patients with AIDS. Their association, possibly coincidental with HIV-1-infected donors who develop AIDS, was extended to a larger population by seroepidemiological studies which included symptomatic and asymptomatic HIV-1-infected individuals. Approximately 70% of these donors, compared to an incidence of 24% in random control samples, were seropositive for HBLV. Similar results were obtained in a smaller study involving HIV-1-seropositive and -seronegative homosexual males.

While the concomitant infection by HIV-1 and HBLV clearly could be coincidental, other observations suggest a possible synergistic role for HBLV in AIDS. For example, the incidence of HBLV seropositivity is similar between symptomatic and asymptomatic donors, although the titer of antibodies to HBLV is consistently higher in patients developing AIDS. In addition, while the initial report of HBLV described its presence in B-cells from infected patients, recent in vitro studies have shown that HBLV can infect a number of other cell types. For example, we reported the infection of fresh peripheral blood lymphocytes with predominant T cell markers, including CD-4 surface antigen. This infection resulted in a dramatic cytopathic effect on these cells. Furthermore, a number of cell lines consisting of T- and B-lymphocytes as well as cells of other origin, e.g., megakaryocytes, could also be infected by HBLV. Many of these same cell lines are also susceptible to infection by HIV-1.

Detailed evaluation of fresh leukocytes and some established cell lines have further demonstrated that individual CD-4 positive T-cells can be infected in vitro by both HIV-1 and HBLV. As a consequence these cells appear to be killed at an accelerated rate compared to those infected by either of the viruses independently. This dual or co-infection of cells was also observed with some established cell lines. For example, the dual infection of a susceptible cell line by HIV-1 and HBLV results in an accelerated loss of viable cells. While these observations are consistent with a possible synergistic activity, the acute mechanisms involved are unknown. Since HBLV is cytopathic for CD-4 T-cells, infection by HBLV could contribute to the development of immune suppression by directly causing the death of CD-4 T-cells. Alternatively, it could contribute indirectly by activating or stimulating HIV-1 expression. It was observed that other human herpesviruses contain regulatory elements capable of interacting with HIV-1 long terminal repeat (LTR) affecting virus expression. Several observations suggest the possibility of HBLV involvement in immune suppressive events associated with infection by HIV-1. Further serological and virus isolation prospective studies should help clarify the possible role of HBLV as a co-factor in the pathogenesis of AIDS.

Isolation of HTLV-II from Patients with a Variety of Neoplastic Diseases

In contrast to HTLV-I there are only a few isolates of HTLV-II, and there is very little information about its incidence or disease association. One reason for the paucity of information on HTLV-II is the difficulty in distinguishing it from HTLV-I by routine serological tests. In a survey of sera from patients with various lymphoproliferative disorders, 20 patients with diseases not routinely ascribed for HTLV-I and HIV-1 were seropositive for HTLV-I/HTLV-II antibodies. Both HTLV-II and HIV-1 were subsequently isolated by passage onto selective target cells. For example, HTLV-II infected selected B-cells as well as T-cells, whereas HIV-1 did not infect the B-cells used. HTLV-II was also isolated from one patient with prolymphocytic T-cell leukemia and from three patients with hairy cell leukemia. Comparison of the restriction enzyme maps of three of these isolates with that of the prototype virus HTLV-II_{Mo} revealed some heterogeneity among the HTLV-II genomes. The findings indicate that HTLV-II is more widely disseminated than previously appreciated; its association with malignancy is not limited to hairy cell leukemia of the T-lymphocyte type; it is B-lymphotropic as well as T-lymphotropic; and it exhibits greater heterogeneity than HTLV-I but less than HIV-1.

Publications:

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Patents:

Ablashi, DV, Salahuddin SZ, Gallo RC. (Pending): Infection of Transmission of HBLV into Glioblastoma and Megakarocytes.

Gallo RC, Markham PD. (Pending): Animal Model, Hylobates Lar for Vaccine/ Infection Study of Human Immunodeficiency Viruses and Related Viruses.

Salahuddin SZ, Nakamura S, Gallo RC. (Pending): Growth Factors and Kaposi's Sarcoma Derived Cloned Cell Lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP07149-05 LTCB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biological Studies on Human Pathogenic Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. Wong-Staal	Research Microbiologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	S. Josephs	Research Chemist	LTCB NCI
	M. R. Sadaie	Senior Staff Fellow	LTCB NCI
	J. Rappoport	Staff Fellow	LTCB NCI
	S. J. Lee	Visiting Fellow	LTCB NCI
	G. Franchini	Guest Researcher	LTCB NCI
	M. L. Bosch	Guest Researcher	LTCB NCI

COOPERATING UNITS *(if any)*

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (R. Franza); Duke University, Durham, NC (W. Greene); Walter Reed Army Institute for Research, Washington, DC (R. Redfield)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Molecular Genetics of Hematopoietic Cells

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
10.0	5.0	5.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are pursuing several broad areas relating to pathogenic human viruses, principally the T-lymphotropic retroviruses and a new DNA herpesvirus, human B-lymphotropic virus (HBLV). There are two distinct groups of human retroviruses: the leukemia viruses (human T-lymphotropic virus type-I [HTLV-I] and HTLV-II) and the human immunodeficiency viruses (HIV-1 and HIV-2). In the past, complementing LTCB's pivotal discovery of HTLV-I and -II, we have contributed to the molecular analysis of these genomes. The major findings can be summarized as follows: (1) all adult T-cell leukemia (ATL) cells contain monoclonally integrated HTLV-I; (2) the site of provirus integra-tion is different from patient to patient, suggesting a transacting mechanism for transformation; and (3) presence of a conserved gene, tax, responsible for transcriptional activation. In collaboration with Warren Leonard (NICHD) and Warner Greene's (Duke University) laboratories, we demonstrated that HTLV-I tax turns on expression of interleukin 2 (IL-2R) and IL-2 in T lymphocytes. The target sequences for tax are distinct from those for antigen/mitogen activation. The major efforts of our group at present are directed at studies on the HIVs. The following areas are addressed: (a) analysis of structure and function of the HIV-1 genome, with emphasis on the novel accessory genes of this virus; (b) analysis of the env gene, in detail, to define epitopes for neutralization, T4 binding, and viral cytopathic effect (CPE). Of relevance is our group's first demonstration of conserved and non-conserved domains in env; (c) molecular approaches to vaccine development. This work is currently carried out in collaboration with several industrial groups; and (d) comparative analysis of the new virus subgroup HIV-2 and the related simian virus, simian T-lymphotropic virus type-IIII (STLV-III).

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

F. Wong-Staal	Research Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
S. Josephs	Research Chemist	LTCB NCI
B. Ensoli	Research Microbiologist	LTCB NCI
M. R. Sadaie	Senior Staff Fellow	LTCB NCI
J. F. Rappoport	Staff Fellow	LTCB NCI
M. L. Bosch	Guest Researcher	LTCB NCI
S. J. Lee	Visiting Fellow	LTCB NCI
S. Colombini	Guest Researcher	LTCB NCI
G. Franchini	Guest Researcher	LTCB NCI
C. Gurgo	Guest Researcher	LTCB NCI
I. Tschachler	Guest Researcher	LTCB NCI
J. F. Zagury	Guest Researcher	LTCB NCI
J. Yourno	IPA	LTCB NCI
A. Buchbinder	Clinical Associate	COP NCI
S. Broder	Associate Director	COP NCI

Objectives:

The major objectives of this project are: (1) analysis of structure and function of the HIV-1 genome with emphasis on the novel accessory genes of this virus; (2) analysis of the HIV-1 env gene, in detail, to define epitopes for neutralization, T4 binding and viral cytopathic effect (CPE); (3) molecular approaches to vaccine development; (4) comparative analysis of the new virus subgroup HIV-2 and the related simian virus STLV-III; (5) molecular cloning and structural analysis of human B lymphotropic virus (HBLV); and (6) molecular mechanism of pathogenesis of Kaposi's sarcoma.

Methods Employed:

Standard virological, molecular biological and recombinant DNA techniques of gene cloning, nucleotide sequencing transfection, nucleic acid isolation, restriction enzyme mapping, Southern and Northern blotting, molecular hybridization and in situ hybridization are utilized in these studies.

Major Findings:

Transfection/Mutagenesis

The availability of full-length molecular clones of HIV which, upon introduction into human T-cells, give rise to replicating cytolytic virus, has provided a model system in which to systematically appraise the functions of different components with the viral genome. The main interests of our group have been to determine which of the multiple open reading frames of the virus are used to encode proteins critical for replication and in vitro cytopathology. In addition to the gag, pol, and env genes (which encode structural components of the

retrovirus), the HIV genome contains at least five other genes: vip, tat, rev, vpr and nef (previously named sor, tat, trs, R and 3'orf). Studies of the transactivator gene of HIV (tat) have shown that this gene is an absolute requirement for virus replication. Mutants in which the coding region of this gene was removed or the splice acceptor (used to generate nature tat mRNA) deleted, were unable to generate virus, or only able to produce low levels of virus, respectively. These defects, we found, could be corrected (at least transiently) by providing a functional tat in trans or the tat protein itself. The functional domain of tat has been localized by site-directed mutagenesis to a central core region that is rich in cysteine residues. This core sequence resembles the metal binding finger proteins which are known to bind nucleic acids in regulating gene expression. We have also shown, by in vitro transcription systems, that tat acts at the transcriptional level.

To further define the functions of the tat and rev genes, translational stop codons were independently generated in the 5' portion of each gene. We have focused on the first coding exon of both genes since this exon has been shown to be necessary and sufficient for tat function.

We evaluated the mutants using various parameters for virus expression and provided definitive evidence that tat has a positive transacting role and is required for transcriptional activation. A chain-terminating mutation early in the rev gene resulted in an increase in transcription of viral mRNA measured by nuclear run-on experiments, but only one major species of viral mRNA (1.8kd) was detected. As a result, little or no viral structural proteins were made. Thus, the rev gene product may have a negative trans-regulatory role in transcription when overexpressed but is essential for expression of viral proteins by promoting accumulation of unspliced mRNA. Recent data suggest that rev may selectively stabilize unspliced mRNA as well as transport it into the cytoplasm.

The role of the nef gene in virus production was investigated. We showed that a major coding region of the nef gene could be removed without adversely affecting the replicative capacities of the virus or its ability to kill human lymphocytes. In fact, the replicative potential of nef mutants appeared higher than that of the wild type. Recent data from other groups suggest nef is a GTP-binding protein and may down-regulate viral as well as cellular genes by signal transduction.

Analysis of the mutant (10-1) in which deletion in the nef extended into the carboxyl terminus of the gp41 (the "small" or transmembrane domain of the HIV envelope) revealed a mutant genome which replicates efficiently but produced virus which was severely compromised in its ability to kill lymphocytes. We suggested that either the carboxyl terminus of the gp41 (the last five amino acids) was directly contributing to in vitro cell killing by HIV, or the modification introduced in gp41 was affecting envelope stability/structure so as to diminish its cytolytic properties.

Studies directed at defining the role of the vip gene which lies between the pol and tat genes of HIV and encodes a 23kd protein have been carried out. We have shown that this gene is crucial in producing infectious virus capable of cell-free transmission; mutants deprived of vip could be transmitted, albeit inefficiently, by coculture with $T4^+$ target cells but failed to establish stable

infection when transmitted in a "cell-free" fashion. Since cells transfected with vip mutant genomes produce normal levels of viral RNA, structural proteins, tat and HIV particles, we currently suspect that the vip gene exerts its effect on virus replication at a post-translational level (perhaps by enhancing assembly of infectious particles). We are also investigating the possibility that vip is a structural component of the virion which acts as a second envelope required for efficient transmission.

DNA/Protein Interactions

A new assay has been developed in collaboration with Dr. Robert Franza at Cold Spring Harbor Laboratory for microscale analysis of DNA binding proteins. The assay involves biotinylation of sequences known to have specific biological importance. The derivitized oligomers are then added to labeled nuclear extracts pretreated with poly GC competitor (to reduce background) and the protein-DNA complex is then bound to streptavidin agarose beads which are centrifuged and washed. The proteins are then visualized after sodium dedocyl sulfate (SDS) polyacrylamide gel electrophoresis and autoradiography. Competition experiments and DNA binding to proteins transferred to nitrocellulose are techniques used to confirm the specificity of binding. We have demonstrated that a protein of 86kd specifically binds to the enhancer of HIV. This protein is constitutively expressed in H9 cells and can be induced by phytohemagglutinin (PHA) stimulation of Jurkat cells. The protein is a candidate for the transcription factor activity as reported by Nabel and Baltimore (Nature 326: 711-13, 1987). Collaborations with Dr. Warner Greene of Duke University have attempted to localize the mitogen response elements within the HIV long-terminal repeat (LTR) in order to address the question of what cellular control events are responsible for HIV latency. These studies show the presence of sequences upstream of -279 which are responsible for a negative regulatory effect by increased mitogen response in LTR-chloramphenicol acetyl transferase (CAT) plasmids deleting this 5' region of the HIV LTR. Sequences homologous to other mitogen inducible genes, such as IL-1, may play a role in the inducibility of the HIV LTR since their deletion diminishes such response. The HIV enhancer and Sp-1 binding sites endow mitogen inducibility in plasmids containing these upstream regulatory regions.

Type-Specific Neutralization Epitopes on Cloned HIV Viruses

One strategy for immunoprophylaxis against acquired immunodeficiency syndrome is the administration of an immunogen intended to elicit protective virus neutralizing antibodies. Neutralizing activity (NA) against HIV-1 has been documented by many investigators using a variety of techniques. Domains within the HIV-1 envelope protein (gp120) and the transmembrane glycoprotein (gp41) have been shown to elicit or absorb NA. Sera from infected patients generally neutralize a broad range of isolates. In contrast, antisera produced in experimental animals by immunization with gp120 or recombinant or synthetic peptides display restricted capacity to neutralize isolates other than that from which the immunogen was derived. Recent observations indicate that individual isolates of HIV-1 are composed of populations of genetically and biologically distinct variants. To determine the implications of such heterogeneity for vaccine development and evaluation, we compared the susceptibility of three viruses molecularly cloned from HTLV-IIIB (HIV-I/NIH/USA/1983/HTLV-IIIB) to the neutralizing effects of a number of antisera.

The results showed that either patients' sera or hyperimmune sera raised against native gp120 or recombinant peptides derived from gp120 of a specific DNA clone from HTLV-IIIB can show close to two log differences neutralization titer against the three cloned viruses. Our data confirm that a single amino acid change in the virus envelope may result in profound changes in recognition by neutralizing antisera, and extend the previous concept of type-specificity of neutralization to differing variants of a single isolate. This suggests that the group-specific NA of some human sera could be directed against a large number of type-specific determinants as well as conserved epitopes. This could have important implications for vaccine development. Inability to demonstrate protection by candidate vaccines after challenge with homologous virus isolates could represent the selection of minor clonal variants not well recognized by the immunized host. Only the use of cloned HIV-1 variants will permit direct evaluation of the hypothesis that neutralizing antibodies are capable of protecting against HIV-1 infection.

Comparative Analysis of HIV-2 and SIV Isolates

Complete nucleotide sequences were obtained for two independent isolates of HIV-2 and one isolate of simian immunodeficiency virus (SIV_{MAC}). Furthermore, infectious clones of these viruses were obtained and cloned viruses inoculated into monkeys. These reagents would be very important for developing an animal model for an HIV vaccine. We have also obtained clear evidence that HIV-2 and SIV contain a novel protein which is virion-associated. This protein (vpx) may be useful as a discriminating reagent for HIV-2 infection.

Mechanisms of Pathogenesis of Kaposi's Sarcoma (KS)

In collaboration with the LTCB cell biology group, who has established in vitro cultures of endothelial cells from KS patients, we have examined the expression of cytokines which may account for the maintenance and expansion of KS cells in vivo. We found a high level expression of several growth factor genes, which can fully account for both autocrine and paracrine modes of growth of cells comprising a typical KS lesion. This result has shed some light on the mechanism of pathogenesis in KS.

Molecular Cloning and Restriction Enzyme Mapping of HBLV

We have now purified the human B-lymphotropic virus DNA by initially banding the virus on sucrose gradients (1.13 to 1.17 gm/ml) followed by gentle lysis and banding on cesium chloride gradients either with or without ethidium bromide. The quantity of virus available has increased due to the ability to grow the virus in susceptible cell lines. Yields of viral DNA from 2.5 to 10 liter cultures are now in the range of 20 ug by these procedures. We have used the HBLV clone, ZVH14, which was obtained by screening DNA obtained from crude virus preparations, to assay for HBLV during purification procedures.

We are screening three plasmid libraries which were prepared from CsCl banded virus DNA. We have obtained over 200 clones which are now being characterized by restriction analyses, gene walking and sequencing to map and analyze the entire HBLV genome.

Numerous probes which cross-react among the previously identified herpesviruses have not hybridized to HBLV DNA with one exception, the Marek's disease virus (MDV). DNA fragments of HBLV, which hybridize to specific fragments of MDV supplied by our collaborator, Dr. Nonayama of Showa Biomedical Research Institute in Florida, have been cloned. We are now defining, in detail, the regions of homology by sequence analyses. Sequencing of the genomic clone, ZVH14, has revealed an open reading frame of 1113 amino acids. This is a candidate open reading frame for the HBLV envelope glycoprotein. Studies of the expression of this open reading frame are being pursued.

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Patents:

Fisher AG, Ratner L, Gallo RC, Wong-Staal F. US Patent (Pending): Non-cytopathic Clone of Human T-Cell Leukemia Virus Type III.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-02 LTCB, Z01CP07148-05 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3711-00)

Title: Procurement of Fresh Cells from Monocytes, Macrophages, and T- and B-Cell Lines

Current Annual Level: $176,869

Man Years: 1.76

Objectives: This contract provides supportive services in the supply of small quantities of T- and B-cells grown in tissue culture, partially purified IL-2, and radiolabelled cells and nucleic acids.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-02 LTCB, Z01CP05535-02 LTCB, Z01CP05536-02 LTCB, Z01CP05539-02 LTCB, Z01CP07148-05 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3725-00)

Title: Preparation and Purification of Viral Components

Current Annual Level: $205,068

Man Years: 1.20

Objectives: The major objectives of this contract are to prepare and supply large quantities of concentrated and purified human type C RNA tumor viruses.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05534-02 LTCB, Z01CP05535-02 LTCB, Z01CP05536-02 LTCB, Z01CP07148-05 LTCB

BIONETICS RESEARCH, INC. (NO1-CP8-7213-00)

Title: Provision of Animal Facilities and Performance of Routine Experiments and Tests

Current Annual Level: $395,118

Man Years: 2.38

Objectives: This contractor provides animal facilities to house small and large animals, including rats, rabbits, goats and monkeys. The animals are used for the preparation of antibodies as well as for inoculation of tumor cells and virus preparations for tumorigenicity testing.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05535-02 LTCB, Z01CP07148-05 LTCB

BIOTECH RESEARCH LABORATORIES, INC. (NO1-CP7-3724-00)

Title: Preparation and Supply of Fresh and Cultured Mammalian Cells

Current Annual Level: $138,843

Man Years: 1.57

Objectives: This contract supplies well-characterized normal and neoplastic mammalian tissue culture cells and receives, processes, and distributes fresh human leukemic cells and tissues. Complete records are maintained on all biological materials handled under this contract.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05535-02 LTCB, Z01CP05536-02 LTCB, Z01CP05537-02 LTCB, Z01CP07148-05 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3722-00)

Title: Provision of Tissues and Cells and Conduct of Routine Tests in Support of Tumor Cell Biology Studies

Current Annual Level: $712,592

Man Years: 4.91

Objectives: The major objectives of this contract are: (1) the use of immunofluorescence and radioimmunoassays to screen human T-cells and T-cell lines for viral structural protein expression; (2) the use of ELISA assays to detect antibodies against human retroviruses in serum from leukemia patients, AIDS and ARC patients, and normal donors; (3) to test culture fluids from short-term and long-term cultured cells for the presence of viral DNA polymerase activity; and (4) to test sera from patients with T-cell leukemia for antibodies to human type C RNA tumor virus (HTLV-I, -II, and -III).

CONTRACT IN SUPPORT OF PROJECTS Z01CP05536-02 LTCB, Z01CP05537-02 LTCB, Z01CP05538-02 LTCB, Z01CP05560-01 LTCB, Z01CP07148-05 LTCB, Z01CP07149-05 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3723-00)

Title: Provision of Hematopoietic Cell Cultures, Growth Factors, and Type C Virus Proteins

Current Annual Level: $620,006

Man Years: 4.06

Objectives: The major objectives of this contract are: (1) to purify and supply factors that promote growth and differentiation of myelogenous leukemic cells and T-cells; (2) to purify the envelope and internal structural proteins of human and nonhuman primate type C RNA tumor viruses; (3) to prepare monoclonal antibodies against the purified structural proteins; (4) to provide cultured T- and B-cells from human cord blood, peripheral blood and leukemic ells; and (5) to prepare and supply radiolabelled cDNA and RNA probes from type C retroviruses.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05535-02 LTCB, ZO1CP05537-02 LTCB, ZO1CP05539-02 LTCB, ZO1CP07149-05 LTCB

BIOTECH RESEARCH LABORATORIES, INC. (NO1-CP6-7731-00)

Title: Support Services in Virology, Tissue Culture, and Immunology

Current Annual Level: $316,323

Man Years: 3.37

Objectives: This contract is to provide supportive services in virology, immunology, and tissue culture. At the present time, these functions include: (1) detailed karyotypic analysis, including Giemsa banding; (2) evaluation of tumorigenicity of various cultured cells by inoculation into nude mice; (3) preparing small quantities of selected cells and retroviruses; (4) testing various tissue cultured cell specimens for mycoplasma contamination; and (5) analyses of sera for HTLV-I or -III related antibodies in sera of patients and normal donors by ELISA and Western blotting techniques.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05537-02 LTCB, Z01CP05538-02 LTCB, Z01CP05560-01 LTCB, Z01CP07148-05 LTCB, Z01CP07149-05 LTCB

BIONETICS RESEARCH, INC. (N01-CP8-7214-00)

Title: Provision of Purified AIDS Virus, Proteins and Subhuman Primate Facilities to Test Immune Response of Viral Antigens

Current Annual Level: $815,302

Man Years: 6.20

Objectives: The major objective of this contract is to supply purified HIV glycoprotein gp160 and gp120 for AIDS vaccine studies.

CONTRACT IN SUPPORT OF PROJECTS Z01CP05538-02 LTCB, Z01CP05539-02 LTCB, Z01CP05560-01 LTCB, Z01CP07149-05 LTCB

REPLIGEN CORPORATION (NO1-CP7-1129-00)

Title: Preparation of Antisera Retrovirus Pool Antigens and Other Viral Components of HTLV-III/LAV

Current Annual Level: $400,000

Man Years: 4.07

Objectives: The major objective of this contract is to clone different regions of the env gene such as tat, rev, nef, in E. coli and baculovirus vector and to produce large quantities of the purified proteins after expression of these genes.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR VIRUS BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

The Laboratory of Tumor Virus Biology (1) identifies and characterizes exogenous viruses associated with the initiation or progression of neoplasia in humans or in animals as models for human neoplasia; (2) elucidates the mechanisms by which viruses associated with naturally-occurring cancers may induce or initiate neoplasia; (3) characterizes and defines the biology and molecular biology of viruses associated with naturally-occurring carcinomas; (4) identifies and characterizes factors involved in viral and cellular gene regulation pertinent to carcinogenesis; and (5) elucidates and defines the cellular and molecular basis of the transformation in carcinogenic progression.

The Viral Oncology Section (1) studies the molecular biology and gene regulation of the papillomaviruses and mechanisms of papillomavirus-induced transformation and carcinogenic progression; (2) develops techniques for DNA-mediated gene transfer; and (3) carries out studies on extrachromosomal plasmid replication, recombination, and partitioning in mammalian cells.

The Cellular Regulation and Transformation Section (1) examines the role of DNA tumor viruses in carcinogenesis and neoplasia; (2) characterizes the cellular and biochemical alterations associated with the oncogenic transformation; (3) analyzes the specific interactions between cellular and viral proteins during oncogenesis; and (4) examines the effect of specialized cellular differentiated functions on viral gene expression.

Among the primary objectives of the Laboratory of Tumor Virus Biology is the evaluation of the potential role of the human papillomaviruses in carcinogenesis. To this end we have investigated the molecular biology of the papillomaviruses. A major focus of the Laboratory has been the molecular biology of the bovine papillomavirus type 1 (BPV-1), which is one of a subgroup of papillomaviruses which readily transform a variety of rodent cells in tissue culture. This virus has served as the prototype for the systematic study of the molecular biology and genetics of the papillomaviruses. The BPV-1 genome encodes two independent transforming genes, one mapping to the E6 open reading frame (ORF) and the second to the E5 ORF. In transformed mouse cells, the viral genome remains as a stable multicopy plasmid, which is faithfully partitioned to the daughter cells at cell division. BPV-1 has characteristics making it an excellent model for the study of the cellular transformation and the viral functions important in a latent infection.

One major pathway which has emerged as being critical for papillomavirus gene expression is that encoded by the E2 open reading frame. E2 transactivation was first described by this laboratory for BPV-1 in 1985. Subsequent studies have gone on to show that this open reading frame encodes at least three distinct proteins. In addition to the full-length E2 transactivator, the open reading frame encodes two transrepressor factors. One of these is expressed from an internal promoter and utilizes a methionine at nucleotide 3089. The second form

of the repressor is initiated from an upstream promoter and a E8/E2 gene product is generated by splicing into the E2 open reading frame at nucleotide 3225. Each of these proteins has now been demonstrated utilizing antisera to the carboxy terminal conserved segment for these proteins. The E2 transregulatory program appears to be a general program for all papillomaviruses. It has been shown now for HPV-16, HPV-18, HPV-6, HPV-11, and the Shope papillomavirus, among others. The effect of these transregulatory proteins on individual viral promoters appears to vary. In BPV-1 it appears that P_{89}, P_{7940}, and P_{2443} are positively activated by the full-length product. Evidence exists from other laboratories that P_{7185} may actually be down-regulated by the full-length E2 product. In HPV-16 and HPV-18, the promoter upstream of E6 and E7, P_{97}, may be down-regulated by the viral E2 gene product.

The targets for transactivation in the BPV-1 long control region (LCR) have been localized using Bal 31. Two E2 responsive elements (E2REs) have been identified. The fragments of the viral genome were assayed for their ability to activate transcription from an enhancer-deleted SV40 early promoter in the presence or absence of the viral E2 gene product. The major target for E2 transactivation ($E2RE_1$) maps to a 196 bp segment between nucleotides 7611 and 7806. This element maps just upstream from the viral promoters, P_{7940} and P_{89}. Deletions which destroy or impair enhancer function revealed that the presence of conserved $ACCN_6GGT$ sequence motifs at either end of $E2RE_1$ are critical components of this element. Primer extension analysis of RNA generated from acute transfections with plasmids containing the BPV-1 LCR upstream of the chloramphenicol acetyltransferase gene revealed that both the P_{7940} and P_{89} promoters are responsive to E2 trans-activation. Subsequent studies have gone on to show that P_{2443} is also responsive to E2 transactivation. The $ACCN_6GGT$ motifs are sites at which the E2 proteins bind directly to DNA. DNA binding is necessary for full tran-scriptional transactivation. One motif is not sufficient for this transcriptional transactivation; a pair of motifs is necessary and sufficient for transcriptional enhancer activity. Full enhancer activity, however, requires the complete $E2RE_1$ which contains a total of four $ACCN_6GGT$ motifs.

The functional domains of the E2 open reading frame have been dissected. The full-length E2 gene product, the E2 repressor protein, and various segments of the E2 gene product have been expressed in a rabbit reticulocyte lysate using RNA-generated in vitro from a prokaryotic promoter. Employing a McKay DNA binding assay and using antisera generated against synthetic peptides corresponding to portions of the E2 protein, the full-length E2 protein and the repressor protein were shown to be able to bind to the $ACCN_6GGT$ motifs. The binding domain was mapped to the terminal 101 amino acids which is a domain present in the full-length E2 protein, the transrepressor mapping downstream from the ATG at base 3089, and the E8/E2 protein which is generated using the splice acceptor at base 3225. The E2 proteins are also capable of forming heterodimers. Dimerization is mediated through sequences in the conserved carboxy terminal 100 amino acids which are also involved in DNA binding. DNA binding is not necessary for dimer formation, however. The conserved 200 amino acids at the amino terminus of E2 appear to be involved in transcriptional transactivation. Deletion of the first 15 amino acids of E2 knocks out the transcriptional transactivation function. Comparison of the putative E2 proteins of a number of sequenced papillomaviruses indicates an internal region which is not highly conserved from virus to virus. This region varies in size and in amino acid composition among the various E2 proteins. This region is referred to as a hinge

region. Deletion mutagenesis has revealed that one can remove the entire hinge region and still have effective DNA binding and effective transcriptional activation. Further deletion into the conserved 200 amino acids at the amino terminus from this hinge region has a drastic negative effect on transactivation.

Collaborative studies have been carried out with Doug Hanahan at Cold Spring Harbor Laboratory studying a transgenic line of mice harboring the complete BPV-1 genome. At eight or nine months of age, these mice routinely develop two types of skin pathology. One of these is characterized by a dramatic but benign thickening of the dermal fibroblastic layer with concomitant atrophy of the epidermis and loss of hair. The general topology of the skin is maintained in these fibromatoses. The second type of lesion is a proturberant dermal tumor, histologically described as a fibrosarcoma. The fibrosarcomas are locally invasive but do not appear capable of metastasizing to internal organs. Both the fibromatoses and the tumors contain extrachromosomal BPV-1 DNA and express BPV-1 RNA. In contrast, the normal skin of young transgenic mice and the normal organs from the older mice contain only integrated viral DNA and the viral genes are not expressed. Thus, the state of the viral DNA and the expression of the viral genomes distinguish normal skin from the fibromatoses and the tumors, but do not differentiate between the two pathologic conditions. The fibrosarcomas are histologically and pathologically distinct from the fibromatoses, and cell lines derived from these two conditions have distinct cell heritable phenotypes in vitro. Thus, alterations in the cellular genes and/or their expression might be involved in the progression to a fibrosarcoma. We have carried out a karyotypic analysis of a series of cell lines derived from the fibromatoses from the fibrosarcomas. The fibrosarcomas but not the fibromatoses show consistent abnormalities of one or both of two chromosomes. In chromosome 8, a trisomy or duplication of a segment of the chromosome has been noted. For chromosome 14, monosomy or a translocation has been noted. These results may direct the search for genes involved in the production of malignancy and suggest that these transgenic mice may be useful in the study and analysis of cytogenetic changes important in papillomavirus-associated tumorigenesis.

Studies with the E5 oncoprotein of BPV-1 have revealed that it contains two distinct domains. The amino terminal two-thirds of the E5 oncoprotein is hydrophobic and essential for biologic function. Insertion of strongly hydrophilic amino acids into this domain abolishes transformation. Yet, one can readily substitute conservative hydrophobic amino acid residues within this domain with no effect. This hydrophobic domain appears to be responsible for the membrane-association of the E5 protein. Site-specific mutagenesis of the 14 amino acids at the carboxy terminus of E5 indicates that this is a separate domain with specific requirements for amino acids at seven of the 14 residues, including the two cysteine residues which are important for E5 dimerization. These cysteines may also be involved in interactions with host proteins. Localization studies utilizing a baculovirus expression vector have indicated that the E5 protein is localized within the cellular Golgi apparatus with a lesser amount present in the plasma membranes. The E5 protein has an unusual asymmetric localization in cell membranes; the carboxy terminus is localized internally in the Golgi apparatus and extracellularly in the plasma membrane. An E5 gene has also been localized in the HPV-16 genome but to date no functional data have revealed whether or not this encodes an important protein for the HPVs.

A new in vitro keratinocyte assay has been developed which demonstrates that HPV types associated with either benign or malignant anogenital lesions can stimulate keratinocytes for cellular proliferation. However, only the HPV types associated with malignant progression are able to alter the cellular response to differentiation signals (such as serum or calcium). The assay is quantitative and preliminary genetic studies implicate the E6/E7 region of the HPV-16 and HPV-18 genomes as being sufficient for the cellular phenotype.

We have extended our detailed genetic analysis of the papillomaviruses to HPV-16. This virus has been strongly implicated as having an etiologic role in some human anogenital malignancies. It is most frequently (greater than 60%) detected in biopsies from cervical carcinoma. In general, the virus is actively transcribed in the cancers and the most abundant transcripts map to the E6 or E7 early open reading frames. We have found that the HPV-16 E7 open reading frame encodes transcriptional transactivation and cellular transformation functions analogous to those of adenovirus E1a proteins. Specifically, the HPV-16 E7 protein can activate the adenovirus E2 promoter and the target for this transactivation maps to the same sequences required for adenovirus E1a activation. The HPV-16 E7 product can also cooperate with an activated ras oncogene to transform primary baby rat kidney cells indicating that, like adenovirus E1a, HPV-16 has cellular immortalization properties. The HPV-16 E7 transforming function differed somewhat from that of adenovirus E1a in that E7 was also able to transform established mouse NIH 3T3 cells. Examination of the primary amino acid sequence of HPV-16 E7 revealed striking similarity of the amino terminus with two distinct conserved domains of adenovirus E1a proteins. These domains in adenovirus E1a are called domain 1 and domain 2 and have been previously shown to be involved in cellular immortalization and in transcriptional repression. The carboxy terminal segment of HPV-16 E7 contains the motif Cys-X-X-Cys repeated twice, a motif which is repeated several times in the conserved domain 3 of the adenovirus E1a 289 amino acid protein. This domain in Ad E1a is involved in transcriptional activation.

We have used a HeLa cell in vitro transcription system to study transcription from the BPV-1 promoters. Five of six BPV-1 promoters which were previously mapped by analysis of the 5′ termini of viral mRNAs, from bovine fibropapillomas and from virally-transformed cells, were found to be active under in vitro transcription conditions. Transcription initiation from each of these promoters was accurate at the nucleotide level as determined by primer extension analysis. The most active promoters in vitro were P_{89} and P_{7185}. P_{89} expression was efficient without the presence of any added E2 factors. In vitro, P_{7185} was efficiently transcribed, suggesting that repression of this promoter does not occur in vitro, raising the possibility that it may be repressed in vivo by labile, viral or cellular factors. The two internal promoters, P_{2443} and P_{3080}, were also transcribed in vitro, providing further evidence that these RNA start sites are actually promoters and not RNA processing sites. The late promoter, which is inactive in BPV-1 transformed cells and active only in productively tissues, is very inefficiently utilized in vitro. Thus, it may be possible to use this in vitro transcription system to assay for trans factors in wart extracts which are capable of activating this promoter.

The block of viral transcription in the late region of BPV-1 appears to be complex. To accurately map the elements and factors involved in transcriptional termination, a series of small subgenomic fragments which cover the entire genome were cloned and used as hybridization probes to quantitate BPV-1 RNA

labelled in vitro in nuclei isolated from virally-transformed C127 cells. These analyses reveal that the rate of transcription of the 3′ early region remains high in the 5′ part of the late region but is then attenuated approximately fivefold about 1 kilobase downstream from the early polyadenylation site. Transcription continues to decrease throughout the rest of the late region and is down at least tenfold by the late polyadenylation site. This tenfold transcription attenuation in the late region would effectively favor the use of the early polyadenylation site over the late polyadenylation site and, thus, is one major component of the block of late transcription in BPV-1 transformed cells and, presumably, is also used in infected fibroblasts and basal epithelial cells of a fibropapilloma. There are still detectable levels of transcription near the late polyadenylation site, however, indicating that additional blocks must exist.

A new series of chloramphenicol acetyltransferase (CAT) expression vectors (pOBCAT) have been designed for mapping negative regulatory elements. The pOBCAT vectors express levels of CAT in transfection assays which are approximately 100-fold higher than those obtained using pSV2CAT vectors. This facilitates transfection analysis in BPV-1 transformed C127 cells which typically give low levels of expression in transient assays and makes these vectors ideal for mapping of negative regulatory elements. A segment of the late region of BPV-1 has been found to inhibit CAT expression by at least 100-fold when cloned into the intron upstream of the CAT gene of the pOBCAT vectors. Since this region of BPV-1 lies immediately upstream of the transcriptional termination described above, it seems likely that the inhibition is due to transcriptional termination. Using the pOBCAT vectors, it has been possible to demonstrate that the BPV-1 polyadenylation site can be efficiently utilized in BPV-1 transformed cells. Thus, it is unlikely that polyadenylation by itself plays a significant role in the regulation of late gene expression. However, sequences immediately upstream of the late polyA site inhibit CAT expression when cloned in the sense-orientation between the CAT gene and the SV40 polyadenylation site of pOBCAT but have little effect when cloned in the same position in the anti-sense orientation. Also, these sequences have little effect when placed in the intron upstream of the CAT gene. The most likely interpretation of these results is that these sequences are involved in the destabilization of mRNA.

Another major area of investigation by this laboratory is the mechanism of transformation by the polyomaviruses. The polyomavirus middle T antigen is the major transforming protein of polyomavirus and has been shown to form a stable complex with the $pp60^{c\text{-}src}$ cellular protein. The relative abundance of $pp60^{c\text{-}src}$ associated with polyoma middle T antigen and the relative abundance of middle T antigen associated with $pp60^{c\text{-}src}$ in a variety of polyoma-transformed rat cells was determined by quantitative immunoblot analyses which detect either of these proteins. The results revealed that approximately 5-10% of the total immuno-precipitable $pp60^{c\text{-}src}$ molecules in polyoma-transformed rat cells are stably associated with middle T antigen. In these same cells, it was found that approximately 10-15% of the detectable middle T antigen was associated with $pp60^{c\text{-}src}$. Approximately 50-75% of the total middle T antigen-associated cellular tyrosine kinase activity potentially represents the enzymatic activity of $pp60^{c\text{-}src}$, while the remaining 25-50% represents the activity of other cellular tyrosine kinases.

The level of $pp60^{c\text{-}src}$ protein kinase activity in a variety of human tumors and human tumor cell lines have been examined. These studies have demonstrated that the tyrosine-specific protein kinase activity of $pp60^{c\text{-}src}$ molecules obtained

from human colon carcinomas and their derived cell lines was found to be elevated over that of normal colon epithelium. The elevated $pp60^{c\text{-}src}$ protein kinase activity in tumor tissues and in cultured colon carcinoma cells did not appear to result solely from an increase in the abundance of $pp60^{c\text{-}src}$, suggesting that the specific activity of the $pp60^{c\text{-}src}$ tyrosine phosphotransferase may be enhanced. The activation of $pp60^{c\text{-}src}$ kinase activity in the colon tumor cell lines was found to be associated with an increase in the turnover rate of the tyrosine phosphates within the carboxy terminal portion of the tumor-derived $pp60^{c\text{-}src}$ molecules.

We have observed in membranes isolated from human colon carcinoma cells an abundant 56 kd protein which contains phosphotyrosine. Peptide analysis of this protein revealed that it represented the gene product of the human lck gene, which is a gene closely related to c-src. The lck gene in humans and mice was thought to be expressed exclusively in cells of lymphoid origin. We confirmed the expression and identity of this gene in human colon carcinomas by cDNA cloning experiments. Further analysis of the expression of lck revealed that significant expression is found only in normal human cells and tissues of lymphoid origin. The gene is frequently expressed at high levels in certain non-lymphoid human tumor cell lines such as cells derived from colon carcinomas and small cell carcinomas of the lung. Interestingly, high levels of lck expression were found in cell lines derived from metastatic sites when compared to those derived from primary tumors. This raises the possibility that the lck gene product may be involved in tumor progression of some non-lymphoid human tumors.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00543-10 LTVB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Papillomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	B. A. Spalholz	Senior Staff Fellow	LTVB	NCI
	R. Schlegel	Chief, CRT Section	LTVB	NCI
	P. Lambert	BioTech Fellow	LTVB	NCI
	P. Hermonat	Guest Researcher	LTVB	NCI
	A. McBride	Visiting Fellow	LTVB	NCI
	J. Byrne	Biologist	LTVB	NCI
	S. Vande Pol	Medical Staff Fellow	LP	NCI

COOPERATING UNITS *(if any)*

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (Doug Hanahan).

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.1	4.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The papillomaviruses are a group of small DNA viruses associated with benign proliferative lesions in a variety of higher vertebrates that occasionally progress to malignancy. There are currently 56 distinct human papillomaviruses and six distinct bovine papillomaviruses. The productive expression of these viruses is linked to the differentiation program of these squamous epithelial cells and, to date, no culture system has yet been developed for the successful propagation of any papillomaviruses in the laboratory. The bovine papillomavirus type 1 (BPV-1) has served as the prototype of the papillomaviruses for unravelling its molecular biology. It is capable of inducing fibroblastic tumors in inoculated rodents and readily transforms a variety of rodent tissue culture cells in the laboratory. The unique feature of the BPV-1 transformation system is that the viral DNA can remain as a stable extrachromosomal plasmid within transformed cells. Our studies are designed to understand the molecular biology of the normal virus host cell interaction with the hope of providing some insight into the viral and cellular factors that may be involved in carcinogenic progression. Within the viral system, the E2 gene products have served as critical factors in regulating viral gene expression. The E2 open reading frame for BPV-1 encodes at least three distinct proteins with DNA binding properties. These proteins have both positive and negative effects on the regulation of viral gene expression. These proteins regulate viral gene expression by binding specifically to ACCN6GGT motifs which are conserved within the viral genome.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB	NCI
B. A. Spalholz	Senior Staff Fellow	LTVB	NCI
R. Schlegel	Chief, CRT Section	LTVB	NCI
P. Lambert	BioTech Fellow	LTVB	NCI
P. Hermonat	Guest Researcher	LTVB	NCI
A. McBride	Visiting Fellow	LTVB	NCI
J. Byrne	Biologist	LTVB	NCI
S. Vande Pol	Medical Staff Fellow	LP	NCI

Objectives:

1. To analyze the molecular biology of the papillomaviruses using the bovine papillomavirus as a model system.
2. To localize and characterize the genes of the bovine papillomavirus involved in cellular transformation.
3. To develop a tissue culture system for the propagation of papillomaviruses.
4. To analyze the viral mRNAs expressed in BPV-1-transformed cells.
5. To localize the transcriptional elements in the viral genome involved in the control of viral gene expression.
6. To determine the factors, both viral and cellular, involved in the control of virus-specific gene expression for the papillomaviruses.
7. To analyze the "early" papillomavirus protein products expressed in transformed cells.
8. To determine the *cis* and *trans* functions required for autonomous extrachromosomal plasmid replication.
9. To determine the nature of the molecular events involved in the progression of a benign papillomavirus lesion into a malignant lesion.
10. To characterize the virally-encoded gene products involved in the transcriptional control of the papillomavirus genome, particularly the E2 transactivator and 3′ E2 repressor proteins.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus hybrid plasmids.
2. Tissue culture.
3. Transcriptional analysis including Northern blotting, cDNA cloning into expression vectors, and nuclear run-off experiments.
4. DNA sequencing.
5. The generation of synthetic peptides based on DNA sequence information.
6. Immunoprecipitation.
7. Immunoblotting and immunofluorescence of viral proteins.
8. Transfer of DNA into mammalian cells using standard calcium precipitation, DEA dextran or electroporation technology.
9. Transgenic mice.

Major Findings:

1. The long control region (LCR) of BPV-1 can function as a conditional transcriptional enhancer which can be specifically transactivated by the viral E2 gene product. Bal 31 endonuclease was used to generate two overlapping series of deleted DNA segments through the LCR to determine the targets for this transactivation. Two distinct E2 responsive elements (E2RE) were located within the LCR. The major target for E2 transactivation ($E2RE_1$) was mapped to a 196 bp fragment between bases 7611 and 7806, just upstream from the promoters, P_{7940} and P_{89}. Further deletion and mutational analysis revealed that the $ACCN_6GGT$ sequence motifs repeated at either end of this responsive element are critical components of this element. Subsequent analysis revealed that two of these motifs, by themselves, are sufficient to serve as a transcriptional enhancer element but that full enhancer activity by the responsive element required all four motifs to be present. A single motif is able to act cooperatively with two remote motifs to augment transcriptional expression from a promoter, suggesting cooperativity between the motifs which is likely to be mediated by the E2 proteins. Primer extension analysis of RNA generated from acute transfections with plasmids containing the BPV-1 LCR driving the chloramphenicol acetyltransferase (CAT) gene revealed that each of the P_{7940} and P_{89} promoters is responsive to E2 transactivation. An additional E2 responsive element maps in the vicinity of p7185, between bases 7200 and 7386. This region of the genome also contains a constitutive enhancer element which is active in bovine cells and in keratinocytes. The role of this E2 responsive element and the constitutive element in the regulation of viral gene expression in BPV-1 is currently under investigation.

2. A negative acting transcriptional regulatory factor(s) encoded by BPV-1 has been identified. This factor inhibits BPV-1 mediated transformation of mouse C127 cells; inhibition is BPV-1 specific and occurs only when the BPV-1 transforming genes are regulated by authentic transcriptional control elements. Plasmids expressing the inhibition function also repress E2 transactivation of the BPV-1 E2-dependent enhancer, and this repression is mediated by the same cis-acting element required for E2 transactivation. Inhibition of transformation may result from down-regulation of E2-dependent viral gene expression. Analysis of a cDNA expressing the inhibition/repression activities maps the function to the 3′ domain of the E2 open reading frame. Additional studies of deletion mutants of a larger cDNA (CW1-28) indicate that a second repressor also has the same activity. This repressor is expressed from an upstream promoter and is generated by fusion of the E8 and E2 open reading frames at the level of splicing. A manuscript describing the E2 carboxy terminal repressor was published in Cell in 1987. A manuscript describing the second repressor is in preparation.

3. The E2 open reading frame of the BPV-1 has been shown to encode both negative and positive acting transcriptional regulatory factors. Studies from several laboratories have shown that the E2 protein of BPV-1 is a DNA binding protein which binds specifically to the motif $ACCN_6GGT$. The DNA binding properties of the E2 encoded factors were analyzed to investigate the mechanism by which they might regulate viral gene expression. Polypeptides corresponding to the full-length E2 product in a shorter protein thought to represent the repressor function were synthesized in vitro by translation of the T7 polymerase-generated transcripts. Using rabbit antisera generated against synthetic

peptides from the E2 open reading frame, it was possible to immunoprecipitate each of these products and show that each was capable of binding the same specific sequence located in the E2 responsive elements of the BPV-1 genome. This DNA binding property was mapped to a carboxy terminal domain of 101 amino acids by analysis of truncated polypeptides synthesized from the E2 open reading frame. A manuscript describing these data was published in the EMBO Journal. This domain involved in DNA binding is present in the three forms of the E2 protein which have been recognized. It is a domain which is common to the full-length protein, to the repressor form of the E2 protein generated from the p3080 promoter, and exists downstream from the splice acceptor at nucleotide 3225.

4. In our attempts to further characterize the various functional domains of the E2 transcriptional regulatory proteins, we have determined domains involved in dimer formation and in transcriptional transactivation. Using in vitro translated polypeptides derived from the E2 open reading frame, we have demonstrated that the E2 polypeptides interact with the $ACCN_6GGT$ binding sites as a dimer. This dimer is stable and formed in the absence of DNA, and dimerization is mediated through the carboxy terminal DNA binding domain. Specific mutations in this domain are in progress to determine the amino acids important for DNA binding and important for dimerization. Comparison of the predicted amino acid sequence of the E2 open reading frames of those papillomaviruses which have been sequenced suggests that the full-length E2 polypeptide consists of three regions. Approximately 220 amino acids at the amino terminus and about 100 amino acids at the carboxy terminus are relatively well-conserved among the E2 proteins. The internal region varies in both length and amino acid composition and is referred to as a hinge region. Deletion analysis of the full-length E2 transactivator revealed that the conserved N terminal domain of the polypeptide is important for transactivation functions. The internal hinge region could be deleted from the full-length polypeptide without affecting its ability to transactivate the BPV-1 enhancer. Thus, the E2 transactivator consists of an N terminal transactivator domain, and a C terminal binding domain linked by a hinge region. A manuscript describing these results is in preparation.

5. A line of transgenic mice harboring the complete BPV-1 genome has been developed by Doug Hanahan at the Cold Spring Harbor Laboratories. These mice develop dermal fibroblastic tumors at approximately eight months of age. These mice also develop large areas of abnormal skin with hair loss due to a generalized proliferation of dermal fibroblasts and atrophy of the skin appendages. The DNA is integrated into the normal tissues of these mice and approximately five to ten copies per diploid genome. The DNA becomes extrachromosomal within the abnormal skin and within the dermal fibroblastic tumors. The tissues and cell lines established from these mice have been analyzed for the state of viral DNA and for expression of the viral genome. BPV-1 transcripts are readily detected in the abnormal appearing skin and in the tumors of older mice. DNA is not present in internal organs of these same mice. No viral transcription is found in the normal tissues or within the tissues of newborn or young mice with no skin abnormalities. Thus, BPV-1 transcriptional activity and extrachromosomal plasmid replication correlate well with the dermal fibroblastic proliferation. These activities, however, are not sufficient for tumor formation. We, therefore, postulate that a second genetic event is necessary for tumor formation. Chromosomal analysis of these lesions indicates a number of specific chromosomal abnormalities which correlate with tumor

formation. These consist of either a duplication of a portion of mouse chromosome 8 and/or a deletion of a portion of mouse chromosome 14. Preliminary experiments indicate that the BPV-1 genome is integrated in mouse chromosome 15. Two manuscripts describing these data have been submitted.

6. Functional analysis of the 3′ open reading frames (ORFs) of the BPV-1 has been complicated by the organization of that part of the genome. A region between nucleotides 3173 and 3551 contains three overlapping ORFs (E2, E3, and E4) as well as a splice acceptor at base 3225. To more clearly assign functions to specific ORFs in this region, specific base substitution mutations were constructed by oligonucleotide-directed site-specific mutagenesis. Mutations were generated which contained translational termination codons specific for each of the three open reading frames. An additional mutation substituted an AC at the 3′ splice junction consensus at nucleotide 3223 changing the sequence from AG to CG. Analysis of these base-specific mutations indicated that the E2- and E4-specific mutants were essentially wild-type in their ability to transform susceptible mouse C127 cells, to replicate as stable plasmids, and to transactivate the E2 LCR. Thus, no essential function for transformation or for plasmid replication can be mapped to the E3 or E4 ORF at this time. The E2-specific termination mutant was defective for plasmid replication, transformation, and transactivation and could not be complemented to higher levels of transformation by a cell line expressing the full-length E2 product. The splice acceptor mutant was also defective for transformation and could not be complemented for transformation in the cell line expressing the full-length E2 product. These data extend previous work and suggest that a spliced E2 product is involved somehow in cellular transformation. Finally, the splice acceptor mutant engineered into a C59 cDNA decreased its transformation efficiency, suggesting that the expression of the E5 transforming protein from the 3′ ORF may utilize the 3′ splice junction at nucleotide 3225. A manuscript describing these results was published in the Journal of Virology.

7. We have found evidence for E2 autoregulation in the bovine papillomavirus. The BPV-1 P_{2443} promoter is located just upstream of the E2, E3, E4, and E5 open reading frames and is active in both transformed rodent cells and in productively infected warts. Analysis of viral RNA functions suggests that transcripts from this promoter encode the E2 transactivator as well as the E5 oncoprotein. To study expression of P_{2443}, the chloramphenicol acetyltransferase (CAT) reporter gene was placed downstream of this promoter, deleting the E2 and E5 ORFs, in a plasmid which contained all BPV-1 upstream sequences, including the LCR. By itself, this plasmid had a low level of activity in transient assays but could be transactivated to a high level by the full-length E2 product. Transactivation of P_{2443} expression by E2 required the LCR in *cis* in an orientation and position-independent manner, suggesting that this transactivation was mediated through the E2 responsive elements located within the LCR. Primer extension analysis of the 5′ ends of the viral RNAs from pooled cells expressing these P_{2443}/CAT plasmids confirmed that E2 transactivation results in an increase in the steady state levels of RNA initiated from the P_{2443} promoter. Furthermore, E2 transactivation of the P_{2443} promoter could be inhibited by the transrepressor encoded by the 3′ portion of the E2 ORF. Thus, expression of the E2 transactivator and the E5 oncoprotein is directly regulated by transcriptional factors encoded by the E2 ORF.

Publications:

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Hermonat PL, Spalholz BA, Howley PM. The bovine papillomavirus P_{2443} promoter is E2 trans-responsive: evidence for E2 autoregulation. EMBO J (In Press).

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Lambert PF, Spalholz BA, Howley PM. A transcriptional repressor encoded by BPV-1 shares a common carboxy terminal domain with the E2 transactivator. Cell 1987;50:69-78.

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Spalholz BA, Baker CC, Lambert PF, Howley PM. BPV-1 E2 transactivation: characterization of the enhancers and promoters in the long control region. Cancer Cells 1987;5:5-13.

Spalholz BA, Lambert PF, Yee CL, Howley PM. Bovine papillomavirus transcriptional regulation: localization of the E2 responsive elements of the long control region. J Virol 1987;61:2128-37.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP00565-06 LTVB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transforming Activities and Proteins of the Papillomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Schlegel	Chief, CRT Section	LTVB	NCI
Others:	V. Bubb	Guest Researcher	LTVB	NCI
	Y. Zhang	Visiting Fellow	LTVB	NCI
	A. Burkhardt	Guest Researcher	LTVB	NCI

COOPERATING UNITS *(if any)*

Department of Human Genetics, Yale University, School of Medicine, New Haven, CT. (Dr. Daniel DiMaio)

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Papillomaviruses induce benign tumors in a variety of vertebrate species including man and, in some cases, these viral-induced lesions can progress to carcinomas. The intent of our laboratory's investigations is to define the mechanisms by which the papillomaviruses "transform" both immortalized or primary cells in vitro and to determine how they contribute to tumorigenesis in vivo. Specifically, we are studying the transforming activities of bovine and human papillomavirus DNA as determined by focus formation, immortalization, and differentiation assays of cultured murine and human epithelial cells. These studies also involve genetic and biochemical analysis of the known viral transforming genes (E6, E7, and E5). In collaboration with Dr. DiMaio's laboratory at Yale, we have been able to demonstrate the E5 transforming protein has two domains essential for biological activity: a hydrophobic, membrane-anchoring domain which has no requirement for specific amino acid residues, and a 14 amino acid hydrophobic, carboxyl terminal domain containing several amino acids (including two cysteine-residue involved in E5 dimer and oligomer formation) which are essential for biological function. We have also shown a correlation between the levels of E5 protein expression and tumorigenicity of bovine papillomavirus type 1 (BPV-1) transformed hamster cells. Using a baculovirus expression vector, we have demonstrated by IF and immuno-electron microscopy that the E5 protein is present predominantly in the Golgi apparatus and to a lesser degree in the plasma membrane. The orientation of the E5 protein is asymmetric and the COOH terminus faces intracellularly in the Golgi and extracellularly on the plasma membrane. We have also demonstrated by DNA sequence analysis that human papillomavirus type 16 (HPV-16), the HPV type most commonly associated with cervical carcinoma, encodes an E5 protein. Finally, we have developed an in vitro assay for the human papillomaviruses which identified two viral activities, alteration of cell proliferation, and cell differentiation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. Schlegel	Chief, CRT Section	LTVB NCI
V. Bubb	Guest Researcher	LTVB NCI
Y. Zhang	Visiting Scientist	LTVB NCI
A. Burkhardt	Guest Researcher	LTVB NCI
C. Gay	Student Volunteer	LTVB NCI

Objectives:

1. Generate specific antisera against the transforming proteins of the human (HPV) and bovine (BPV) papillomaviruses.
2. Isolate and characterize the biochemical and biological properties of the papillomavirus transforming proteins.
3. Perform mutagenesis studies of isolated transforming genes to determine the functional domains of the viral transforming proteins.
4. Establish an in vitro assay with human keratinocytes to evaluate the biological consequences of human papillomavirus infection.
5. Determine whether papillomavirus transforming genes cooperate with known cellular oncogenes to initiate tumor formation or tumor progression.
6. Determine if any of the early viral proteins can function as TSTA molecules or whether they might be useful for immunoprophylaxis.

Methods employed:

1. Keratinocyte cell culture.
2. Electroporation and CaPO4 transfection of keratinocytes.
3. Animal tumorigenicity assays and in vitro cytotoxicity assays.
4. Recombinant DNA methodology for the construction of subgenomic BPV and HPV molecules.
5. Synthesis of mutant papillomavirus genes with oligonucleotides.
6. Expression of HPV and BPV transforming proteins in baculovirus expression vectors for protein purification and characterization.
7. Immunoprecipitation, immunoblotting, and immunofluorescence of viral proteins.
8. DNA and RNA hybridization.
9. Fluorescent activated cell sorter (FACS) analysis of cell surface tumor antigens and cellular DNA content.
10. Polyacrylamide and agarose gel electrophoresis.
11. DNA sequencing.
12. Site-specific mutagenesis using M13 vectors.
13. High pressure liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) of viral and cellular proteins.
14. Two dimensional gel electrophoresis.
15. Microinjection of tissue culture cells.

Major Findings:

1. The E5 transforming protein of BPV-1 contains two distinct, but essential domains.

In collaboration with Dr. DiMaio's laboratory at Yale, we have been able to demonstrate that in the hydrophobic NH_2-terminal, two-thirds of the E5 molecule is essential for biological function. Insertion of strongly hydrophilic amino acids into this domain abolishes transformation, although one can readily substitute conservative amino acid residues without effect, indicating that a specific amino acid sequence is not required. This hydrophobic domain appears to be responsible for the membrane-association of E5 protein. Site-specific mutagenesis of the 14 amino acids present at the E5 COOH-terminus indicates that this domain has requirements for specific amino acids at seven positions, including the two cysteines that mediate E5 dimerization. As might be anticipated, mutation of both cysteine residues to serine results in abolishment of both transformation and dimerization. Interestingly, however, mutation of either cysteine alone abolishes transformation but not dimerization. This suggests that the E5 COOH may participate in important protein interactions other than homodimer formation, such as complexing with intracellular regulatory protein.

2. The E5 transforming protein of BPV-1 is localized predominantly in the Golgi apparatus.

Using a baculovirus expression vector and an isolated mammalian cell line which overexpresses E5 protein, we demonstrated by immunofluorescence (IF) and immuno-electron microscopy that the E5 protein is present predominantly in the cellular Golgi apparatus and that a lesser amount is present in the plasma membranes. The E5 protein has an unusual asymmetric orientation in cell membranes; the COOH-terminus faces intraluminally in the Golgi and extra-cellularly in the plasma membrane, suggesting that its important COOH-terminal domain may interact with growth factor receptors or other intrinsic membrane proteins which participate in signal transduction.

3. HPV-16 also encodes an E5 protein.

We sequenced a new isolate of HPV-16, the HPV type most commonly found in cervical carcinoma, and found that it encoded an E5 open reading frame (ORF) which was similar to those described for other HPV types such as HPV-6, HPV-11, and HPV-18. The predicted E5 molecule exhibited a trimodal hydrophobic structure but did not show any specific conservation of amino acid residues. We also observed that a mistake had been made in sequencing the original isolate of HPV-16 and that this isolate also contained an E5 ORF.

4. An in vitro assay identifies the HPV types associated with cervical carcinoma and detects two distinct viral activities.

We have developed an in vitro keratinocyte assay which demonstrates that HPV types associated with either benign or malignant lesions can stimulate cellular proliferation to a similar degree. However, only the types associated with

cervical carcinoma initiated an altered cellular response to differentiation signals (i.e., serum or calcium). The assay is quantitative and preliminary genetic studies indicate that the E6/E7 region of HPV-18 and the E7 gene of HPV-16 are sufficient to achieve this cellular phenotype. In addition, the assay can evaluate the effect of non-viral factors such as glucocortoroids which were shown to increase the frequency of cellular transformation.

Publications:

Bubb V, McCance DJ, Schlegel R. DNA sequence of the HPV-16 E5 ORF and the structural conservation of its encoded protein. Virol 1988;163:243-6.

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Schlegel R. Probing the function of viral fusion proteins with synthetic peptides. In: Sowers A, ed. Cell fusion. New York: Plenum, 1987,33-43.

Schlegel R. Papillomaviruses. Arch Dermatol 1987;123:537.

Schlegel R, Wade-Glass M. The E5 transforming protein of bovine papillomavirus. In: Steinberg B, Brandsma J, Taichman L, eds. Cancer cells. New York: Cold Spring Harbor Laboratory Press, 1987;87-91.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP00898-05 LTVB

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Role of Human Papillomaviruses in Human Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	C. C. Baker	Senior Investigator	LTVB	NCI
	W. Phelps	IRTA Fellow	LTVB	NCI
	L. Cowsert	BioTech Fellow	LTVB	NCI
	K. Munger	Visiting Fellow	LTVB	NCI
	H. Romanczuk	IRTA Fellow	LTVB	NCI
	C. Yee	Biologist	LTVB	NCI
	J. Byrne	Biologist	LTVB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Tumor Virus Biology

SECTION
Viral Oncology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.2	3.7	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The papillomaviruses are associated with naturally-occurring cancers in a variety of animal species, including man. There are now 56 distinct human papillomaviruses (HPVs) which have been identified. Approximately a dozen of these have now been associated with human genital tract lesions. Of these, HPV-6 and HPV-11 have been associated with a high percentage of benign genital lesions, and HPV-16, HPV-18, and HPV-33 have been found in a high percentage of cervical carcinomas. A number of human cervical carcinoma cell lines exist containing integrated HPV DNAs which are transcriptionally active. Integration of the viral genome in these cell lines and in tissues obtained directly from cervical carcinomas have demonstrated that often the HPV genomes are integrated in the malignant lesions. This integration event does not appear to demonstrate specificity with regard to the host chromosome but does indicate some specificity with regard to the viral genome. Integration often occurs in the E1 or E2 open reading frame, such that it disrupts expression of the E2 open reading frame. The E2 open reading frame of the papillomaviruses encodes a DNA binding protein which is involved in the transregulation of the viral promoters. Integration into the E2 open reading frame, therefore, results in the loss of this regulatory factor and the deregulation of the promoter upstream of the E6 and E7 open reading frames. The E6 and E7 open reading frames have been shown to be expressed on a regular basis in cervical carcinomas. The E7 open reading frame encodes a transcriptional transacting function which can activate the adenovirus E2 promoter. It can also cooperate with ras in the transformation of primary rat embryo cells. There are regions of the E7 gene of HPV-16 and of the E7 proteins of other genital associated HPVs which are strikingly similar to domains of the adenovirus E1a gene product.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB NCI
C. C. Baker	Senior Investigator	LTVB NCI
W. Phelps	IRTA Fellow	LTVB NCI
L. Cowsert	BioTech Fellow	LTVB NCI
K. Münger	Visiting Fellow	LTVB NCI
H. Romanczuk	IRTA Fellow	LTVB NCI
C. Yee	Biologist	LTVB NCI
J. Byrne	Biologist	LTVB NCI

Objectives:

1. To analyze human squamous cell carcinomas from a variety of sites for the presence of HPV DNAs and the expression of HPV-specific mRNAs.
2. To characterize the HPV RNAs expressed in HPV-associated carcinomas and to generate full-length cDNA clones of such transcripts.
3. To determine which viral genes are being expressed within the cervical carcinomas and cervical carcinoma cell lines.
4. To determine whether hybrid viral-host cell RNAs are expressed within the positive carcinoma lines.
5. To characterize the HPV-16 and HPV-18 genes which can transactivate transcriptional regulatory sequences within the viral genome and within the host cell.
6. To identify and characterize the HPV-16 and HPV-18 gene products involved in transformation and immortalization.
7. To determine the viral promoters active in benign HPV-16 and HPV-18 associated lesions and in cervical carcinomas.
8. To determine the cellular factors involved in regulating viral gene expression.
9. To identify the cellular factors which interact with the viral transcriptional, transregulatory, and transforming gene products.

Methods Employed:

1. Standard recombinant DNA technology.
2. Northern blot analysis of RNAs.
3. cDNA cloning using expression vectors.
4. Immunoblotting and immunofluorescence of viral proteins.
5. DNA sequencing.
6. In situ hybridization.
7. Transient and stable DNA transfection techniques.
8. Cellular transformation using primary and established cell lines.

Major Findings:

1. The DNA and RNA from autopsy specimens of a 26 year old male with juvenile onset laryngotracheobronchial papillomatosis who developed squamous cell carcinoma of the lung have been examined. This patient had no history of

radiation therapy, chemotherapy, or a history of smoking. DNA hybridization analysis revealed HPV-11 DNA in high copy number in the tracheal papillomas, in the primary squamous cell carcinoma of the lung, and in a lymph node metastases. In addition to a small amount of monomeric DNA, an altered extrachromosomal form of HPV-11 DNA with a tandem repeat of the long control region of the HPV-11 genome was present in a metastatic lesion in the liver. A slot blot analysis of the RNA prepared from the tissues showed that it was positive for HPV-11 sequences and demonstrated that the HPV-11 genomes were transcriptionally active. This represents the first study of a case of a squamous cell carcinoma arising in a patient with laryngotracheal papillomatosis in which the tumor has been analyzed for HPV DNA. The demonstration, in this case, of HPV-11 DNA in the primary lung tumor and in the metastatic lesions indicates that the HPV type usually associated with laryngeal papillomas can be associated with malignant transformation. Malignant transformation of laryngeal papillomas may not, therefore, be associated with the same group of HPVs which have been associated with carcinogenic progression in cervical and penile lesions (i.e., HPV-16 and HPV-18). A paper describing these data was published in the New England Journal of Medicine. We have cloned the HPV-11 DNAs from the tissues. These DNAs are being structurally analyzed using restriction endonucleases. They are also being functionally assessed in transformation and transfection assays utilizing primary rodent cells and primary human foreskin epithelial cells.

2. In a continuation of our analysis of HPV-16 encoded transcriptional regulatory factors, a novel papillomavirus transactivating function was detected that could activate the adenovirus E2 early promoter. This function was genetically mapped to the E7 open reading frame of HPV-16 and was found to be distinct from the HPV-16 E2 transregulatory function, which this laboratory had previously described. The target in the adeno E2 promoter for this novel HPV-16 function was similar to that required for adenovirus E1a activation, suggesting that these two different viral factors might act as transcriptional activators through a common mechanism. Studies looking at functional similarities between the HPV-16 E7 gene and the adenovirus E1a gene were extended, and we were able to show that like adenovirus E1a, the HPV-16 E7 gene could cooperate with an activated ras oncogene to transform primary baby rat kidney cells. This E7 transforming function differed somewhat from that of adenovirus E1a in that E7 was, by itself, sufficient to transform established mouse cells. Examination of the amino acid sequence of HPV-16 E7 revealed striking similarities with the conserved domains 1 and 2 of adenovirus E1a proteins. These two domains of adenovirus E1a have been previously shown to be important in cellular transformation and in the transcriptional transrepression phenotypes. The carboxy terminal domain of HPV-16 E7 contains the motif Cys-X-X-Cys repeated two times. This motif is also present in domain 3 of adenovirus E1a which is involved in transcriptional transactivation. A manuscript describing these data was published in Cell.

3. The HPV-16 E6 function has also been analyzed. NIH 3T3 cells were cotransfected with plasmids which express all of the early open reading frames of HPV-16 or all but the E6 open reading frame of HPV-16. Each of these plasmids was readily able to form foci on NIH 3T3 cells. When analyzed for anchorage independence in soft agar or for tumorigenicity in nude mice, only those cells transformed by the plasmids in which the E6 open reading frame was intact was capable of scoring positive in these assays. Pooled populations of NIH 3T3 cells

transfected with these plasmids were tested for growth in soft agar, and deletion of the E6 ORF showed a pronounced effect in that the size of the anchorage independent colonies were reduced markedly. Plasmids in which the E6, E6 star, and E7 ORFs have been individually expressed from the Rous sarcoma virus LTR, and cells harboring E6 or E6 star alone, formed large colonies in soft agar, whereas cells containing the E7 alone did not. When injected into nude mice, cells containing E6 and E6 star were more efficient in the induction of tumors than were cells containing E7. We, therefore, conclude that in the NIH 3T3 assay system, the E6 and the E7 ORFs each encode independent oncogenic functions. A manuscript describing these data is in preparation.

Publications:

Byrne JC, Tsao MS, Fraser RS, Howley PM. The presence and expression of human papillomavirus DNA in a patient with chronic laryngotracheobronchial papillomatosis and metastatic squamous cell carcinoma of the lung. N Engl J Med 1987;317:873-8.

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Phelps WC, Yee CL, Münger K, Howley PM. The human papillomavirus type 16 E gene encodes transactivation and transformation functions similar to adenovirus Ela. Cell 1988;53:339-47.

Phelps WC, Yee CL, Münger K, Howley PM. Functional and sequence similarities between HPV16 E7 and adenovirus E1a. In: Current topics in microbiology and immunology. Stuttgart: Springer-Verlag (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05420-04 LTVB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transformation by Polyomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. B. Bolen	Microbiologist	LTVB	NCI
Others:	V. DeSeau	Biologist	LTVB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The polyomaviruses comprise a class of small DNA tumor viruses within the papovavirus group of DNA viruses. Members of the polyomavirus class include polyomavirus (Py) of mice, simian virus 40 of monkeys, hamster papovavirus, and JC and BK viruses of humans. Of these viruses, Py has been most thoroughly characterized with respect to the genetic elements and proteins involved in oncogenic transformation of mammalian cells. Oncogenic transformation of rodent cells by Py requires the continued expression of the Py-encoded middle tumor antigen (MTAg). The MTAg is a membrane-associated phosphoprotein with an associated tyrosine-specific protein kinase activity that has been demonstrated to be, at least in part, a property of the c-src gene product. The importance of MTAg-associated tyrosine-specific protein kinase activity is suggested by the finding that all known transformation-competent strains of Py encode MTAg molecules which possess this associated activity.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Microbiologist	LTVB NCI
V. DeSeau	Biologist	LTVB NCI

Objective:

Role of polyomavirus middle tumor antigen and pp60$^{c\text{-}src}$ in polyomavirus transformation.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of chimeric plasmids.
2. Cell culture.
3. Cloning of mammalian cells.
4. Transfer of DNA into mammalian cells.
5. Transcriptional analyses of RNA including Northern, S1, and RNase protection.
6. Immunoprecipitation analysis and protein kinase assays.
7. Two-dimensional phosphoamino acid analysis.
8. Immunoblot analysis.
9. High and medium pressure liquid chromatography of proteins and amino acids.
10. One and two-dimensional gel electrophoresis.
11. Generation of peptide-specific polyclonal antisera.

Major Findings:

The relative abundance of pp60$^{c\text{-}src}$ molecules associated with Py MTAg and relative abundance of MTAg associated with pp60$^{c\text{-}src}$ in a variety of Py transformed rat cells was determined by quantitative immunoblot analyses which detect pp60$^{c\text{-}src}$ or Py MTAg. The results showed that approximately 5-10% of the total immunoprecipitable pp60$^{c\text{-}src}$ molecules in Py-transformed rat cells are stably associated with MTAg. In these same cells, it was found that approximately 10-15% of the detectable MTAg molecules are stably associated with pp60$^{c\text{-}src}$. Other results showed that approximately 50-75% of the total MTAg associated cellular tyrosine kinase activity potentially represents the enzymatic activity of pp60$^{c\text{-}src}$, while the remaining 25-50% represents the activity of other cellular tyrosine kinases.

Publications:

Bolen JB, DeSeau V, O'Shaughnessy J, Amini S. Analysis of middle tumor antigen and pp60$^{c\text{-}src}$ interactions in polyomavirus-transformed rat cells. J Virol 1987;61:3299-305.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05481-03 LTVB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Regulation of Tyrosine Protein Kinases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. B. Bolen	Microbiologist	LTVB	NCI
Others:	A. Veillette	Guest Researcher	LTVB	NCI
	V. DeSeau	Biologist	LTVB	NCI
	I. D. Horak	Medical Staff Fellow	MB	NCI

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The protein products of proto-oncogenes possess functions (e.g., enzymatic activity, nucleic acid binding activity) that are believed to play a role in the regulation of normal cellular growth and differentiation. Thus, analysis of the functional state of proto-oncogene encoded proteins represents one experimental approach that may provide insights into the biochemical alterations within cells that contribute to oncogenic transformation. While the biochemical functions of most proto-oncogene products are not known, several have been shown to be tyrosine-specific protein kinases. Of the proto-oncogene-encoded tyrosine kinases, the most extensively characterized are the members of the src family of tyrosine protein kinases. This gene family includes c-src, c-yes, c-fgr, fyn, hck, lyn, lck, and tkl.

We have analyzed the abundance and activity of several of the members of the protein products encoded by this gene family in a variety of human tumor cell lines and tissues as well as normal human and rodent tissues and cells. Our results indicate that the control of expression and gene product function of this group of cellular proteins is altered in several different types of human tumor cells. However, our data also suggest that a variety of other human tumor cell types maintain normal control mechanisms which govern the expression and function of the src family of tyrosine protein kinases implying that selected tumor cell systems may provide useful cellular models for analyzing the normal functions of this class of enzymes.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Microbiologist	LTVB	NCI
A. Veillette	Guest Researcher	LTVB	NCI
V. DeSeau	Biologist	LTVB	NCI
I.D. Horak	Medical Staff Fellow	MB	NCI

Objectives:

1. Analysis of the src kinase family members in normal and transformed human and rodent cells and tissues.
2. Mechanism of regulation of src kinase family members in normal and transformed human and rodent cells and tissues.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of chimeric plasmids.
2. Cell culture.
3. Cloning of mammalian cells.
4. Transfer of DNA into mammalian cells.
5. Transcriptional analyses of RNA including Northern, S1, and RNase protection.
6. In vitro transcription and translation of RNA.
7. Construction and analysis of cDNA libraries.
8. Immunoprecipitation analysis and protein kinase assays.
9. Two-dimensional phosphoamino acid analysis.
10. Immunoblot analysis.
11. High and medium pressure liquid chromatography of proteins and amino acids.
12. One and two-dimensional gel electrophoresis.
13. Generation of peptide-specific polyclonal antisera.

Major Findings:

We evaluated the level of $pp60^{c\text{-}src}$ protein kinase activity in a variety of human tumor tissues and human tumor cell lines and estimated the abundance of $pp60^{c\text{-}src}$ in several of these tissues and cell lines. Our studies demonstrated that the tyrosine-specific protein kinase activity of $pp60^{c\text{-}src}$ molecules obtained from human colon carcinoma tissues and tumor-derived cell lines were elevated over that from normal colon tissues or cultures of normal colon mucosal cells. The elevated $pp60^{c\text{-}src}$ protein kinase activity in tumor tissues and in cultured colon carcinoma cells did not appear to result solely from an increase in the abundance of $pp60^{c\text{-}src}$, suggesting that the specific activity of the $pp60^{c\text{-}src}$ tyrosine phosphotransferase is enhanced. The activation of $pp60^{c\text{-}src}$ kinase activity in the colon tumor cell lines was found to be associated with an increase in the turnover rate of tyrosine-phosphates within the carboxy terminal portion of the tumor derived $pp60^{c\text{-}src}$ molecules.

We compared the level of phosphotyrosyl phosphatase activity in lysates from normal human colon mucosal cells and human colon carcinoma cells and analyzed the effect of incubating these cells with sodium orthovanadate on the relative abundance of acid-stable phosphotyrosine and on in vitro protein kinase activity of $pp60^{c\text{-}src}$. Additionally, we compared the effect of lysing these cells in buffer containing only nonionic detergents with radio-immune precipitation assay (RIPA) buffer, which contains both sodium dodecyl sulfate and deoxycholate, on the in vitro kinase activity of $pp60^{c\text{-}src}$. Our results showed that the level of detectable phosphotyrosyl phosphatase activity in lysates derived from normal colon cells and colon carcinoma cells is very similar. Additionally, the abundance of acid-stable phosphotyrosine in these cells in the absence or presence of vanadate is not significantly different. However, incubation of these cells with vanadate significantly stimulates the activity of $pp60^{c\text{-}src}$ derived from the normal colon cells, while having no detectable effect on the activity of $pp60^{c\text{-}src}$ from the colon tumor cells.

In membranes isolated from human colon carcinoma cells, we observed an abundant 56 kilodalton protein which contained phosphotyrosine. Peptide analysis of this protein revealed that it represented the gene product of the human lck gene which is a gene closely related to the c-src gene. The lck gene in humans and in mice was thought to be expressed exclusively in cells of lymphoid origin. We confirmed the expression and identity of this gene in human colon carcinoma cells by cDNA cloning experiments. Further analysis of the expression of the lck gene revealed that significant expression is found only in normal human cells and tissues of lymphoid origin. However, this gene is frequently expressed at high levels in certain non-lymphoid human tumor cells such as cells derived from colon carcinomas and small cell carcinomas of the lung. Interestingly, higher levels of lck expression were found in cell lines derived from metastatic tumor sites when compared to those derived from primary tumors. This raises the possibility that the lck gene product may be involved in tumor progression of some non-lymphoid human tumors.

The tyrosine kinase gene lck is primarily expressed in cells and tissues of lymphoid lineage, more specifically in T-lymphocytes. High levels of the lck gene product ($p56^{lck}$) can also be detected in some non-lymphoid human tumor cell lines such as those derived from colon carcinomas. We have found that in response to the phorbol ester, 4ß-phorbol 12-myristate (PMA), as well as the diacylglycerol analogue 1,2-dioctanoyl-sn-glycerol (diC8), the lck protein is extensively modified into a product migrating at approximately 60 kilodaltons (kDa) (p60) on sodium dodecyl sulfate-polyacrylamide gels. This modification is associated with a marked increased in serine phosphorylation within a 15 kDa amino-terminal fragment demonstrating the altered electrophoretic mobility. These modifications are rapidly induced following treatment with these agents and appear to be completely reversible within 60 minutes of treatment with dioctanoylglycerol (diC8) which has been shown to cause a short-lived activation of protein kinase C. We also found that these changes are associated with a small but reproducible decrease in the ability of the lck protein to be phosphorylated in immune complex kinase assays. These alterations of the lck gene product might play an important role in the activation of T-lymphocytes in response to antigen recognition. However, they are also induced independently of T-cell activation, suggesting that they are not necessarily implicative of this process.

Additionally the constitutive presence of similar modifications of the lck protein in some neoplastic cells suggests that they might also be implicated in the transformed phenotype.

We have compared in different human neuroblastoma cell lines and human glioblastoma cells the expression level, structure, and tyrosine-specific protein kinase activity of $pp60^{c\text{-}src}$. Our results show that not all human neuroblastoma cell lines express $pp60^{c\text{-}src}$ molecules with amino-terminal structural alterations. In neuroblastoma cells which possess $pp60^{c\text{-}src}$ with altered gel migration, the diminished polyacrylamide gel mobility of $pp60^{c\text{-}src}$ was found not to be dependent upon amino-terminal phosphorylations since extensive treatment of these molecules with phosphatase did not significantly change their gel migration properties. Similar differences in gel migration were observed when RNA from the various neuroblastoma and glioblastoma cells was translated in vitro using either rabbit reticulocyte or wheat germ lysates. While the level of c-src mRNA in the different cells analyzed was found to be similar, the abundance of $pp60^{c\text{-}src}$ in these same cells was found to vary by as much as 12-fold. This suggests that the abundance of $pp60^{c\text{-}src}$ in human neuroendocrine tumors is regulated through post-transcriptional and/or post-translational events which may be related to the stage of neuronal differentiation of the cells. Based upon determination of $pp60^{c\text{-}src}$ abundance by immunoblot analysis, we demonstrate that $pp60^{c\text{-}src}$ molecules derived from human neuroblastoma and glioblastoma cells have very similar in vitro protein kinase activities.

Publications:

Bolen JB, Veillette V, Schwartz AM, DeSeau V, Rosen N. Analysis of $pp60^{c\text{-}src}$ in human colon carcinoma and normal human colon mucosal cells. Oncogene Res 1987;1:149-68.

DeSeau V, Rosen N, Bolen JB. Analysis of phosphotyrosyl phosphatase activity in human colon carcinoma and normal colon mucosal cells. J Cell Biochem 1987;35: 113-28.

DeSeau, V, Rosen N, Bolen JB. Analysis of phosphotyrosyl phosphatase activity in human colon carcinoma and normal colon mucosal cells. In: Lippman M, ed. Growth regulation in cancer. New York: Alan R Liss, 1987;36-54.

Herlyn M, Mancianti ML, Jambrosic J, Bolen JB, Koprowski H. Regulatory factors that determine growth and phenotype of normal human melanocytes. J Exp Cell Res (In Press).

O'Shaughnessy, J, DeSeau V, Amini S, Rosen N, Bolen JB. Analysis of the c-src gene product structure, abundance, and protein kinase activity in human neuroblastoma and glioblastoma cells. Oncogene Res 1987;2:1-18.

Thiele CJ, Cazenave L, Bolen JB, Israel MA. Developmentally regulated genes in neuroblastoma. Adv in Neuroblast Res (In Press).

Veillette A, Foss FM, Sausville EA, Bolen JB, Rosen N. Expression of the lck tyrosine kinase gene in human colon carcinoma and other non-lymphoid human tumor cell lines. Oncogene Res 1987;1:357-74.

Veillette, A, Horak ID, Bolen JB. Post-translational alterations of the tyrosine kinase $p56^{lck}$ in response to activators of protein kinase C. Oncogene Res (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05482-03 LTVB

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of Papillomavirus Late Transcription

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. C. Baker	Senior Investigator	LTVB	NCI
Others:	L. M. Cowsert	Biotechnology Fellow	LTVB	NCI
	U. Linz	Visiting Fellow	LTVB	NCI
	J. S. Noe	Biologist	LTVB	NCI

COOPERATING UNITS *(if any)*

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Laboratory of Tumor Virus Biology

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Viral Oncology Section

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the spece provided.)*

The papillomaviruses cause benign and malignant lesions of squamous epithelia in higher vertebrates. The complete lytic cycle of these viruses (including late gene expression) occurs only in the differentiated cells of the squamous epithelium. Malignant lesions and infected cells in culture do not produce virus. An understanding of the transcriptional regulation of the papillomaviruses and its relationship to the control of epithelial cell differentiation is necessary for the elucidation of the role of the papillomaviruses in carcinogenesis. We have used bovine papillomavirus type 1 (BPV-1) as a model system for the study of papillomavirus late transcription and its control. A strong viral transcriptional promoter (called the late promoter) was previously identified in this laboratory and is active only in productively infected epithelium. Transcription from BPV-1 promoters was studied in a HeLa cell in vitro transcription system and all but one early promoter (P7940) were active. The late promoter showed only very weak activity in this system; thus the in vitro system may provide a good assay for transcription factors extracted from productively infected tissues. In vitro keratinocyte culture systems are also being developed for the study of late viral transcription. Nuclear run-off analysis of BPV-1 transcription in transformed C127 cells indicated that transcription of the BPV-1 genome is attenuated greater than tenfold between the early and late polyadenylation sites. In addition we have mapped a negative element to the 3′ L1 ORF and 3′ untranslated region which inhibits chloramphenicol acetyltransferase (CAT) expression in an orientation-dependent manner when cloned into the 3′ untranslated region of a CAT expression vector. This factor may selectively destabilize late mRNAs.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. C. Baker	Senior Investigator	LTVB NCI
L. M. Cowsert	Biotechnology Fellow	LTVB NCI
U. Linz	Guest Researcher	LTVB NCI
J. Noe	Biologist	LTVB NCI

Objectives:

1. To study the control of late transcription of papillomaviruses using bovine papillomavirus type 1 (BPV-1) as a model system.
2. To set up an in vitro transcription system which faithfully utilizes BPV-1 promoters and to use this system to assay cellular and/or viral transcription factors.
3. To determine the viral and/or cellular factors involved in the trans-activation of the major late viral transcriptional promoter.
4. To identify the cis-acting sequence elements involved in the control of the major late promoter.
5. To determine if transcription termination between the early and late polyadenylation sites plays a role in the control of BPV-1 late gene expression.
6. To construct eukaryotic expression vectors suitable for the identification and mapping of cis-acting regulatory elements which lie within and 3′ to transcription units.
7. To identify the cis-acting sequence elements in the late region of BPV-1 which may control late transcription through transcription termination, polyadenylation, and/or mRNA stability.
8. To identify the viral and/or cellular trans-acting factors which interact with late region sequence elements to control late transcription.
9. To develop an in vitro keratinocyte culture and differentiation system which is capable of supporting BPV-1 productive infection and to use this system to study regulation of late gene expression.

Methods Employed:

1. Tissue culture for the preparation of viral mRNA from non-productively infected cells.
2. Inoculation of calves by BPV-1 to generate fibropapillomas for the isolation of mRNA and factors produced during productive infection.
3. Standard isolation of DNA and RNA from cells and tissue.
4. Transcriptional analysis by cDNA cloning, Northern blotting, primer extension, nuclease S1 protection and nuclear run-off analysis.
5. Construction of eukaryotic expression vectors using standard recombinant DNA technology.
6. Control element mapping by the generation of successive deletions in viral control regions using the exonuclease III deletion system and subcloning of these deletions into eukaryotic expression vectors.

7. Functional analysis in vivo of transcriptional control elements by the transfer of recombinant plasmids into cells and the assay in vitro for recombinant gene products.
8. Selection of stable cell lines containing recombinant expression vectors for the analysis of transcription termination by nuclear run-off analysis and for the analysis of mRNA stability by actinomycin-D chase analysis.
9. Functional analysis of transcriptional promoter sequence elements and trans-acting factors using in vitro transcription systems.
10. Preparation of crude nuclear extracts from bovine fibropapillomas and BPV-1 transformed cells to assay for trans-acting factors in the in vitro transcription system.
11. Identification of cDNAs encoding trans-activation factors by transfection into cell lines containing BPV-1 genomes and assaying for promoter activation by primer extension analysis.
12. Culture of bovine keratinocytes on rafts to allow full differentiation in vitro.
13. Analysis of the effects of keratinocyte differentiation on BPV-1 late gene expression in the in vitro raft system.

Major Findings:

1. We have used a HeLa cell in vitro transcription system to study transcription from BPV-1 promoters. Five of six bovine papillomavirus type 1 (BPV-1) promoters, which were previously mapped by determining the 5′ termini of viral mRNAs from bovine fibropapillomas and BPV-1 transformed cells, were found to be active under in vitro transcription conditions. Transcription initiation at each of these promoters was accurate at the nucleotide level as determined by primer extension analysis. The most active promoters in vitro were P_{89} and P_{7185}. P_{89}, which requires $E2RE_1$ in cis and the E2 transactivator protein in trans for in vivo activity, is efficiently transcribed in vitro in the absence of these factors. P_{7185} is the weakest BPV-1 promoter in vivo and is probably repressed by a labile repressor. In vitro, however, P_{7185} is efficiently transcribed, suggesting that repression of this promoter does not occur in vitro. The two internal promoters, P_{2443} and P_{3080}, are also transcribed in vitro, providing further evidence that these internal RNA start sites are actually promoters and not RNA processing sites. The late promoter, which is inactive in BPV-1 transformed cells and active only in productively infected tissues, is very inefficiently utilized in vitro. Thus it should be possible to use the in vitro transcription system to assay for trans factors in wart extracts which are capable of activating this promoter. Initial experiments failed to activate P_L, however. Activity of P_{7940} has not been observed in vitro. A manuscript describing these results is in press in the Journal of Virology.

2. In order to identify host cell and/or viral specific trans factors capable of activating the BPV-1 late promoter (P_L), we have transfected a fibropapilloma cDNA expression library into cells carrying BPV-1 genomes. No activation of P_L was observed in initial studies using BPV-1 transformed C127 cells. However, primer extension analysis suggested weak activation of P_L with selected cDNA sublibraries and BPV-1 containing fibroblasts isolated from a bovine fibropapilloma. We are now developing BPV-1 infected bovine fibroblast, keratinocyte and conjunctival cell lines to use in these experiments. In preliminary studies, we have been able to demonstrate the in vitro infection of

bovine keratinocytes with purified BPV-1. Southern and Northern blot analysis of infected keratinocytes has demonstrated the presence of transcriptionally active episomal BPV-1 DNA in these cells. Experiments are also being carried out to determine cell culture conditions that will allow complete differentiation of bovine keratinocytes in vitro. Briefly, keratinocytes are plated on a collagen matrix containing irradiated feeder cells. Cultures are grown submerged in cell culture media for seven days and then raised such that the cells are exposed to air and nutrients are transported through the collagen matrix. In preliminary experiments using human keratinocytes, we have been able to induce stratification and partial differentiation. This system will be used with infected bovine keratinocytes to study the effects of differentiation on transcription from BPV-1 promoters with emphasis on the late promoter.

3. To more accurately map transcription termination within the late region of BPV-1, a series of small subgenomic fragments which cover the entire genome were cloned. These subgenomic clones were used as hybridization probes to quantitate BPV-1 RNA labeled in vitro in nuclei isolated from BPV-1 transformed C127 cells. These analyses revealed that the rate of transcription of the 3′ early region remains high in the 5′ part of the late region and then is attenuated approximately fivefold about 1 kb downstream from the early polyadenylation site. Transcription continues to decrease throughout the rest of the late region and is down at least tenfold by the late polyadenylation site. This tenfold transcription attenuation in the late region would effectively favor the use of the early polyadenylation site over the late polyadenylation site and thus is one major component of the block to late transcription in the BPV-1 transformed cell and presumably also in the infected fibroblasts and basal epithelial cells of a fibropapilloma. There are still detectable levels of transcription near the late polyadenylation site, however, indicating that additional blocks must exist. A manuscript describing these results is in preparation.

4. A new series of chloramphenicol acetyl transferase (CAT) expression vectors (pOBCAT) has been designed for the mapping of negative regulatory elements. The pOBCAT vectors express levels of CAT in transfection assays which are approximately 100-fold higher than those obtained using pSV2CAT vectors. This facilitates transfection analysis in BPV-1 transformed C127 cells which typically give low levels of expression in transient assays and make these vectors ideal for the mapping of negative regulatory elements. An additional property of the pOBCAT vectors is an absolute requirement for a polyadenylation signal for efficient CAT expression, making them useful for assaying for the utilization of a polyadenylation signal. The pOBCAT vectors are also being used in a collaboration with the laboratory of Dr. David Levens, Laboratory of Pathology, DCBD, NCI, to study negative regulatory elements involved in the control of c-myc transcription.

5. An approximately 1 kbp fragment (n. 4452-5433) of the BPV-1 late region inhibits CAT expression by at least 100-fold when cloned into the intron upstream of the CAT gene in the pOBCAT vectors. Since this region of BPV-1 lies immediately upstream of the transcription termination described in (3), it is likely that this inhibition is due to transcription termination. The BPV-1 fragment has been cloned into a polylinker designed for the generation of unidirectional deletions using Exonuclease III. This system will be used to map the cis element(s) involved in transcription termination. Once the cis element

is mapped, we will identify cellular or viral factors which interact with this element and attempt to determine how transcription termination is regulated during productive infection.

6. Using the pOBCAT vectors, it was demonstrated that the BPV-1 late polyadenylation signal can be efficiently utilized in the BPV-1 transformed C127 cell. Thus, it is unlikely that polyadenylation plays a significant role in the regulation of late transcription. However, sequences immediately upstream of the late polyadenylation signal inhibit CAT expression when cloned in the sense orientation between the CAT gene and the SV40 polyadenylation signal of pOBCAT, but have little effect when cloned in the same position in the antisense orientation or when cloned in an intron upstream of the CAT gene in pOBCAT or downstream of the polyadenylation signal in pOBCAT. The most likely mechanism of action of these sequences is destabilization of mRNA. Experiments are currently in progress to confirm this mechanism. Consistent with this hypothesis, however, is the observation that the sequence elements which are responsible for the rapid turnover of many unstable mRNAs (GM-CSF, c-myc, etc.) are present in the 3' untranslated regions of these mRNAs and are AT rich. The negative element which we have identified in BPV-1 has been mapped to a region which includes only the extreme 3' part of the L1 ORF and the 3' untranslated region. In addition, the late 3' untranslated regions of BPV-1 and the other papillomaviruses which have been examined are all very AT rich (70-80% AT). This suggests that similar negative elements may exist for all papillomaviruses and RNA stability may play a role in the maintenance of viral latency.

Publications:

Linz U, Baker CC. Promoters of bovine papillomavirus type 1: in vitro activity and utilization. J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05518-02 LTVB

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October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transformation and Gene Regulation of the Hamster Papovavirus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. B. Bolen	Microbiologist	LTVB	NCI
Others:	P. M. Howley	Chief	LTVB	NCI
	J. Pyper	Guest Researcher	LTVB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The hamster papovavirus (HaPV) was originally isolated from skin epitheliomas originating from hair follicle epithelial cells in Syrian hamsters. The HaPV virions are found in the keratinized layer of the epithelium from infected animals, but are not found in the basal layers. Thus, the maturation of this virus is limited to terminally differentiated keratinocytes, thereby resembling the tissue-specific tropism of the papillomaviruses. However, the morphology of HaPV virions, the DNA sequence of the HaPV genome, and the genetic organization of the HaPV genome clearly show that this virus is a member of the polyomavirus family. In contrast with other members of the polyomaviruses and papillomaviruses, HaPV injection into newborn hamsters produces rapid and acute lymphomas and leukemias which are thought to be of T cell origin. Thymectomy of the animals severely reduces the incidence of this disease but results in formation of sarcomas at the site of injection. Thus, the HaPV is capable of inducing tumors of lymphoid, mesenchymal, and epitelial origin in its natural host. The viral genes responsible for this broad tumor potential and the control of the expression of these genes are currently unknown.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Microbiologist	LTVB	NCI
P. M. Howley	Chief	LTVB	NCI
J. Pyper	Guest Researcher	LTVB	NCI

Objectives:

1. To identify and characterize the HaPV encoded proteins required for oncogenic transformation and tumor formation in hamster and other rodent cells.
2. To identify and characterize the HaPV DNA cis-acting elements responsible for the control of early and late gene expression in different types of rodent cells and to determine what trans-acting viral encoded proteins are required for HaPV gene expression.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of chimeric plasmids.
2. Cell culture.
3. Transfer of DNA into rodent cells.
4. Transcriptional analysis of viral RNAs.
5. Southern analysis of viral DNA.
6. Synthesis of oligonucleotides for site-specific mutation of viral genomes.
7. Generation of virus-specific antisera.
8. Immunoprecipitation analysis and peptide mapping analysis.

Major Findings:

This first full year of this project has been primarily devoted to the development of appropriate molecular, cellular, and immunological reagents. Since all of the HaPV transfected rodent cell lines previously generated in the laboratory were found to express very low levels of HaPV-specific RNA and protein, one initial goal was to establish rodent cell lines expressing higher levels of HaPV mRNA and early proteins. One way to accomplish this objective was to provide a potentially more active promoter-enhancer element upstream of the putative transforming protein coding region than is normally present in the HaPV genome. The element initially chosen was the well-characterized SV40 promoter-enhancer which has been shown to provide relatively strong transcriptional stimulation of a variety of exogenous genes in rodent and mammalian cells. The SV40-specific DNA sequences present in this region also contain the viral origin of replication which has been shown to act in cis as a signal for DNA replication in the presence of SV40 large T antigen, thereby providing an additional means to amplify downstream exogenous genes. Immunoprecipitation of HaPV proteins recognized by sera from rats bearing HaPV-induced tumors from 35S-methionine-labeled cos-1 cells (these cells constitutively produce SV40 large T antigen) 48 hours following transfection with the recombinant SV40/HaPV plasmid revealed that HaPV-specific gene expression was, indeed, significantly elevated over that

previously observed with the intact HaPV sequences alone. However, while the intact HaPV expressed predominantly a single size class of protein in the range expected for the HaPV large T antigen, the SV40-HaPV construct generated additional protein species which did not correspond to the predicted sizes of open reading frames in the HaPV DNA. Additionally, it was found that stable cell lines derived from either cos cells or cos cells expressing a temperature-sensitive large T antigen did not significantly overexpress HaPV-specific proteins.

The SV40/HaPV chimeric plasmid was subsequently co-transfected into FR3T3 (Fischer rat 3T3) cells along with a neomycin-resistance expression vector and G418 resistant colonies were selected at random. The cell clones were then assayed for the production of HaPV proteins by immunoprecipitation of metabolically labeled cells with HaPV rat T sera. There were several positive FR3T3 cell lines in which a strong putative large T band could be detected and also a possible middle T band. Two cellular clones were selected for further analysis (clone 1-1 and clone 2-9). Both the 1-1 and 2-9 cell clones grew rapidly in tissue culture and morphologically resembled polyomavirus (Py)-transformed rat cells. The potential T antigen profiles of these clones were then compared to those of a Py-transformed FR3T3 cell line. Analysis of these cells yielded immunoprecipitable proteins with the sizes of T antigens predicted from the DNA sequences in that the putative HaPV large and middle T proteins were slightly smaller than the corresponding Py proteins. Peptide mapping of the various proteins was then conducted in order to confirm the potential identities of the individual species. The results of these experiments suggested, however, that all of the detectable proteins yielded phosphate, methionine, and cysteine peptides that were too similar to be encoded by different reading frames, thereby raising the possibility that no detectable HaPV middle T antigen was being produced in these cells. The peptide mapping studies were more consistent with the concept that all the detected proteins represented truncated forms of large T antigen. Additional evidence to suggest that these rat cell clones were not synthesizing a full complement of HaPV early proteins was provided by the fact that following injection of 50 Fischer rats with approximately 1×10^7 2-9 cells, 0/50 rats developed tumors after three months.

The results presented above suggested that the HaPV non-coding enhancer-promoter region might be necessary for proper expression of HaPV proteins. These results also raised the possibility that proper HaPV early gene expression may require cell-type specific and/or species-specific co-factors.

To examine whether HaPV expression could be elevated or whether the proteins synthesized were dependent upon hamster specific cellular factors, a variety of hamster cell lines were transfected with HaPV DNA, the cells were metabolically labeled 48 hours later, and immunoprecipitation analysis conducted. In these assays the HAK and BHK-21 cells appeared to produce the highest levels of HaPV specific proteins. These two cell lines were subsequently used to establish cell lines following transfection with HaPV DNA along with a neomycin-resistance expression vector and G418 selection. Of the 60 G418 resistant colonies isolated, nine of 30 BHK-21 cell lines and one of 30 HAK cell lines showed the same set of HaPV anti-T sera reactive proteins not found in the G418 control cell lines.

Interestingly, these putative HaPV encoded proteins were distinguishable from the reactive proteins synthesized in the FR3T3-derived HaPV cell lines. Both HaPV positive and negative HAK and BHK-21 representative cell lines are currently being analyzed for the presence of HaPV DNA and HaPV-specific transcription.

It is anticipated that we will soon be able to define an appropriate cell culture system to use for the generation of cDNAs and also to use for rapid assessment of the possible functions of the early HaPV proteins. Additionally, specific antisera which will enable us to evaluate the proteins expressed in different cells should soon be available. The injection of HaPV DNA into hamsters should result in generating a series of tumors and a relevant model system which will allow us to assess more accurately the potential importance of various regions of the HaPV genome in the genesis of the diverse types of tumors which this virus is known to cause.

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 through September 30, 1988

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has as its charge the planning, development, implementation, and coordination of research programs on the etiology and mechanisms of carcinogenesis. In the past, particular emphasis was placed on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts were conducted on virus-host relationships in virus-induced cancers, focusing on the detection and characterization of oncogenic viruses, their mechanisms of genetic integration and expression, and their modes of transmission in animals and man. The rapid technological development of eukaryote genetics and molecular biology has resulted in a synthesis of viral and cellular gene action during neoplastic processes. As a result, the research emphasis of the Laboratory has converged on the elucidation of regulatory events which are operative in human cancers and homologous animal models. In addition, the combined maturation of human genetics and molecular biology of infectious disease have permitted the implementation of experiments designed to study interaction of viruses and genetic structure of human populations. The multidisciplinary understanding of the neoplastic process, combining knowledge and technology from immunology, pathology, physiology, molecular biology, and genetics is the common character of the various research projects of the Laboratory, with a constant consideration of therapeutic opportunities. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

SCIENTIFIC BACKGROUND AND SIGNIFICANCE: The combination of technological advances, and a number of coordinate, empirical observations, has dramatically altered the thinking of the scientific community on the molecular and genetic mechanisms of carcinogenesis. The procedures of molecular cloning, DNA sequencing, hybridoma production and somatic cell genetics have pushed the study of eukaryote genetic analysis from a speculative and interpretive discipline to the level of viewing gene action directly. A revolution in biological thinking and analysis is upon us, and these methodologies are being applied as rigorously to the dissection of carcinogenesis as to any other biological process. The contributions to the generalized journals (i.e., Science, Nature, and Cell) have experienced a quantum increase in definitive studies on the mechanism of carcinogenesis and transformation.

Several major advances are responsible for our changes in thinking, and these concepts and developments have influenced, and in part been influenced by, the research effort of the LVC, NCI. Among these generalized advances are: (1) The development of the concepts and properties of vertebrate "oncogenes." These loci are normal cellular genes which were initially described as transduced RNA segments in transforming retrovirus genomes and have also been discovered by focus induction after transfection of mouse 3T3 cells with

genomic DNA extracted from human tumors. The limited number of proto-oncogenes (circa 40) described to date has attracted considerable research emphasis over the past few years as an experimental opportunity to study neoplastic transformation directly from both genetic and molecular perspectives. (2) The normal functional role of several of these oncogenes during tissue development has recently been elucidated in several systems, including erb-B, fms, sis, jun, fos, ras, and a role in signal transduction, phosphorylation and in stimulation of cellular differentiation or proliferation. (3) Virtually all of the human oncogene homologs have now been chromosomally mapped to specific positions on the human gene map. These are in addition to the nearly 4,000 different loci comprising the human genetic map. In addition to the proto-oncogene loci, approximately 20 additional described loci are thought to participate in neoplastic transformation in man (e.g., growth factors, cell surface antigens, retroviral receptors, and integration sites, etc.). A dramatic advance has been the demonstration that certain human tumors with specific chromosomal rearrangements can be interpreted to involve the modulated regulation of cellular oncogenes by normally distant DNA regulatory elements which have been placed adjacent to the oncogenes by chromosomal rearrangement. (4) Despite a long history of unsuccessful attempts to isolate human type C retroviruses in a valiant effort by the former NCI Virus Cancer Program, two human diseases have been associated with exogenous human retroviruses. These are adult T-cell leukemia, which is etiologically associated with human T-cell leukemia virus-I and -II, and acquired immunodeficiency syndrome (AIDS), which has been serologically correlated with the development of antibodies to a retrovirus designated human immunodeficiency virus (HIV). (5) The study of animal models of certain of the homologous diseases in primates, rodents, and cats has provided terrific opportunities for reconstruction and analysis of the initiation and progress of mammalian tumors. For example, the AIDS models in primates (simian acquired immunodeficiency syndrome [SAIDS]) and in FeLV-infected cats (feline acquired immunodeficiency syndrome [FAIDS]) have modified significantly our interpretation of retroviral pathology to include broad levels of immune impairment in addition to (and possibly, in combination with) leukemogenesis.

The most significant findings of the LVC during this year are discussed below.

1. Expansion of the human gene map using physical mapping methods on genes which influence human tumorigenesis. The combination of two gene mapping technologies, somatic cell hybrid analysis and in situ hybridization, has been used in collaborative studies to genetically map 35 distinct human structural genes at the LVC. The rodent x human hybrid panels were developed using isozyme markers and high resolution G-trypsin banding from a group of over 1,000 hybrid clones. Southern analysis of hybrid DNA and in situ hybridization of tritiated probes to metaphase chromosomes have resulted in chromosomal localization of nearly 30% of the proto-oncogenes on the human gene map, plus loci for several growth factors, growth factor and retrovirus receptors, endogenous retroviral segments, and retroviral integration sites. A previously unknown cluster of nine structural loci related to hematological development was discovered on human chromosome 5q and was found to be related to the $5q^-$ anemia, a syndrome characterized by several abnormalities in blood cell production. The collaborative gene mapping studies have served as the basis for several ongoing projects which relate to the genetic events involved in neoplastic transformation.

2. Progress in the search for human genetic loci which influence infectivity, disease progression and immune response after exposure to HIV. The epidemic of human AIDS has resulted in a massive research effort to understand the virology, epidemiology, and molecular biology of HIV, the etiologic agent. A striking feature of the epidemiology is the observation that only a small fraction of the exposed individuals actually have clinical AIDS. Although the temporal data must await several years before a definitive conclusion can be reached, it is probable that many of the exposed individuals will never develop AIDS. There are three simple explanations for heterogeneous response to virus exposure based upon model systems from other disease outbreaks. These include (1) functional genetic variability in the virus population; (2) cofactor requirement or a stochastic event such as a second virus, a somatic mutational event, blastogenic transformation of latently infected cells, etc.; and (3) genetic polymorphism in host populations for alleles which delimit viral infection and effect. The focus of this project is the search for human genes in category three. We shall employ the human restriction fragment length polymorphism (RFLP) markers dispersed throughout the human genome to detect distortion in population genetic equilibrium of disease-resistance/sensitivity loci. The combination of rapid advances in AIDS research, human gene mapping, population genetics, and epidemiology makes the timing appropriate for this analysis. We anticipate the use of approximately 400 gene probes and 5,000 patients from different disease categories in about 12 collaborative cohorts. At this writing we have collected 392 probes and 730 patients from 10 AIDS cohorts. Their genetic characterization is in progress.

3. Demonstration of the interactive consequences of population genetic structure and disease outbreaks in animal populations. Population genetic analysis of human and animal populations has been used to study the genetic health and disease susceptibility of several species. The south African subspecies of cheetah was shown to be genetically depauperate insofar as it is monomorphic at 52 isozyme loci. Unrelated cheetahs also accepted skin grafts, a situation without precedent among outbred mammalian species. A devastating epizootic of feline infectious peritonitis (an RNA-containing coronavirus) in cheetahs was hypothesized to result from abrogation of a major histocompatibility complex haplotype in T-cell stimulation. We have hypothesized the occurrence of two severe population bottlenecks in the recent history of the species. The first probably occurred toward the end of the Pleistocene approximately 10,000 years ago; the second was possibly a consequence of trophy hunting around the late 1900s. A molecular phylogeny of the great and lesser apes and man was derived based on genetic distance of 383 different proteins resolved by two-dimensional gel electrophoresis (2DE). A molecular phylogeny of the 37 species of the Felidae was constructed based on several molecular measures of evolutionary distance. Similarly, a consensus phylogeny of the Urisidae, Ailuropoda (giant panda) and Ailurus (red panda), was derived from distance matrices derived from three distinct molecular measures of genetic distance plus a cladistic analysis of isozyme and 2DE character data. A comparative analysis of cytological and linkage maps of mammals has indicated a noncontinuous tempo of chromosomal evolution in certain lineages (e.g., primates, felids) that are highly conserved in their chromosomal presentation, while others (rodents, lesser apes, canids) are chromosomally shuffled as if rapid saltatory cytological rearrangements occurred during the speciation events. A reconstruction of cytological rearrangements which have occurred during carnivore evolution has been achieved with particular emphasis on Canidae, Felidae, and Ursidae.

4. Functional characterization of the raf oncogene family in man. This research is an ongoing project which has led to the identification of four chromosomally distinct loci, some of which are transcriptionally modified in human tumors. Among the highlights of the past year's results are: (1) Characterization of genomic DNA of human A-raf-1 and its pseudogene(s). (2) Dissection of the transcriptional control region of c-raf-1, identification of alternate splice products and alteration of c-raf-1 in human lung and renal carcinoma. c-raf-1 is a clone marker of Von Hippel Lindan disease, a genetic cancer syndrome. (3) Experiments to functionally map c-raf-1 and A-raf-1 products in the signal transduction pathway of growth factors which showed that both genes appear to act downstream of protein kinase C (PKC), tyrosine kinase and ras oncogenes, making them the end of a chain that includes most other peripheral cytoplasmic and membrane-associated oncogenes. Thus, raf protein kinases and their regulation would appear to be ideal targets for the tailoring of modulating drugs with a potential for therapeutic significance. (4) The c-raf-protein-associated serine/threonine-specific kinase was further characterized. N-terminal truncation and specific point mutations increase kinase activity as well as transforming ability. (5) There is a division of labor between members of the raf family; c-raf-1 appears to have a basic regulatory role in most tissues, i.e., it is expressed everywhere, albeit at varying levels, whereas A-raf-1 is more restricted in its expression with highest levels in the epididymis. (6) Biochemical evidence was obtained for a role of c-raf-1 in all histological types of lung carcinoma. The gene is expressed at unusually high levels in 60-80% of all tumors as determined by Northern, immunoblot and immunohistochemical techniques. Moreover, c-raf-1 protein kinase is constitutively activated in these tumors. We are currently evaluating the role of c-raf in transformation of these cells. To aid in this evaluation, an animal model system was developed for rapid (5-13 weeks), high frequency induction of lung adenocarcinoma in mice. The tumors contain transforming DNA as judged by DNA transfection and express uniformly high levels of c-raf RNA and protein. Attempts to modulate tumor induction and promotion in these mice by stimulation of an anti-raf protein-directed immune response yielded promising results since the latency, but not the final incidence of tumor development in mice promoted with butylated hydroxytoluene, was almost doubled.

5. Biological and molecular genetic dissection of the synergistic aspects of raf and myc oncogenes in tumorigenesis. Highlights of our work on raf-myc synergism, function and regulation of expression of myc are as follows: (1) v-raf and v-myc oncogenes act synergistically in vivo in the mouse for induction of carcinoma as well as of lymphoid/hematopoietic tumors and in vitro in the transformation of chondrocytes, macrophages, myeloid stem cells, and B-cells from primary hematopoietic stem cell cultures. The basis for this synergism is a combination of signals required early (competence) and late (progression) in the G1 phase of the cell cycle and are thought to involve activation of myc or equivalent nuclear factors by raf-mediated phosphorylation. (2) High level expression of v-myc can abrogate the requirement of hematopoietic/lymphoid and fibroblastic cells for a variety of c-myc or, more generally, competence gene-inducing growth factors including interleukin (IL)-3, IL-2, and platelet-derived growth factor. Thus, myc appears to serve as a second messenger function for competence-inducing growth factors. (3) Evidence for an autoregulatory mechanism in c-myc transcription regulation was established in three different lineages of mouse cells infected with recombinant retroviruses expressing high levels of v-myc. High level expression of

the full complement of v-myc sequences invariably shut off c-myc expression in these cells and analysis of v-myc deletion mutants has defined elements within v-myc controlling nuclear localization, and c-myc suppression and induction. Moreover, we have extended this observation to include N-myc in the cross-regulation of c-myc expression. (4) A role for tyrosine kinase genes in IL-2 signal transduction and regulation of c-myc expression was established in IL-3-dependent murine FDC-P1 myeloid cells. Specifically, using retroviruses harboring wild-type and temperature-sensitive versions of the v-abl tyrosine kinase we have shown that conditional expression of function abl correlates with the ability of this oncogene to abrogate IL-2 dependence and trans-activate expression of c-myc.

6. Characterization of promotion sensitivity genes and transforming genes in mouse and human cells. Two genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been previously cloned. These putative genes, termed pro 1 and pro 2, have been sequenced and are being characterized with respect to mode of activation, regulation of expression, and nature of the gene products. Unique pro 1-hybridizing transcripts have been identified in mouse cytoplasmic RNA. These transcripts are polymerase III transcripts and contain B1-alu sequences. Polymerase III transcripts can play a role in regulating mRNA splicing, stability, transport, or translation. An aberrant polymerase III transcript, such as a P^+ active one, may cause altered post-transcriptional regulation of other genes. DNA from the human nasopharyngeal carcinoma cell line, CNE_2, has been shown to be P^+ active. This activity is, at least in part, attributable to activated homologs of mouse pro 1, as shown by screening a CNE_2 genomic library with a mouse pro 1 probe and testing the homologs for P^+ activity after transfection into resistant mouse cells. Coincidence of P^+ activity and homology to mouse pro 1 has been demonstrated in a 1-kb fragment. Inactive pro 1 homologs isolated from a normal human library and from the CNE_2 library are being compared with activated CNE_2 pro 1 to ascertain the mode of activation. Sequencing and assay of chimeric constructs of sequences from human pro 1 that is P^+ active or inactive is expected to elucidate sequences critical to biological activity. Transforming genes whose activity is detectable in JB6 P^+ cells (without tumor promoter), but not in NIH 3T3 cells, are being cloned from human nasopharyngeal carcinoma and colon carcinoma cell lines. In both cases transforming activity appears to be (1) attributable to a non-ras gene, and (2) dependent on the presence of an activated pro gene.

7. The role of a C kinase substrate and of a C kinase-regulated trans-acting factor in promotion of neoplastic transformation. Both activation of PKC and the subsequent loss of PKC activity may be on the signal transduction pathway for 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted transformation. A C-kinase substrate of 80 kDa has been found to be differentially phosphorylated in P^-, P^+, and neoplastically transformed JB6 cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. This p80 is postulated to be a tumor suppressor. Western analysis indicates that the progressive decrease of phosphorylated p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. Recent studies on TPA-inducible genes have focused on those regulated by the trans-acting transcriptional factor, AP-1 (jun oncogene). TPA induces AP-1 regulated gene expression in P^+ but not P^- JB6 cells. This indicates that AP-1 regulated gene expression (1) may be required for tumor promoter-induced transformation, and (2) may be, in turn, controlled by activated pro genes

found in P^+ but not in P^- cells. Finally, pro 1 and pro 2 transfectants show cotransfer with pro genes of AP-1 inducibility and sensitivity to calcium-regulated promotion and antipromotion, indicating that similar pathways are specified by pro 1 and pro 2.

8. Characterization of retroviruses (type-D and SIVs) isolated from primates. Three distinct primate lentiviruses (simian immunodeficiency viruses, SIV) have been isolated from a macaque (M. nemestrina) housed at the University of Washington Primate Center (SIV/Mne), from a wild-caught mangabey (SIV/Cat) and from a colony-housed African green monkey (SIV/Cae). The relatedness of SIV/Mne to other lentiviruses was examined by amino acid sequence analysis of gag proteins. Of 125 residues sequenced, 92% of the SIV/Mne amino acids were identical to predicted residues of SIV/mac (an SIV isolate from the New England Primate Center), 83% were identical to HIV-2, and 41% to HIV-1. SIV/Mne has been inoculated into six macaques and two baboons. All six macaques became viremic and died 15 to 120 weeks after inoculation, with immunologic abnormalities including a marked decrease in CD4+ peripheral blood lymphocytes. The baboons were antibody- and virus-negative and remained healthy. A full-length molecular clone of SIV/Mne has been obtained; a 1.7-kb fragment at the 3′ end of the virus has been sequenced and shown to be 92% homologous to SIV/mac. This molecular clone has also been inoculated into macaques, and elicits an antibody response.

A protein designed p14 and purified from SIV/Mne virus preparations was shown to be the translational product of the X-open reading frame (X-ORF) of SIV. A homologous protein (16K) was also shown to be present in HIV-2; there is no X-ORF in HIV-1. Thus, SIV/Mne and HIV-2 appear to be the first known examples of retroviruses to contain substantial amounts of viral proteins that are not products of the gag, pro, pol, or env genes. Purified p14 binds to single-stranded nucleic acids in vitro. Diagnostic procedures to be developed based on this protein will distinguish HIV-2/SIV from HIV-1. This will facilitate epidemiological studies to more accurately evaluate the spread of these viruses and their role in the pathological manifestations accompanying AIDS.

9. Characterization of HIV mutants defective in gag gene processing. Single-cell clones of HIV-1-infected HuT 78 cells have been obtained which produce virus with no mature gag proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analyses of proteins associated with this virus show only large amounts of the HIV-1 gag precursor, Pr55. Electron microscopy reveals aberrant "immature" virus particles which contain an electron-lucent core surrounded by a semielectron-dense, incomplete ring of ribonucleoproteins. Purified and lysed virus preparations lack an intact protease; the addition of partially purified protease isolated from a "wild-type" virus results in the cleavage of Pr55 to proteins that comigrate and cross-react antigenically with the mature HIV-1 gag proteins, p26 and p17. This in vitro assay for HIV protease using its natural substrate, Pr55, is being used to identify HIV protease-specific inhibitors that may have therapeutic applications in treating HIV-infected patients.

The proteolytic cleavage products of the gag precursor of a related virus, simian immunodeficiency virus (SIV/Mne), have been purified and their amino acid sequences partially determined. The SIV/Mne gag precursor (Pr60gag) is cleaved to proteins with the order p16-p28-p2-p1-p6. p16 is the 5′ gag protein, and p8 was identified as the nucleic acid binding protein. Peptide

bonds cleaved during proteolytic processing of the SIV gag precursor are similar to bonds cleaved during processing of HIV-1 gag precursors, suggesting that the SIV and HIV viral proteases have similar cleavage site specificities.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05367-04 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Genetic Structure of Natural Populations of Past and Present

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Hector Seuanez	Visiting Scientist	LVC	NCI
	Thomas Fanning	Expert	LB,DCBD	NCI

COOPERATING UNITS *(if any)* LCS, ALC, NIH, Beth. MD (D.Goldman); Natl. Zool. Park, Wash. DC (D.Wildt, M.Bush); Natl. Museums Kenya, Nairobi, Kenya, E. Africa (R.Leakey); PRI, Frederick, MD (W.Modi, D.Janczewski); Dept. Ecol.Behavior, Minneapolis, MN (C. Packer); H&W Cytogenetic Serv., Inc., Lovettsville, VA (W. Nash)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Population genetic analysis of human and animal populations has been used to study the genetic health and disease susceptibility of several species. The south African subspecies of cheetah was shown to be genetically depauperate insofar as it is monomorphic at 52 isozyme loci. Unrelated cheetahs also accepted skin grafts, a situation without precedent among outbred mammalian species. A devastating epizootic of feline infectious peritonitis (an RNA-containing coronavirus) in cheetahs was hypothesized to result from abrogation of a major histocompatibility complex haplotype in T-cell stimulation. We have hypothesized the occurrence of two severe population bottlenecks in the recent history of the species. The first probably occurred toward the end of the Pleistocene approximately 10,000 years ago; the second was possibly a consequence of trophy hunting around the late 1900s. A molecular phylogeny of the great and lesser apes and man was derived based on genetic distance of 383 different proteins resolved by two-dimensional gel electrophoresis (2DE). A molecular phylogeny of the 37 species of the Felidae was constructed based on several molecular measures of evolutionary distance. Similarly, a consensus phylogeny of the Urisidae, Ailuropoda (giant panda) and Ailurus (red panda), was derived from distance matrices derived from three distinct molecular measures of genetic distance plus a cladistic analysis of isozyme and 2DE character data. A comparative analysis of cytological and linkage maps of mammals has indicated a noncontinuous tempo of chromosomal evolution in certain lineages (e.g., primates, felids) that are highly conserved in their chromosomal presentation, while others (rodents, lesser apes, canids) are chromosomally shuffled as if rapid saltatory cytological rearrangements occurred during the speciation events. A reconstruction of cytological rearrangements which have occurred during carnivore evolution has been achieved with particular emphasis on Canidae, Felidae, and Ursidae.

GPO 914-918

Project Description

Names Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Hector Seuanez	Visiting Scientist	LVC	NCI
Thomas Fanning	Expert	LB,DCBD	NCI

Objectives:

(1) Use of molecular procedures to determine phylogenetic affinities and relationships between extant species of hominoid primates, felids, and selected carnivores. The derived topologies have important implications for heterologous embryo transfer and more generally for the ultimate resolution of the natural history of interacting gene systems that drive development and carcinogenesis. (2) Development of molecular procedures for assessing the genetic status of natural populations and for use in studying heritability of disease susceptibility, both congenital and etiologic. (3) The biologic resolution of adaptive strategies employed by rarely studied mammalian populations for defense against neoplastic and infectious etiologic agents that affect human populations.

Methods Employed:

The following techniques were employed: (1) cell culture procedures, (2) isozyme electrophoresis, (3) two-dimensional gel electrophoresis (2DE), (4) microcomplement fixation using heterologous rabbit antisera, (5) high resolution cytogenetics procedures, (6) gene mapping procedures using somatic cell hybrids, (7) virological procedures, (8) surgical skin grafting, and (9) statistical analysis of phylogenetic algorithms, DNA.

Major Findings:

1. Resolution of the human-chimpanzee-gorilla evolutionary trichotomy using phenetic and phylogenetic analysis of 383 fibroblast protein variants resolved by 2DE. A molecular phylogeny for the hominoid primates was constructed by using genetic distances from a survey of 383 radiolabeled fibroblast polypeptides resolved by 2DE. An internally consistent matrix of Nei genetic distances was generated on the basis of variants in electrophoretic position. The derived phylogenetic tree indicated a branching sequence, from oldest to most recent, of cercopithecoids (Macaca fascicularis), gibbon-siamang, orangutan, gorilla, and human-chimpanzee. A cladistic analysis of 240 electrophoretic characters that varied between ape species produced an identical tree. Genetic distance measures obtained by 2DE are largely consistent with those generated by other molecular procedures. In addition, the 2DE data set appears to resolve the human-chimpanzee-gorilla trichotomy in favor of a more recent association of chimpanzees and humans.

2. Molecular and cytological evolution in Carnivora: Canidae (dog family). The dog family, Canidae, consists of approximately 40 extant species which are generally classified in 10 to 12 genera. They have a worldwide distribution and their taxonomic relationship has been disputed. We have collected blood and tissue culture samples from 17 species representing each of the genera and used these materials for molecular evolutionary distance estimations. The products of 51 genetic loci for each species were analyzed by isozyme gel electrophoresis to generate matrices of genetic distance and character states. In addition, phylogenetic trees based upon parsimonious arranging of cytological exchanges were derived. Cladistic and phenetic evolutionary trees were produced with computer assistance. The results indicate that the Canidae can be divided into several monophyletic groups within which there are various degrees of phylogenetic similarity: the wolf-like canids, the South American canids, the Vulpes-like canids (foxes), Urocyon, Nyctereutes, and Otocyon.

3. Molecular evolution of Carnivora: Ursidae (bears) and Ailuropoda (giant panda). The taxonomic status of the giant panda and the lesser panda has been a biological puzzle since their description by western naturalists a century ago. We applied four independent molecular and one cytological method to resolve the phylogenetic position of the giant and red pandas, of the seven living Ursid species, and a few representative procyonids. The conclusions of these studies are summarized as follows. Between 30 and 50 million years before present (MYBP), the progenitors of the modern ursids and procyonids split into two lineages. Within 10 million years of that event (possibly at its inception), the procyonid group split into Old World procyonids and the New World procyonids. The red panda and giant panda clearly do not share a common ancestor after the ursid-procyonid split, emphasizing that the morphological similarities of the panda are probably the result of parallel retention of ancestral characters that may have been lost (for example, in the bear) after their divergence from the main line. At about 18 to 25 MYBP, the ancestor of the giant panda diverged from the ursid line. This event was at least 20 million years after the initial divergence of the ursid and procyonid split. Near the time that the orangutan diverged from the African ape-human line (13 to 16 MYBP) the earliest true bear, Tremarctos (spectacled bear), split from the ursid line. The genus Ursus began its radiation into the ursine bears (brown, black and sun bears) 6 to 8 million years later (8 to 12 MYBP). A retrospective analysis of the karyology for this group indicated that the lineages leading to Tremarctos and Ailuropoda experienced a comprehensive fusion event of the primitive ursine acrocentric chromosomes.

4. The evolution of chromosome morphology in Carnivora is conservative, with the exceptions of global rearrangements in two families, Ursidae and Canidae. We have prepared high resolution G-banded karyotypes of over 90% of the species in three carnivore families, Ursidae, Canidae and Felidae, plus representative members of the other families. Within the Felidae, the 37 species all have either 18 or 19 pairs of chromosomes. Fifteen of these are invariant in all cats and 14 of these are found intact in all other carnivore families. This means we can derive an ancestral "carnivore" karyotype which we have done and it is very similar to the feline karyotype. We can also use the four variable feline chromosomes to generate a minimum distance cladistic evolutionary tree based on chromosome morphology. When this was attempted, the derived trees were consistent with the major clades predicted by the albumin immunological

distance (AID) and allozyme genetic distance trees. When other carnivore families were examined the karyotypes were very homologous to the Felidae with the exception of Ursidae (discussed in Number 3) and the Canidae. The extensive chromosomal arrangements in the Canidae were not, however, beyond resolution. We have used the chromosomal morphology to reinterpret Canid evolution and a minimum distance evolutionary tree was derived by cladistic methods.

5. Demographic analysis of the captive cheetah population in North American zoological facilities reveals a population headed for extinction. The African cheetah has been bred in North American zoological facilities since 1956. The captive population has since grown to around 200 animals due to a combined increase in importation plus captive births. From 1982 to 1986, the captive birth rate declined by 50%, primarily due to a low frequency of breeding individuals in the population. The 1986 population had an effective breeding size of 28.1 in a total population of over 193 cheetahs. The incidence of infant mortality has been high (36.7%) relative to other zoo-bred species, perhaps as a consequence of the previously observed genetic impoverishment of the species. The combination of low fecundity, high infant mortality, and population dynamics indicates that the North American captive cheetah population is neither a self-sustaining nor a theoretically "viable population" as defined by Soule et al. (1986). Possible recommendations for improving captive cheetah propagation have been developed and communicated.

6. Lions as a species show abundant genetic variation and reproductive fitness, but isolated relict lion populations display discernible genetic consequences. In the study of three distinct lion populations, a direct correlative relationship was observed between the extent of genetic variability and the physiology of reproduction. African lions from the vast outbred populations of the Serengeti Plains had abundant genetic variation, a low percentage of sperm abnormalities per ejaculate (24.8%), and high circulating levels of testosterone. Asiatic lions descended from the isolated Gir Forest of western India had no detectable allozyme variation, a high percentage of sperm pleiomorphisms (66.2%), and comparatively low testerone concentrations. African lions recently experiencing a population bottleneck in the Ngorongoro Crater, adjacent to the Serengeti Ecosystem, had less genetic variation than the surrounding founder population, while sperm abnormalities (50.5%) and peripheral testerone levels were similar to Asiatic lions. These results support the assertion that reproductive function of free-ranging mammals can be impaired as a result of demographic contraction followed by inbreeding.

7. A genetic forensic study revealed genetic introgression of the entire captive population of Asiatic lions. The Asiatic lion (Panthera leo persica) exists in the wild as a single relict population of approximately 250 individuals in the protected Gir Forest Sanctuary in western India. In 1981, a species survival plan (SSP) for the Asiatic lion was established by the American Association of Zoological Parks and Aquariums to manage the 200+ descendants of Asiatic lions in captivity in western zoological facilities. This captive population was derived from seven founders. Pedigree analysis of the genetic transmission of these three biochemical loci demonstrated that two of the five primary founder animals of the SSP Asiatic lion population were descendants of the African subspecies. Three other founder animals were pure

Asian. A retrospective SSP pedigree analysis of two morphologic characters (prominent abdominal fold and pairing of infraorbital foramen) that are partially diagnostic for persica vs leo was consistent with this conclusion as well.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05382-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genes Involved in Preneoplastic Progression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Others:	John Seed	Special Volunteer	LVC	NCI
	W. Karol Dowjat	Visiting Fellow	LVC	NCI
	Cao Ya	Guest Researcher	LVC	NCI
	Glenn A. Hegamyer	Health Science Officer	LVC	NCI
	Paul Woolley	IPA Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

Hunan Med. College, Hunan, China (K.-T. Yao); Cancer Res. Lab., Univ. W. Ontario, Canada (D. Denhardt); Dept. Radiation Oncology, Univ. Arizona Med. Sch., Tucson, AR (G.T. Bowden); PRI, Frederick, MD (R. Garrity)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
7.4	4.4	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The aim of this research is to identify and characterize genes that specify susceptibility to tumor promoter-induced neoplastic transformation in mice and humans. Evidence suggesting the involvement of such genes in animal and human systems has come from the observation that animals can be bred for sensitivity to tumor promotion. Two genes that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been previously cloned. These putative genes, termed pro 1 and pro 2, have been sequenced and are being characterized with respect to mode of activation and regulation of expression. Unique pro 1-hybridizing transcripts have been identified in mouse cytoplasmic RNA. These transcripts are polymerase III transcripts and contain B1-Alu sequences. Polymerase III transcripts can play a role in regulating mRNA splicing, transport, or translation. P+ active pro 1 homologs have been isolated from a human nasopharyngeal carcinoma cell line, CNE2. Coincidence of P+ activity and homology to mouse pro 1 have been demonstrated in a 1-kb fragment. This activity is, at least in part, attributable to activated homologs of mouse pro 1, as shown by screening a CNE2 genomic library with a mouse pro 1 probe and testing the homologs for P+ activity after transfection into resistant mouse cells. Inactive pro 1 homologs isolated from a normal human library and from the CNE2 library are being compared with activated CNE2 pro 1 to ascertain the mode of activation. Sequencing and assay of chimeric constructs of sequences from human pro 1 that is P+ active or inactive is expected to elucidate sequences critical to biological activity. Transforming genes whose activity is detectable in JB6 P+ cells, but not in NIH 3T3 cells, are being cloned from human nasopharyngeal carcinoma and colon carcinoma cell lines. In both cases transforming activity appears to be attributable to a non-ras gene.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
John Seed	Special Volunteer	LVC	NCI
W. Karol Dowjat	Visiting Fellow	LVC	NCI
Cao Ya	Guest Researcher	LVC	NCI
Glenn A. Hegamyer	Health Science Officer	LVC	NCI
Paul Woolley	IPA Fellow	LVC	NCI

Objectives:

To elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans. To clone the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells. To elucidate the structure and mode of activation of these genes. To learn how expression of these genes is regulated. To learn the functions of these genes and their products. To clone and characterize activated human homologs of promotion sensitivity genes. To clone and characterize a novel transforming gene and its regulation by pro genes from both mouse and human tumors.

Methods Employed:

The following techniques are being utilized: (1) gene cloning techniques using sib selection and hybridization search routines; (2) calcium-phosphate DNA transfection followed by assay of sensitivity to promotion of anchorage-independence by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA); (3) use of ligated dominant selectable markers; (4) generation of new recombinant DNA constructs; (5) restriction mapping; (6) sequencing the cloned genes by the Maxam and Gilbert or dideoxy technique; (7) computer-aided analysis of their structure and possible function; (8) Southern and Northern transfer techniques to analyze genome organization and expression of the pro genes; (9) RNase and S-1 protection to analyze size and sequence of RNA transcripts; (10) purification of nuclear and messenger RNA; (11) construction of genomic and cDNA libraries; (12) screening of libraries for pro gene homologs, for P^+-active sequences, and for transforming sequences using JB6 recipient cells; (13) calcium phosphate DNA transfection followed by assay of anchorage-independent, transforming activity (without TPA); and (14) deletion analysis to define minimum biologically active sequences.

Major Findings:

1. pro 1 appears to encode not a polymerase II transcript, but a small B1-Alu-containing polymerase III transcript. The use of single-stranded RNA probes for detection of pro 1 hybridizable RNA by Northern analysis revealed that a probe specific for detecting a predicted polymerase III-catalyzed transcript (but not one specific for a polymerase II transcript) hybridized to a small RNA band. Presence of a B1-Alu fragment on the pro 1 probe is essential for

detection of this small RNA. A small RNA such as this could play a role in transcriptional or translational regulation of other genes.

2. B1-Alu fragment of pro 1 is essential for P^+ biological activity. Bal-31 deletion analysis revealed that the B1-Alu fragment of pro 1 is essential for its biological activity. pro 1 was subcloned into pGEM, linearized by restriction at the 3' end and digested with Bal-31. Pools of the digested recombinant containing fragments of similar size were then tested for P^+ biological activity in a transfection assay. The fragments capable of transferring the promotion-sensitive phenotype to P^- cells were restricted to those which preserved the integrity of the B1-Alu segment of pro 1. Further studies to define the limits of the P^+ active sequence are utilizing a series of cloned Exo-III deletion mutants that have been constructed.

3. Isolation of promotion-sensitive transfectant cell lines. Transfectant cell lines carrying marker sequences have been developed. In these studies, pro 1 was subcloned into pSV2neo, a plasmid carrying a selectable marker which confers resistance on eukaryotic cells to the toxic effects of geneticin. This recombinant was transfected into P^- cells and the recipients were then selected for resistance to geneticin. Another recombinant was constructed that contains an additional marker sequence (a 194-bp fragment of x174) in the B1-Alu sequence of pro 1. Despite the insertion of this sequence, the plasmid retains the full biological activity of pro 1 in transfection assays. Transfectants carrying this recombinant will be used to assess the possible movement of pro 1 sequences within the genome following TPA treatment as well as to assess pro 1 expression.

4. A pro 2-homologous cDNA. A mouse epidermal carcinoma cDNA library obtained from G.T. Bowden of the University of Arizona Medical School was screened using suitable pro 2 probes yielding a 900-bp fragment that was then subcloned, sequenced and compared to a pro 2 genomic clone. There was 79% homology (234/295) over a 295-bp region. The region of highest homology is between nucleotides 1620 and 1910 of pro 2. This region is within the repeated sequence of nucleotides 1524 to 2060 of pro 2.

5. Coincidence of P^+ activity and homology to mouse pro 1 in human nasopharyngeal carcinoma (NPC) pro 1. Restriction mapping and Southern hybridization analysis revealed structural polymorphism among pro 1 hybridizable NPC cell CNE_2 clones. Three structural classes, termed b, m, and d have been distinguished with clones belonging to a given class indistinguishable by restriction enzyme analysis. For each class, the minimum P^+ active fragments thus far identified are a 4.4-kb EcoRI fragment for clones belonging to class m, and a 5.5-kb EcoRI fragment of clone d. The most extensively studied clones of class b appear to have P^+ activity localized in the 1-kb fragment generated by Alu-1. For all classes, P^+ activity and homology to mouse pro 1 were found to reside within the same restriction fragments. This observation implies the requirement of structural homology to mouse pro 1 for P^+ activity of CNE_2 clones.

6. Comparison of P^+ active and inactive human NPC sequences. One out of ten tested CNE_2 pro 1 homologous clones turned out to be inactive. This particular clone j belongs to class b and, so far, cannot be distinguished from active homologs i and b by restriction site polymorphism, heteroduplex formation, or Southern blot analysis using mouse pro 1 and various human molecular probes. This raises the possibility that discrete changes, rather than gross rearrangements, may determine the P^+ activation of these pro 1 homologous sequences. The complete sequence analysis of activated and non-activated clones is expected to establish the mechanism of activation. Sequencing of the 1-kb fragment of an active clone i and corresponding to the 1-kb fragment of an inactive clone j is presently underway.

7. Cloning of the transforming gene from JB6 RT101 cells. A unique transforming gene from transformed JB6 cells is being cloned by sib selection. A genomic library of 400,000 recombinants in pUC19 was prepared from size selected (3-12 kb), HindIII digested, RT101 cell DNA. This library was divided into 20 pools each containing approximately 20,000 recombinants. Each pool was assayed for the presence of a transforming gene by transfection of plasmid DNA from the pool into JB6 clone 41 cell recipients and assay of DNA-dependent, anchorage-independent transforming activity. A single pool capable of conferring the transformed phenotype on JB6 P^+ recipient cells was identified. This pool of recombinants was subsequently divided into ten equal fractions and one positive pool from this subdivided fraction was identified. This pool has been further subdivided and tested for activity, with the intent of isolating a single most active transforming fragment for further study.

8. Cloning of a transforming gene from the NPC cell line, CNE_2. NPC DNA transfers an anchorage-independent transforming activity to mouse JB6 P^+ cells. DNA from primary transfectants that is human Alu positive has been used to generate secondary transfectants. A high proportion of these secondary transfectants show three characteristics: (1) human Alu positivity of DNA, (2) transforming activity of DNA on transfection, and (3) transformed phenotype of transfectants. A genomic library of tertiary transfectants is being screened to isolate the transforming sequence(s). DNA from secondary transfectants shows the absence of any characteristic human restriction fragments of Ki-ras, H-ras or N-ras, as well as lack of transforming activity in the NIH 3T3 focus assay. Thus, a non-ras transforming gene is expected to be isolated from cells of NPC, a major form of cancer in the world.

9. DNA from human colon adenocarcinoma cell lines transfers a non-ras transforming activity. We have shown that DNA from the human colon adenocarcinoma HT-29 and DLD1 cell lines transforms P^+ JB6 clone 41 cells. Some 25 clonal transfectant cell lines were established from the primary round of transfection. Of those containing human Alu sequences, a high proportion showed a transformed phenotype and DNA that transferred anchorage-independent transforming activity. These are being used as the source of DNA for secondary transfections. This transforming activity is probably not due to the ras family because (1) DNA of neither parental cell line transforms NIH 3T3 cells; and (2) Western blot analysis showed that the p21 protein in the parent cells

showed no overexpression and no change in migration, i.e., no clear evidence of altered ras. This transforming activity is being cloned.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05383-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membrane Signal Transduction in Tumor Promotion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Others:	Stephanie Simek	IRTA Fellow	LVC	NCI
	Lori Bernstein	Biologist	LVC	NCI
	John Seed	Special Volunteer	LVC	NCI
	Bonita M. Smith	Special Volunteer	LVC	NCI
	Michael H. Antecol	Visiting Fellow	LVC	NCI

COOPERATING UNITS (if any)

Inst. Med. Sci., Univ. of Tokyo, Tokyo, Japan (T. Kuroki); Swiss Inst. for Exp. Cancer Res., Lausanne, Switzerland (P. Cerutti)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.2	1.5	1.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of these studies is to determine the required biochemical events that occur between tumor promoter-receptor interaction and the activation of effectors of neoplastic transformation. Candidate second messengers include protein phosphorylation, reactive oxygen generation, and calcium mobilization. Both activation of protein kinase C (PKC) and the subsequent loss of PKC activity may be on the signal transduction pathway for 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted transformation. A C-kinase substrate of 80 kDa has been found to be differentially phosphorylated in P-, P+, and neoplastically transformed JB6 cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. This pp80 is postulated to be a tumor suppressor. Western analysis indicates that the progressive level of p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. Recent studies on TPA-inducible genes have focussed on those regulated by the *trans*-acting transcriptional factor, AP-1 (*jun* oncogene). TPA induces AP-1-regulated gene expression in P+ but not P- JB6 cells. This indicates that AP-1-regulated gene expression (1) may be required for tumor promoter-induced transformation, and (2) may be, in turn, controlled by activated *pro* genes found in P+ but not P- cells. Finally, *pro* 1 and *pro* 2 transfectants show cotransfer with *pro* genes of sensitivity to calcium-regulated promotion and antipromotion.

DHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Stephanie Simek	IRTA Fellow	LVC	NCI
Lori R. Bernstein	Biologist	LVC	NCI
John L. Seed	Special Volunteer	LVC	NCI
Bonita M. Smith	Special Volunteer	LVC	NCI
Michael H. Antecol	Visiting Fellow	LVC	NCI

Objectives:

To determine the required biochemical events that occur between the interaction of tumor promoters with the plasma membrane and the activation of intracellular effectors of neoplastic transformation. In the case of phorbol diester tumor promoters, a major aim is to identify promotion-relevant events that are closely coupled to phorbol ester receptor binding. Candidates for such second messengers or signal transduction events include protein kinase C (PKC)-catalyzed protein phosphorylation, reactive oxygen generation, calcium mobilization, and PKC-regulated trans-acting transcriptional factors AP-1, AP-2, etc. Tumor promoter-inducible AP-1-dependent gene expression will be analyzed for promotion relevance. An overall aim is to understand the nuclear gene regulation events triggered by activation of plasma membrane PKC. Finally, a PKC substrate of 80 kDa postulated to be a tumor suppressor will be analyzed.

Methods Employed:

(1) Assay of calcium-dependent, phospholipid-dependent protein kinase (C kinase) activity; (2) assay of the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) on rate of synthesis and phosphorylation of proteins in intact promotion-sensitive (P^+) or -resistant (P^-) cells; (3) immunoprecipitation and Western blotting with antisera to PKC substrates or other P^+-related proteins; and (4) assay of a PKC-regulated trans-acting factor function such as AP-1-dependent gene expression.

Major Findings:

1. PKC substrate p80 shows progressive decrease in expression during progression from the early preneoplastic to the neoplastic state. A PKC substrate of 80 kDa (p80) was found to be progressively less phosphorylated in early preneoplastic (P^-), later preneoplastic (P^+), neoplastically transformed (Tx), and JB6 cells. Recent Western analysis using antibody to p80 has revealed a progressive decrease in the amount of p80 expressed in P^-, P^+, and Tx cells, with the P^- cells containing the highest basal level and the transformed cells containing little or no detectable p80. TPA treatment enhances the level of phosphorylation of p80 in P^- cells at 1 to 4 hours after treatment, whereas synthesis of pp80 in P^- cells remains unaffected by TPA treatment. It is possible that the function of pp80 is directly regulated

through phosphorylation by C kinase. The differential expression of pp80 in three cell lines that differ in their stage of neoplastic progression suggests that this protein may play a role in transformation suppression.

2. AP-1/jun function is differentially regulated in promotion-sensitive and promotion-resistant mouse epidermal JB6 cells. A growing body of evidence indicates that genes transcriptionally inducible by TPA encode proteins which implement tumor promotion events. Recently, it has been discovered that AP-1, a trans-acting protein factor, induces the expression of some of these genes in cells exposed to TPA. The AP-1 protein itself is encoded by a proto-oncogene, recently discovered and designated as c-jun. We hypothesize that AP-1-regulated gene expression is a required event in the promotion of neoplastic transformation, and that an activated pro gene, in turn, can play a critical role in functional regulation of AP-1. This hypothesis has been tested using the JB6 cell system of promotion-sensitive and -resistant variants. JB6 cell variants have been transfected with a construct that contains an AP-1 TPA-responsive cis-enhancer element attached to a transcriptional reporter gene encoding chloramphenicol acetyltransferase (CAT). JB6 clonal P^+ variants show inducible CAT synthesis in response to TPA; in contrast, two independently derived clonal P^- variants are not inducible by TPA. This differential inducibility is also displayed by P^+ and P^- cells treated with epidermal growth factor, another transformation promoter for JB6 P^+ cells. These data are consistent with the hypothesis that elevated expression of tumor promoter-inducible genes controlled by AP-1 is required for promotion, and that activated pro genes can dictate function of the AP-1 trans-acting protein factor.

3. Transfer of activated pro 1 or pro 2 cotransfers sensitivity to calcium modulating promoters and antipromoters. Sets of pro 1 or pro 2 transfectants (into P^- cells) were produced. These showed both P^+ phenotype and presence of the transfected gene as indicated by an indirect assay for vector hybridization. Like parental P^+ cells, these transfectant cells were sensitive to induction of transformation not only by TPA but also by calcium-analog lanthanides. In addition, these transfectants were, like parental cells, sensitive to antipromotion by ethyleneglycolbis (β-aminoethylether)-N,N-tetra acetic acid-lowered extracellular calcium. Thus, the phenotype transferred by pro 1 or pro 2 is similar to the parental P^+ phenotype and similar to each other.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05384-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Analysis of Human Cellular Genes in Neoplastic Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Takis S. Papas	Chief	LMO	NCI
	Joost Oppenheim	Chief	LMI	NCI

COOPERATING UNITS *(if any)* PRI,Fred.,MD(M.Dean);BRI,Fred.,MD(G.VandeWoude);CHB,NHLBI, Beth.,MD (N.Anagnou);Johns Hopkins Hosp.,Balt.,MD(B.Vogelstein);Rorer Biotech., Hoesham,PA(M.Jaye);Albert Einstein Coll.Med.,Bronx,NY(R.Burke);LMG,NINCDS (R. Lazzarini);LDBA,NIDR(P.Killen);CBMB,NICHD(R.Klausner); Univ.Chicago,IL(R.Burke)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland, 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The combination of three gene mapping technologies, somatic cell hybrid analysis and in situ hybridization and reverse fragment length polymorphism analysis of human pedigrees, has been used in collaborative studies to genetically map 35 distinct structural genes in man. The rodent x human hybrid panels were developed using isozyme markers and high resolution G-trypsin banding from a group of over 1,000 hybrid clones. Southern analysis of hybrid DNA and in situ hybridization of tritiated probes to metaphase chromosomes have resulted in chromosomal localization of nearly 30% of the proto-oncogenes on the human gene map, plus loci for several growth factors, retrovirus and growth factor receptors, endogenous retroviral segments, and retroviral integration sites. A previously unknown cluster of nine structural loci related to hematological development was discovered on human chromosome 5q and was found to be related to the 5q- anemia, a syndrome characterized by several abnormalities in blood cell production. The collaborative gene mapping studies have served as the basis for several ongoing projects which relate to the genetic events involved in neoplastic transformation.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Takis S. Papas	Chief	LMO	NCI
Joost Oppenheim	Chief	LMI	NCI

Objectives:

1. The augmentation of the human gene map with loci that have direct or indirect connections to the processes of neoplastic transformation in man or other vertebrate model systems. The specific genes under study fall into six general groups: (1) cellular proto-oncogene loci, (2) genes which code for growth factors, (3) genes coding for receptors for growth factors and for retroviruses, (4) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (5) chromosomal integration sites for chronic transforming retroviruses, and (6) restriction genes that delimit retroviral replication.

2. Genetic analysis of cooperative and sequential gene actions in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

3. The development of new approaches to the understanding of genetic control of carcinogenesis. This goal involves the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

4. The resolution of the sequences and tissue specificities of distinct cellular genes in different human malignancies. Thus, neoplastic pathways of genetic networks can be dissected by genetic analysis in protocols reminiscent of dissection of metabolic enzyme pathways in early microbial studies.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures including radio-immunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays, including RIA, cytotoxicity, fluorescent antibody procedures and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription *in vitro*, solution hybridization, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis, and molecular cloning of eukaryotic genes; (7) *in situ* hybridization to metaphase chromosomes; and (8) reverse fragment length polymorphism linkage analysis of human pedigrees and populations.

Major Findings:

1. The proto-oncogene family in man. Transforming genes, or proto-oncogenes, represent a class of conserved cellular genes that may play an important role in tumorigenesis. They were initially described as transduced RNA segments in transforming retrovirus genomes. They have also been discovered by focus induction after transfection of mouse NIH 3T3 cells with genomic DNA extracted from human tumors.

In collaboration with laboratories within and outside the NIH, we have mapped over one-third of the proto-oncogene loci in man. Several have included pseudogenes (ras and raf). Others have shown evidence of gene fusion (ets, met-tpr, trk, fgr). Separation of these components using hybrid panels has resolved the fusion-fission events in the evolutionary history of the oncogenes.

2. The dihydrofolate reductase (DHFR) locus and the growth factor cluster on human chromosome 5q. The human DHFR (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) gene family includes a functional gene (hDHFR) and at least four intronless genes. Three intronless genes (hDHFR-ψ2, hDHFR-ψ3, and hDHFR-ψ4) are identified as pseudogenes because of DNA sequence divergence from the functional gene with introns, while one intronless gene (hDHFR-ψ1) is completely homologous to the coding sequences of the functional gene. Analysis of genomic DNA from our panels of human x rodent somatic cell hybrids with specific molecular probes showed that the five genes are dispersed in the human genome to five different chromosomes. The functional gene, hDHFR, was assigned to chromosome (chr) 5q11.1-22 by somatic cell hybrid analysis and in situ hybridization. We have further determined the pseudogene chromosomal positions to be: hDHFR-ψ1-chr 18, hDHFR-ψ2-chr 6, and hDHFR-ψ4-chr 3.

The hDHFR-ψ1 DNA sequence is identical to the sequence of the consecutive coding exons of the functional gene, suggesting that the transposition event which produced hDHFR-ψ1 occurred rather recently in human evolution. But the hDHFR-ψ1 gene is not transcriptionally active, similar to other pseudogenes of DHFR. The hDHFR-ψ1 pseudogene exhibits a novel form of polymorphism in humans. The pseudogene sequence is present in the DNA of some chromosomes and is completely absent in others. The allelic frequency of the hDHFR-ψ1-containing chromosomes was found to be 94% in Caucasians of Mediterranean descent, 77% in Asian Indians, 67% in Chinese, 57% in southeast Asians, and 32.5% in American blacks. These data suggest that the transposition of this "perfect" pseudogene occurred rather recently, perhaps prior to the divergence of the human racial groups.

The placement of the functional DHFR locus on chromosome 5 allowed us to design a selective system based upon the requirement for active DHFR in hybrids crossed to mutant $DHFR^-$ rodent cells. Hybrids grown in the absence of both hypoxanthine and thymidine selectively retain human chromosome 5. Because the human FMS proto-oncogene had also been mapped to chromosome 5, we investigated the role of this gene (which had been shown by C. Sherr's group at the St. Judes Children's Research Hospital to encode the cell receptor for colony stimulating factor, CSF-1) in the $5q^-$ anemia, a syndrome characterized by

several abnormalities in blood cell production. Virtually all of the 5q$^-$ patients showed hemizygous dosage dependence for the FMS gene, indicating that these patients had somatically developed a deletion for FMS, which raised the possibility that a FMS product depression contributed to the 5q$^-$ anemia. This seemed particularly reasonable because CSF-1 is expressed in macrophages and in other lymphoid cells which are known to produce growth factors which augment hematological development.

At this point an interesting series of experimental events occurred. First, we discovered that two additional growth factor loci, endothelial cell growth factor (ECGF) and interleukin-3 (IL-3), or multi-CSF, mapped right on top of the FMS locus on 5q. Then other laboratories rapidly reported the localization of other loci also coding for growth factors or growth factor receptors (granulocyte macrophage-CSF, platelet-derived growth factor receptor, macrophage CSF, and the beta andronergic receptor) which are also in the 5q region. The occurrence of seven or eight different loci all related to hematopoietic and neoplastic processes in a narrow region of the human genome is perhaps significant. The only other regions of the mammalian genome known to us which exhibit such functional clustering are the immunoglobin genes and the HLA complex. The significance of this ordering and its relationship to those genes associated with disorders resulting from chromosome 5 rearrangements are under investigation in several laboratories, including the LVC.

3. Genetic characterization of the GLI family of proto-oncogenes which encodes a zinc finger protein in man. A gene, termed GLI, was identified that is amplified more than 50-fold in a malignant glioma. The gene is expressed at high levels in the original tumor and its derived cell line, and is located at chromosome 12 position (q13 to q14.3). The GLI gene is a member of a select group of cellular genes that are genetically altered in primary human brain tumors. Further characterization of GLI revealed the presence of five tandem "zinc fingers" related to those of Kruppel (Kr), a Drosophila segmentation gene of the gap class. We have used GLI cDNA as a molecular probe to isolate related sequences in the human. Partial characterization of six related loci, including sequence determination, expression studies, and chromosome localization, revealed that each locus could encode a separate finger protein. The proteins all had similar "H-C links," i.e., a conserved stretch of seven amino acids connecting the C-terminal histidine of one finger to the N-terminal cysteine of the next. Two of the loci, GLI2 and GLI3, can encode fingers which are nearly identical to those of GLI (89% and 84% amino acid similarity, respectively). Thus, the predicted proteins could be placed into one of two subgroups: the GLI subgroup (consensus finger $^{Y}/_{F}XCX_4CX_3FX_5LX_2HX_{3-4}H^{T}/_{S}GEKP$) or the Kr subgroup (consensus finger $^{Y}/_{F}XCX_2CX_3FX_5LX_2HX_3HTGEKP$). Unlike GLI or Kr, most of these loci were expressed in many adult tissues. The predicted proteins likely function to control the expression of other genes, and by analogy with Kr and GLI, may be important in human development, tissue-specific differentiation, or neoplasia.

4. Genetic localization of the human α2(IV) collagen chain locus in a position adjacent to α1(IV) collagen gene on 13q34. A 2.1-kb cDNA clone was isolated from a human placental library encoding the α2 chain of type IV collagen, a major structural protein of basement membranes. The DNA sequence encoded 445 amino acids in the triple-helical domain and 227 amino acids for the entire

carboxyl-terminal globular domain. The latter is composed of two homologous subdomains which are highly conserved (65%) between the α1 and α2 chains. The triple-helical domain contained seven interruptions of the Gly-X-Y repeat and these interruptions were, in general, larger than their counterpart in the α1 chain. DNA from human x rodent hybrid cell lines was analyzed under conditions in which there was no cross hybridization of the α2(IV) cDNA probe with the gene for the α1(IV) collagen chain. An EcoRI fragment characteristic of the α2 chain had a concordance of 0.97 with chromosome 13. This result was confirmed and extended with in situ localization of the gene at 13q34. Since the α1(IV) gene has previously been localized to 13q34, the two type IV collagen genes reside on the same chromosome, possibly in a gene cluster. The presence of the genes for type IV collagen chains on chromosome 13 appears to exclude a primary role for these genes in X-linked forms of hereditary nephritis and adult polycystic kidney disease.

5. Genetic analysis of the kappa light chain immunoglobulin deleting element: duplicated and dispersed forms. Human light chain genes are used in a κ before λ order. Accompanying this hierarchy is the rearrangement of a κ-deleting element (Kde) which eliminates the κ locus before λ gene rearrangement. In approximately 60% of the rearrangements the Kde recombines at a conserved heptamer within the J_κ-C_κ intron. We demonstrated that aberrant V/J rearrangements possessing apparent "N" nucleotides existed 5′ to the J_κ-Kde rearrangements. This suggests that the Kde may selectively eliminate nonfunctional V/J allelles. A κ-producing cell that displayed the unusual finding of λ gene rearrangement demonstrated a rearranged Kde. The rearrangement was a Vκ/Kde recombination and the heptamer-11 bp spacer-nonamer flanking the Vκ is the target site of the Kde 40% of the time. The mouse possesses a counterpart to the Kde (recombining sequence [RS]) and the highly conserved regions surround the heptamer-spacer-nonamer signals. No complete protein product was predicted from the germline Kde near its breakpoint and no consistent fusion product was predicted from either the V/Kde or V/J-Kde rearrangements. A distal portion of the Kde is duplicated and is present at 2q11 as well as 2p11. The evolutionary conservation of the κ-elimination event, and the duplication and maintenance of the Kde indicates that it has a function. A portion of the Kde may still prove to encode a trans-acting factor that directly affects λ rearrangement. A certain role for the Kde is its site-specific rearrangement, which destroys ineffective κ genes and sets the stage for λ gene utilization.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05385-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Genetic Analysis of Feline Cellular Genes: A Comparative Approach

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David Derse	Senior Staff Fellow	LVC	NCI
	Naoya Yuhki	Visiting Fellow	LVC	NCI
	Raoul E. Benveniste	Medical Officer	LVC	NCI
	Hector Seuanez	Visiting Scientist	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)* PRI, Frederick, MD (D.A. Gilbert, W.S. Modi); H&W Cytogenetics Services, Inc., Lovettsville, VA (W.G. Nash); Univ. of CA, San Diego, CA (J.S. O'Brien); NIAID, NIH, Bethesda, MD (C. Kozak); National Zoological Park, Washington, DC (D.E. Wildt)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A panel of over 200 rodent x cat somatic cell hybrid clones was employed to construct a genetic map of the domestic cat (Felis catus). The present map consists of over 70 biochemical (isozyme or DNA clone) loci. A remarkable extent of linkage homology between the feline and human maps was discovered that was three to four times more conserved than the mouse-to-human genetic synteny (linkage homology). Nearly 35% of the human cytological map can be aligned, band-for-band, with syntenically homologous feline chromosomes. This degree of linkage homology was used to estimate chromosomal location of feline homologs of proto-oncogenes and to test for transposition of the proto-oncogene family during the over 80 million years of evolution which has elapsed since man and cat shared a common ancestor. The organization of three distinct endogenous retroviral families was studied and found to resemble endogenous retroviral families in other mammalian species, including man. Genetic loci, which encode a series of lysosomal enzymes involved in feline models of human neurological storage diseases, have been localized. A molecular phylogeny of the Felidae family has been derived based upon three methodologies, and a cytogenetic description of Felidae evolution was developed.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David Derse	Senior Staff Fellow	LVC	NCI
Naoya Yuhki	Visiting Fellow	LVC	NCI
Raoul E. Benveniste	Medical Officer	LVC	NCI
Hector Seuanez	Visiting Scientist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI

Objectives:

(1) The development and expansion of the genetic map of the domestic cat (Felis catus) with particular emphasis on molecular genetic loci involved in neoplastic transformation. (2) The understanding of the genomic and developmental organization of feline loci involved in cancer. (3) The description of the comparative structure of the cat genome relative to other felids, to other carnivores, and to other mammals, specifically, mouse and man. (4) The development of the gene delivery technologies for treatment of feline models of human inborn errors.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide, and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures, including radio-immunoassay (RIA), reverse transcriptase assay, and viral cloning; (5) immunological assays, including RIA, cytotoxicity, fluorescent antibody procedures, immunoprecipitation, microcomplement fixation, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription in vitro, molecular cloning, gene splicing, DNA and RNA blotting; and (7) in situ DNA hybridization to metaphase chromosomes.

Major Findings:

1. Constructing the gene map of the cat: Extensive conservation of linkage arrangement to the human genetic map. A research emphasis on the construction of the genetic map of the cat has been a major focus in our laboratory for several years. The cat gene map now consists of over 70 loci including some 30 proto-oncogene loci. With the exception of a preliminary synteny map for the domestic dog, the only other carnivore gene-chromosome map was derived by the Russian geneticist, O. L. Serov and his colleagues for the American mink (Mustela vision). In nearly all cases where they could be compared, the mink and cat linkages agree, thereby confirming the cytological indication of homology. When we first compared linkage maps of cat and man, we were struck by the high degree of syntenic homology which existed between the two species,

especially when we considered that cat and man were in different mammalian orders. In most cases, syntenic groups located on human chromosome arms were also syntenic in cats and in the case of at least five human chromosomes (1, 6, 11, 12, and X), gene homologs from both arms are also syntenic in cats, suggesting conservation of large portions of these chromosomes. By contrast, the mouse gene map is three to four times more rearranged, compared with man, than is that of the cat.

The striking linkage homology between cat and man, combined with certain advantageous cytological characteristics of primate and feline chromosomal evolution, prompted us to search for cytological homology between syntenically homologous chromosomes in the two species. High-resolution, G-banded preparations of homologous chromosomes were carefully examined, and several regions of band-for-band homology were identified (HSA1p:FCAC1, HSA2p:FCAA3, HSA2q:FCAC1, HSA11:FCAD1, and HSA12:FCAB4). In all, we could align between 30 and 35% of the human karyotype band-for-band with the feline karyotype despite the passage of over 80 million years since these species shared a common ancestor.

2. Conservation of proto-oncogene chromosomal location in the cat. Transforming genes, or proto-oncogenes, represent a class of evolutionarily conserved cellular genes which may play an important role in tumorigenesis. The physiological function of the majority of normal proto-oncogenes has only just begun to be clarified, although the fact that some encode growth factors (sis) or receptors (erbB, fms) and that all are precisely regulated during development suggests a critical role for these genes. Because of the prevalence of feline leukemia virus (FeLV) in pet cats, this species has been the source of more retroviral oncogene isolations than any other mammal. The identification of homologous chromosomal segments between cat and man, discussed above, combined with the accumulated genetic and molecular information of the vertebrate oncogenes, provide a good opportunity to search for transposition of cellular genes during the more than 80 million years since primates and carnivores shared a common ancestor. Oncogenes are prime candidates for such a consideration because they are the best-known targets for retroviral recombination.

There are about 30 proto-oncogene loci mapped to various chromosomal positions in man. Approximately 30 oncogenes and growth factor loci have been chromosomally assigned in the cat. Nearly all of these fall in chromosomal linkage groups which would be predicted by the human-cat syntenic map (for example, rel and the structural gene for interleukin-2).

3. Molecular evolution of the Felidae. Because of a special fascination that zoologists and naturalists have for cultural, aesthetic, and scientific aspects of the Felidae, a large variety of literature has accumulated regarding their taxonomy. Unfortunately, there is little agreement among students of felid systematics. A variety of classification schemes, which have been based primarily on morphological, ethological, and physiological considerations of the 37 extant species, range from a "lumping" into two genera (Felis and Acinonyx) to a "splitting" into as many as 20 genera within the Felidae.

Over the past five years, in collaboration with numerous zoos and wildlife preserves, we have collected blood samples and skin biopsies (for establishing cell cultures) from 34 of the 37 extant species of Felidae in anticipation of molecular evolutionary studies (see Project Z01CP05367-04 LVC). We applied the clock-like metric of albumin immunological distance to the cat family. Rabbit antisera raised against purified serum albumins from ten cat species were utilized in a quantitative microcomplement fixation assay of 34 felid species. We used a variety of phylogenetic algorithms developed by evolutionary theorists to construct a phylogenetic tree based on the principles of the molecular clock hypothesis. We have also built a similar phylogenetic tree based upon genetic distance estimates of over 50 isozyme loci of the same 34 species. The trees derived from the two molecular methods were largely concordant. An important conclusion derived from the molecular topology was the resolution of felid evolution into three major lineages.

4. Genetic loci for feline lysosomal enzymes: Model for human storage diseases. The lysosomal storage diseases of man are fatal disorders of the nervous system resulting from inherited defects in catabolism of gangliosides and other complex glycolipids and glycoproteins. The specific enzyme defects responsible for a variety of human lysosomal storage diseases have been identified and characterized, and more recently, the normal genes coding for many lysosomal enzymes have been isolated. Several of these diseases have model systems in the domestic cat which render the species of potential use for developing gene therapy. An initial step in this project was to employ the feline mapping panel to genetically map the feline genetic loci which encode the affected enzymes. A panel of 42 rodent x cat somatic cell hybrids has been used to assign eight structural genes for lysosomal enzymes to specific chromosomes in the domestic cat. The assignments include alpha-glucosidase (GANAB) to chromosome D1, alpha-galactosidase (GLA) to the X chromosome, beta-galactosidase-1 (GLB1) to chromosome B3, beta-glucuronidase (GUSB) to chromosome E3, alpha-mannosidase A (MANA) to chromosome B3, alpha-L-fucosidase (FUCA) to chromosome C1, hexose-aminidase A (HEXA) to chromosome B3, and alpha-L-iduronidase (IDUA) to chromosome D4. In all cases, the feline lysosomal enzyme genes were located in linkage groups which were syntenic with their homologous position in the human gene map. These assignments expand the genetic map of the cat and reaffirm the extensive syntenic homology between the chromosome maps of man and cat.

Publications:

Dean M, Kozak C, Robbins J, Callahan R, O'Brien S, Vande Woude GF. Chromosomal localization of the met proto-oncogene in the mouse and cat genome. Genomics 1987;1:167-73.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05389-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Reproductive-Endocrine-Genetic Strategies in Animal Species

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David E. Wildt	Special Volunteer	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Leslie Johnston	IRTA Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

Dept. of Animal Health, National Zoological Park, Smithsonian Institution, Washington, DC (A.M. Miller, J.G. Howard, M.C. Schiewe, S. Hurlburt, M. Bush); Veterinary Resources Branch, DRS, NIH, Bethesda, MD (P.M. Schmidt)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland, 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.9	1.7	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to study and define the biological prerequisites, including the influence of genetics, on reproductive function of mammalian species. Findings are of relevance in the propagation of valuable laboratory animal models, endangered species and in developmental studies associated with the delivery of molecularly cloned genes into early stage pre-implantation embryos. Areas of effort in the female primarily focus on hormonal induction of ovulation, oocyte or embryo recovery, in vitro fertilization, and embryo transfer or cryostorage. Recent work focuses on oocyte maturation in vitro and the development of gene delivery techniques into embryos allowing studies of the mechanisms associated with transformation and inborn errors in early development. Efforts are complemented by multidisciplinary research in male reproduction, particularly in the fields of sperm cell integrity and function tested by in vitro fertilization systems using both homologous and heterologous oocytes. Emphasis is applied to the gametes and embryos of two laboratory species, the mouse and cat, both of which serve as model systems for human disease and rare or endangered species. Progress to date in the mouse has allowed (1) the routine production of offspring following embryo freeze-thawing, culture, and transfer to foster mothers; (2) the detailed analysis of the various factors, including the impact of genotype, on the efficiency of embryo cryopreservation; and (3) pilot studies to develop a system to mature and in vitro fertilize immature, ovarian oocytes. Progress using the cat model now permits (1) the routine recovery of viable, follicular oocytes from hormonally induced females; (2) successful in vitro fertilization and cleavage of approximately 70-80% of all mature oocytes; (3) the production of live, healthy young from in vitro fertilized and surgically transferred embryos; and (4) culture of two-cell embryos to the morula stage in vitro. Currently, these procedures are being applied to pilot studies directed at introducing genes exogenously into pre-implantation embryos using retroviral vectors.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David E. Wildt	Special Volunteer	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Leslie Johnston	IRTA Fellow	LVC	NCI

Objectives:

To increase and integrate the reproductive-endocrine-genetic data base, thereby permitting improved propagation, gamete and embryo cryopreservation, embryological manipulation and gene transfer technology in the domestic cat and related species.

Methods Employed:

The following techniques were used: (1) hormonal induction of ovarian activity; (2) laparoscopic recovery of ovarian follicular oocytes; (3) electroejaculation and laboratory processing for sperm recovery and induction of capacitation; (4) programmable freezing for cryopreservation of gametes and embryos; (5) light and electron microscopy for evaluation of spermatozoal function and integrity; (6) in vitro oocyte maturation, fertilization, and embryo culture; (7) surgical procedures for embryo transfer; (8) biochemical genetic procedures for determining extent and character of genetic variation, and for monitoring paternity and maternity exclusion after embryo transfer; and (9) retroviral infection of pre-implantation embryos.

Major Findings:

1. Variables influencing embryo cryopreservation. Previous research in our laboratories has concentrated on analyzing the many factors which impact upon the efficiency of mouse embryo cryopreservation. These studies have determined that, in addition to type of cryoprotectant, container, freezing unit, plunge temperature and post-thaw dilution approach, a primary variable affecting the success of embryo cryobanking is animal genotype. One additional technical factor which influences embryo quality and developmental competence, either in a fresh or thawed in vitro culture system, is the source of hormone used for follicle stimulation and maturation. Studies found that the pregnant mare's serum gonadotropin (PMSG) source affected the ability of an embryo to survive in culture either immediately after collection or after frozen storage. This effect was genotype-specific with some mouse strains being relatively insensitive to PMSG source, whereas gonadotropin source played a major role in determining in vitro viability in others. Together, the data indicated that in vitro development of thawed embryos is influenced not only by genotype, but also by the source of the gonadotropin used to promote follicle and oocyte maturation.

A major collaborative effort was continued with the Veterinary Resources Branch of the NIH to maintain an embryo cryopreservation bank for numerous genotypes of inbred, outbred and congenic laboratory mice. To date, a minimum of 1,000 embryos has been permanently stored from each of 29 genotypes.

2. Hormonal induction of follicle activity, oocyte recovery and in vitro fertilization. In vitro fertilization (IVF) offers a valuable approach for studying the processes of gamete interaction and early embryonic development as well as species-specific variables which affect gamete fusion. Domestic cats, particularly models with inbred genotypes valuable for human-related health research, do not reproduce well under laboratory conditions. A reliable IVF system would be useful for ensuring adequate propagation of these animals.

To date, studies have emphasized establishing a consistently reliable IVF system for the domestic cat. Cats are given PMSG hormone followed by human chorionic gonadotropin (hCG) to stimulate intrafollicular oocyte maturation. Follicular oocytes are recovered by an atraumatic, laparoscopic approach, mixed with electroejaculated, processed spermatozoa, and then cultured in a CO_2, humidified environment. Initial studies determined that IVF in the cat was dependent on the interval between the PMSG and hCG stimuli which appeared to be related to intrafollicular oocyte maturation. Recovering oocytes at 84 hours after PMSG injection currently results in an overall fertilization and cleavage rate of 79%. Two- and four-cell embryos surgically transferred to the oviducts of six oocyte donors resulted in five pregnancies and the production of one to four kittens/litter. To date, these studies have defined the importance of intrafollicular oocyte maturation for IVF, established the capacitation interval for ejaculated spermatozoa, demonstrated the ability to routinely produce cat embryos in the laboratory, and unequivocally established the developmental competence of carnivore embryos produced by IVF.

3. Influence of sperm quality and swim-up processing on penetration of heterologous or homologous oocytes. A fascinating aspect of reproduction in cats is the high numbers of morphologically abnormal spermatozoa found in ejaculates. To determine the influence of teratospermia on fertilization, two populations of domestic cats which consistently produce either high or low numbers of normal sperm forms were studied. The penetration of zona pellucida-free hamster ova has been used to measure the functional competence of spermatozoa to penetrate an ovum in vitro and undergo nuclear decondensation within the vitellus in a variety of species. Electroejaculates from cats in these two populations were collected, processed to provide the same number of normal-appearing spermatozoa/inseminant and added to either zona-free hamster oocytes or zona-intact, immature domestic cat oocytes. Penetration was defined either by the presence of a decondensed sperm head with a corresponding flagellum (in the hamster assay) or by the sperm head entering through at least the outer layer of the zona (in the cat assay).

Spermatozoa from both cat populations were capable of penetrating both types of oocytes, but at markedly different overall rates. For hamster ova, penetration rate varied from 2.2% (low normal sperm) to 14.9% (high normal sperm). For cat oocytes, from 11.3% (low normal sperm) to 67.1% (high normal sperm) were penetrated. Penetration of hamster ova and cat ova by ejaculates containing low numbers of abnormal sperm was superior to ejaculates containing higher

numbers of pleiomorphic cells in the original ejaculate. Furthermore, within populations, there appeared to be major differences among male cats in the ability of spermatozoa to penetrate heterologous or homologcus oocytes. These results suggest that the pre-processing, morphological integrity of the spermatozoan is a major factor dictating membrane penetration and/or nuclear decondensation within the felid oocyte. These findings demonstrate a relationship between sperm integrity and fertilization potential in the domestic cat and offer strategies for processing ejaculates to maximize IVF attempts or promote other artificial breeding approaches.

4. In vitro maturation and fertilization of immature, ovarian oocytes. In vitro maturation of antral oocytes followed by IVF has been achieved in several species and has resulted in the birth of live-young. The ability to routinely produce cat embryos from oocytes recovered from ovariohysterectomy material would have a major impact on the utility and practicality of the species for molecular biology studies. This study begins to explore the conditions for promoting carnivore oocyte maturation in vitro. To maximize efficiency, pilot studies have been initiated to repeat studies by earlier investigators who pioneered the mouse system. Immature, follicular mouse oocytes have been matured in vitro, successfully subjected to IVF and have resulted in cleaved embryos which have cultured to the blastocyst stage of development. Pilot studies with cat ovaries collected from spay clinics also have been initiated. Approximately 20-80 oocyte-cumulus cell complexes are recoverable per pair of ovaries. Hoescht staining analyses of oocytes have revealed that nuclear condensation begins as early as 6 hours in culture with the appearance of metaphase I at approximately 20 hours.

5. Gene transfer into cat embryos via retroviral vectors. There are several domestic cat models for specific lysosomal storage diseases applicable to gene therapy in humans. This project is designed to determine the effectiveness of introducing exogenous genes in the domestic cat embryo using retroviral vectors carrying non-viral genes. The success of infection will be evaluated by testing for integrated virus using the polymerase chain reaction method. Two retroviral vectors are scheduled for use, PA12 (derived from a Moloney murine leukemia virus DNA clone with the env gene replaced with the amphotropic virus clone 4070A counterpart) and cistorneo (derived from a DNA clone of the amphotropic virus 4070). Exogenous genes scheduled for use are non-pathogenic (i.e., globin gene) or will represent the functional human cDNA of any of the specific lysosomal genes. To date, 16 cat embryos (2- to 8-cell) have been infected and cultured to the 16-cell to morula stage of development.

Publications:

Goodrowe KL, Howard JG, Wildt DE. Comparison of embryo recovery, embryo quality, oestradiol-17β and progesterone profiles in domestic cats at natural and induced-oestrus. J Reprod Fertil 1988;82:553-61.

Goodrowe KL, Miller AM, Wildt DE. Capacitation of domestic cat spermatozoa as determined by homologous zona pellucida penetration. In: Boland MP, ed. Proceedings of the international congress on animal reproduction and artificial insemination. Dublin: University College Dublin (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05414-05 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of Retroviruses (Type-D and SIVs) Isolated from Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	David Derse	Senior Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)* Univ. of Washington, Seattle, WA (W. Morton, C.-C. Tsai); Oncogen, Seattle, WA (S.-L. Hu); Bionetics Research, Inc., Frederick, MD (L. Henderson, S. Oroszlan); USAMRIID, Frederick, MD (P.B. Jahrling); Program Resources, Inc., Frederick, MD (P. Dorn, M. Gonda)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Leukemia and Lymphoma Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.1	0.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Three distinct primate lentiviruses (simian immunodeficiency viruses, SIV) have been isolated from a macaque (M. nemestrina) (SIV/Mne) housed at the University of Washington Primate Center, from a wild-caught mangabey (SIV/Cat) and from a colony-housed African green monkey (SIV/Cae). The relatedness of SIV/Mne to other lentiviruses was examined by amino acid sequence analysis of gag proteins. Of 125 residues sequenced, 92% of the SIV/Mne amino acids were identical to predicted residues of SIV/mac (an SIV isolate from the New England Primate Center), 83% were identical to human immunodeficiency virus-2 (HIV-2), and 41% to HIV-1. SIV/Mne has been inoculated into six macaques and two baboons. All six macaques became viremic and died 15 to 120 weeks after inoculation, with immunologic abnormalities including a marked decrease in CD4+ peripheral blood lymphocytes. The baboons were antibody- and virus-negative and remained healthy. A full-length molecular clone of SIV/Mne has been obtained; a 1.7-kb fragment at the 3′ end of the virus has been sequenced and shown to be 92% homologous to SIV/mac. This molecular clone has also been inoculated into macaques, and elicits an antibody response.

A protein designed p14 and purified from SIV/Mne virus preparations was shown to be the translational product of the X-open reading frame (X-ORF) of SIV. A homologous protein (16K) was also shown to be present in HIV-2; there is no X-ORF in HIV-1. Thus, SIV/Mne and HIV-2 appear to be the first known examples of retroviruses to contain substantial amounts of viral proteins that are not products of the gag, pro, pol, or env genes. Purified p14 binds to single-stranded nucleic acids in vitro.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
David Derse	Senior Staff Fellow	LVC	NCI

Objectives:

To characterize primate retroviruses, with emphasis on the type-D and lentivirus (simian immunodeficiency virus, SIV) classes. To determine, by molecular hybridization and immunological techniques, the homology between these viruses and other primate retroviruses.

New isolates will be characterized by examining their host range for various cells in vitro, by obtaining molecular clones, restriction enzyme maps, and DNA sequence data. In addition, viral proteins will be purified, N-terminal amino acid sequences determined, and antisera to the individual proteins raised in rabbits in order to develop specific immunological reagents and to examine the extent of antigenic and molecular similarities to human AIDS viral isolates. To determine the pathogenicity of these isolates in various primate species in order to develop a suitable animal model for AIDS. To prevent disease in primates by vaccination with various sub-particulate viral preparations. To examine the effect of various genes on pathogenicity by performing site-directed mutagenesis and rechallenging primates with these new variants.

To determine the prevalence of these primate viruses in various primate colonies and in feral populations by examining sera for the presence of cross-reactive antibodies and peripheral blood lymphocytes for the presence of viruses.

Methods Employed:

Virus isolation was attempted using cell lines that have previously been employed for the isolation of other primate and human retroviruses. Fresh tumors, whole blood, or sera obtained from primates were cocultivated with various cells and the supernatant assayed weekly for reverse transcriptase activity. Cloned retroviral DNA was used as a probe for detection of related DNA sequences in primates. Viral proteins were purified by high pressure liquid chromatography. Antigens and antibodies were detected by radio-immunoassays, ELISA assays, and by Western immunoblot techniques.

Major Findings:

1. Isolation and molecular characterization of lentiviruses (SIV class) from primates. A retrovirus has been isolated on the human T-cell line, HuT 78, after cocultivation of a lymph node from a pig-tailed macaque (M. nemestrina) that had died with malignant lymphoma in 1982 at the University of Washington Regional Primate Research Center. This isolate, designated simian

immunodeficiency virus (SIV/Mne), shows the characteristic morphology of a lentivirus and replicates to high titers in various lymphocyte lines of human and primate origin. The relatedness of SIV/Mne to other lentiviruses was examined immunologically and by amino acid sequence analysis of gag proteins. A total of 125 residues of SIV/Mne amino acid sequence was compared to the predicted amino acid sequences for the same gag regions of SIV/mac (an isolate from the New England Primate Center) and HIVs. In the compared regions, 92% of the SIV/Mne amino acids were identical to predicted residues of SIV/mac; 83% were identical to HIV-2, and 41% to HIV-1. SIV/Mne is thus closely related to HIV-2, the West African AIDS isolate.

A molecular clone has been obtained by screening a genomic library constructed in lambda EMBL3 from a single cell clone of SIV/Mne-infected HuT 78 cells. One full-length molecular clone was shown to be infectious in vitro. A 1.7-kb fragment at the 3′ end of SIV/Mne (including a portion of env and the long terminal repeat) has been sequenced and shown to possess a 92% homology to the published sequence of SIV/mac.

Another SIV isolate has been obtained by cocultivating human peripheral blood lymphocytes (PBLs) with minced spleen tissue from a mangabey (Cercocebus atys) that was caught in West Africa in 1969. This isolate, designated SIV/Cat, shows no restriction enzyme site similarity with SIV/Mne (10 sites examined). Preliminary characterization of SIV/Cat viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a major viral gag protein of 26 kilodaltons (p26) that can be distinguished from the major gag protein of HIV-I (p24) or SIV/Mne (p28). This new isolate will be cloned and its pathogenicity in primates examined.

A third SIV isolate has been obtained by cocultivating PBLs from apparently healthy African green monkeys (Cercopithecus aethiops) housed at the U.S. Army Medical Research Institute of Infectious Diseases. This isolate, designated SIV/Cae, differs markedly from SIV/Mne and SIV/Cat in its in vitro host range and will also be cloned and its proteins characterized.

2. Identification of a unique viral protein in SIV/Mne and HIV-2. A protein designated p14 purified from SIV/Mne was shown by amino acid sequence analysis to be nearly identical to the predicted translational product of an open reading frame, designated X-open reading frame (X-ORF), in the proviral nucleotide sequences of HIV-2. Antisera to SIV p14 detects p14 in purified SIV/Mne and a homologous protein (16K) in purified HIV-2. These data clearly show that the X-ORFs of SIV/Mne and HIV-2 are translated and that the protein products copurify with the viruses. Quantitative analysis reveals that the molar amount of p14 found with purified SIV/Mne is approximately equal to the molar amounts of structural proteins derived from the gag gene. It was estimated that the molar ratio of the X-ORF protein to the gag proteins in purified HIV-2 is similar to that found for p14 in SIV. Thus, SIV/Mne and HIV-2 appear to be the first known examples of retroviruses to contain substantial amounts of viral proteins that are not products of the gag, pro, pol, or env genes. Purified p14 binds to single-stranded nucleic acids in vitro. HIV-1 does not contain an X-ORF and antisera to SIV/Mne p14 does not detect a

homologous protein in purified HIV-1. Diagnostic procedures based on detection of the X-ORF protein thus may be useful for distinguishing among closely related immunosuppressive viruses.

3. Infection of primates with SIV/Mne. SIV/Mne was endpoint diluted on HuT 78 cells and inoculated intravenously into three juvenile rhesus monkeys (M. mulatta), three juvenile pig-tailed macaques (M. nemestrina) and two juvenile baboons (Papio cynocephalus). All six macaques became viremic by 3 weeks after inoculation, whereas neither of the baboons developed viremia. One pig-tailed macaque died at 15 weeks with a suppurative peritonitis secondary to an ulcerative, necrotizing colitis. Immunologic abnormalities included a marked increase in CD4+ PBLs. Although five macaques mounted an antibody response to SIV/Mne, the animal that died at 15 weeks remained antibody negative. All five other macaques died 66 to 120 weeks after inoculation after exhibiting progressive weight loss, anemia, and diarrhea. Histopathologic findings at necropsy included various manifestations of immune deficiency and nephropathy, pancreatitis, adenocarcinoma, and lymphoid atrophy.

SIV/Mne could be readily isolated on HuT 78 cells from spleen and lymph nodes of all necropsied macaques. By using human PBLs as the indicator cell line, virus was also isolated from samples of bone marrow, pancreas, liver and cerebrospinal fluid. SIV antigens were localized by avidin-biotin immunohistochemistry to pancreatic islet cells, to bone marrow endothelial cells, and to brain macrophages. The data suggest that African baboons may be resistant to infection by SIV/Mne, while Asian macaques are susceptible to infection with this pathogenic primate lentivirus.

The molecular clone of SIV/Mne, which is infectious in vitro, has also been inoculated into two pig-tailed macaques and two baboons; an antibody response has been elicited in the two macaques, showing that the cloned virus is replicating in these animals.

4. Development and testing of vaccines to protect against SAIDS-D/Washington (SAIDS-D/W) retrovirus infection. A type-D retrovirus (SAIDS-D/W) associated with retroperitoneal fibromatosis at the University of Washington Primate Center has been molecularly cloned and shown to be infectious in vitro and in macaques in vivo. In collaboration with Dr. Shui-lok Hu, a portion of this clone containing the envelope and transmembrane proteins (gp70 and p22E, respectively) has been recombined with vaccinia virus and inoculated into four macaques. All four animals developed neutralizing antibody to SAIDS-D/W virus. Since greater than 90% of colony macaques at the University of Washington are infected with SAIDS-D/W virus, the development of an effective vaccine will have important implications in terms of colony management.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05417-04 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Characterization of raf Oncogenes in Normal and Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Thomas W. Beck	Biotechnology Fellow	LVC	NCI
	Ulrich Brennscheidt	Special Volunteer	LVC	NCI
	Jong-Eun Lee	Special Volunteer	LVC	NCI
	Gunamani Sithanandam	IRTA Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	John L. Cleveland	Senior Staff Fellow	LVC	NCI
	Berton Zbar	Chief, Cellular Immunity Section	LI	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.7	2.1	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In both mouse and man, two active oncogenes related to v-raf have been identified. c-raf-1 has been localized to mouse chromosome 6 and to human chromosome 3p25 near sites specifically altered in small cell lung carcinoma (SCLC), familial renal cell carcinoma (RCC), and mixed parotid gland tumors. The human c-raf-1 gene contains 17 exons and spans more than 50 Kbp. It is expressed as a 3.4-Kb mRNA which is found in most mouse tissues and cell lines at various levels and encodes a cytoplasmic phosphoprotein of 648 amino acids (73 Kd) which possesses serine/threonine-specific protein kinase activity. Restriction fragment length polymorphism analysis of SCLC (paired and unpaired) and non-SCLC DNAs indicate that one allele of c-raf-1 is lost in all informative SCLC cases (46 total cases). Linkage analysis has demonstrated that the c-raf-1 is located within 13 cM of an autosomal dominant gene(s) which is phenotypically displayed as inherited susceptibility to certain cancers including RCC (Von Hippel-Lindau disease). A-raf-1 has been localized to the X chromosome, representing the first active human oncogene on a sex chromosome, at position p11 in man. Alterations of the X chromosome are rare in human cancers although certain translocations involving the X chromosome have been described in human leukemias. A-raf-1 is a more compact gene than c-raf-1 which may explain why it has not registered as a DNA-transfection-activated oncogene. The A-raf mRNA is 2.6 Kb in both mouse and man, it encodes a 606 amino acid phosphoprotein (67.5 Kd) which shows 60% homology with c-raf-1 and displays a more restricted pattern of tissue expression than c-raf-1, with highest levels in the epididymis.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Thomas W. Beck	Biotechnology Fellow	LVC	NCI
Ulrich Brennscheidt	Special Volunteer	LVC	NCI
Jong-Eun Lee	Special Volunteer	LVC	NCI
Gunamani Sithanandam	IRTA Fellow	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
John L. Cleveland	Senior Staff Fellow	LVC	NCI
Berton Zbar	Chief, Cellular Immunity Section	LI	NCI
John D. Minna	Chief	NMOB	NCI

Objectives:

The specific objectives of these studies are (1) to molecularly characterize c-raf-1 and A-raf-1 genes in normal and tumor tissues, (2) to determine how these genes are regulated in normal and transformed cells, (3) to identify and characterize the genes and gene products which regulate raf expression in normal and transformed cells, and (4) to define the genetic mechanism(s) by which the raf family of proto-oncogenes becomes activated and causes transformation.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and subclone raf genomic and cDNA sequences from bacteriophage lambda gt10 libraries. raf clones were sequenced using dideoxy and Maxam and Gilbert techniques, and were computer analyzed. Specific cDNA clones were inserted into expression vectors, and high levels of synthesis of raf polypeptides in E. coli were demonstrated by protein gel and immunoblot analyses. raf-specific transcripts were identified by Northern blot analyses of poly(A)+ RNA preparations from tumors or established cell lines. cDNA libraries from specific cell lines were made by standard techniques, cloned into lambda gt10, and specific raf cDNAs were isolated. Protein analyses of raf in normal and transformed cells were carried out by metabolic labeling, immunoprecipitation and gel analyses, and raf-associated kinase activity was demonstrated using our established protocol.

Major Findings:

1. The functional human c-raf-1 gene is the most characterized raf family gene. The human c-raf-1 cDNA isolated from a human fetal liver cDNA library is 2.97 Kb. It contains a single large open reading frame of 1944 nucleotides coding for a phosphoprotein of 648 amino acids and a predicted molecular weight of 73.0 Kd. Using additional cDNA libraries, we have isolated more 5′ extensions and identified six overlapping genomic DNA clones for the human c-raf-1 locus. These span more than 50 Kb and contain all 17 exons of the gene.

2. Using restriction fragment length polymorphisms within the c-raf-1 locus, we have examined DNA from a total of 83 human lung carcinomas. In an analysis of 10 paired (normal versus tumor) small cell lung carcinoma (SCLC) DNA samples, informative cases were observed, all of which showed a loss of heterozygosity at the c-raf-1 locus. A Bgl I polymorphism detectable with c-raf-1 DNA probes shows 50% heterozygosity in a normal population. This was used to analyze 73 unpaired lung carcinoma DNAs. Out of 31 non-SCLC samples, 15 showed heterozygosity as predicted for a normal population; however, none of the 42 SCLC samples were heterozygous. This striking loss of heterozygosity at c-raf-1 locus in SCLC, but not non-SCLC, indicates that one allele of the c-raf-1 locus is deleted in SCLC.

3. c-raf-1 gene expression has been examined by Northern hybridization in adult and embryonic mouse tissues and in established cell lines using a variety of mitogens and growth inhibitors. The gene is uniformly expressed in most cells and tissues examined, albeit at different levels. The mRNA size is 3.4 and 3.1 Kb in human and mouse, respectively. We have only observed altered c-raf mRNAs in one mouse cell line containing the long terminal repeat-activated c-raf-1; however, altered cDNAs which result from differential splicing have been identified which would not be predicted to deviate significantly from the normal size (3.4 kb) c-raf-1 mRNA. c-raf RNAs are uniformly expressed at very high levels in chemically induced mouse lung carcinomas and lymphomas, in transformed murine and human tumor cell lines, and in chemically induced rat liver preneoplastic nodules, but not in the resulting rat hepatomas.

4. Antisera prepared against v-raf 30K and C-terminal SP63 peptide react with the gag-raf fusion protein (p79) of 3611-MSV-transformed NIH 3T3 cells, and also identify the 74-Kd, normal c-raf-1 gene product from a variety of rodent and human cell lines, thus confirming the size of the c-raf protein based on the cDNA sequence.

5. The A-raf-1 locus has been mapped in both mouse and man to the X chromosome. The human A-raf-1 locus has been regionally mapped to position p21 to q11 near the locus for testicular feminization syndrome and Menkes syndrome, which are linked in both mouse and man. In the mouse, the A-raf-1 locus has been mapped to a region 10-17 cM proximal to the hypoxanthine phosphoribosyl transferase gene between the DXPas4 and the DXPas7 loci. This localization is compatible with the presence of the A-raf-1 oncogene on the short arm of the human X chromosome between the centromere and Xp21. Although no specific translocations involving the X chromosome have been described for any particular human cancer, rare translocations of the X chromosome with autosomes have been reported and X chromosome loss is a frequent occurrence in certain types of acute lymphocytic leukemia.

6. A near full-length human A-raf cDNA was isolated from the T-cell library which is 2.46 Kb and contains a single long open reading frame of 1818 nucleotides coding for a protein of 606 amino acids and a molecular weight of 67.5 Kd. The predicted amino acid sequence shows 60% identity with the c-raf-1 and, taking into account conservative amino acid substitutions, the homology is greater than 85%.

7. Southern blotting experiments and genomic cloning show that the A-raf-1 gene is less than 37 Kb and that very short introns intersperse the coding sequences in the 5′ half of the gene. The smaller size of this gene relative to c-raf-1 may explain why A-raf-1 has not been detected as an oncogene in DNA transfection experiments.

8. A-raf gene expression has been examined in embryonic and adult mouse tissues, and in a variety of murine and human cell lines. The A-raf mRNA is 2.6 Kb in both rodents and humans. However, in certain human and murine T-cell lines a 1.3-Kb A-raf hybridizing mRNA has also been observed. In contrast to c-raf-1, A-raf-1 shows a restricted tissue distribution of expression with the highest levels observed in the epididymis and no detectable expression in the cerebrum.

9. The human A-raf-1 cDNA was incorporated into an E. coli expression vector and the purified protein, as well as A-raf synthetic peptides, were used to generate A-raf-specific antisera. These antisera react with the gag-A-raf fusion protein of A-raf-MSV transformed mouse cells and also identify a 67-Kd protein in A549 cells and a chemically induced mouse T-cell lymphoma, thus confirming the predicted size of the A-raf protein.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05418-04 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of raf and myc Oncogenes in Transformation In Vivo and In Vitro

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	John L. Cleveland	Senior Staff Fellow	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI
	Michael Potter	Biologist	LG	NCI
	Jacalyn H. Pierce	Microbiologist	LCMB	NCI

COOPERATING UNITS *(if any)*

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Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.8	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to evaluate the target cell range for transformation by v-raf, as well as to determine whether v-raf is capable of inducing transformation by itself or requires interaction with a second oncogene, myc, a series of recombinant viruses was constructed with either or both viral oncogenes on the 3611-murine sarcoma virus (MSV) background. A combination of both oncogenes in an infectious murine retrovirus (J-2) induces hematopoietic neoplasms, in addition to less prominent fibrosarcomas and pancreatic adenocarcinomas 1 to 3 weeks after inoculation. The hematologic neoplasms consist of immunoblastic lymphomas of T- and B-cell lineages, and erythroblastosis. In parallel to the synergistic action of both oncogenes on hematopoietic cells in vivo, we find that synergism also exists for transformation of primary hematopoietic, myeloid, epithelial cells, and chondrocytes in vitro. The synergism can be exploited for the immortalization/transformation of rare stem cells and the study of lineage relationships. Towards this end we have uncovered a close relationship between myeloid and B-cell lineages as well as effects of constitutive oncogene expression on "determination" within these lineages. From growth factor abrogation experiments, complementation studies with mutant cell lines and antibody microinjections, we conclude that the mechanism underlying synergism includes a combination of signals required early and late in the cell cycle which connect via activity modulation of myc and/or other nuclear factors by direct or indirect phosphorylation involving raf protein kinases.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
John L. Cleveland	Senior Staff Fellow	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
Robert Nalewaik	Microbiologist	LVC	NCI
Michael Potter	Biologist	LG	NCI
Jacalyn H. Pierce	Microbiologist	LCMB	NCI

Objectives:

As part of our efforts to determine normal and pathological raf function, the consequences of constitutive raf expression (v-raf, various forms of c-raf, and A-raf-1) are being examined in a variety of cell types. For gene transfer, a series of isogenic retroviruses has been constructed which carry various raf genes alone, or in combination with v-myc. The specific aims of this project are: (1) To determine whether there is a distinct target cell preference for transformation by wild-type and mutant raf and myc oncogenes in vivo and in vitro. (2) To determine, as a first step in delineating pathways of function for raf relative to myc oncogenes, whether raf and myc act synergistically in transformation. (3) To assess the individual contribution of raf and myc in such a possible synergism. For this purpose, the single oncogenes in their wild-type and mutant forms will be incorporated into retroviruses and examined for their ability to abrogate growth factor requirements of cells, alter their morphology, their differentiation program, or their proliferative potential. (4) To use appropriate virus constructs to immortalize rare cell types in culture for lineage studies and to allow for biochemical analysis of their differentiated products. (5) In the future, to adapt the virus constructs for efficient and controlled expression in mouse and human cells. (6) To widen the range of oncogenes that is being tested for synergism with raf and with each other. Specifically, we now have included other serine protein kinases such as protein kinase C.

Methods Employed:

RNA and DNA from human tumors were analyzed using established protocols. Standard recombinant DNA technology was utilized in the construction of new murine retroviruses, and high titer stocks of these viruses were prepared after transfection onto NIH 3T3 cells. Expression of recombinant retroviruses and cellular proto-oncogenes in induced murine tumors and transformed cell lines was assessed using standard criteria for Northern hybridization and Western blotting.

Major Findings:

1. In vitro transforming potential of v-raf and v-myc in primary cultures. Having established the in vivo tissue preferences for transformation of v-myc and v-raf, we examined, in detail, the effects of these two oncogenes

alone, or in combination, on the transformation of primary hematopoietic cultures. Additionally, we have tested the potency of these viruses in transforming primary chondrocyte cultures. The results can be summarized as follows: (A) v-myc and v-raf synergize in the transformation of primary hematopoietic and chondrocyte cultures. Regardless of tissue source or culture regimen, the dual oncogene virus, J-2, has been demonstrated to be a powerful agent in the immortalization of a diverse set of cell lineages from primary cultures, including the isolation of several rare stem cell lines. In contrast, the transforming potential for v-myc or v-raf alone appears to be quite limited (see below). These J-2-immortalized cells generally appear to behave similar to their primary counterparts in their responses to differentiation or growth-promoting agents. Moreover, where characterized, these cell lines have been found to produce biologically active modifiers including growth factors and antibodies. Therefore, these cells represent sources for the production of rare biological products. (B) Transformation of primary hematopoietic cultures by v-raf and v-raf + myc viruses. We have examined the transforming potential of the v-raf, v-myc, and the v-raf + v-myc viruses in primary hematopoietic cell cultures using four different culture regimens. In each system tested, the viruses carrying only v-myc failed to show any demonstrable effect on primary cultures and the potential of v-raf viruses was quite limited with respect to the J-2 virus. The results can be summarized as follows: (1) Immortalization of macrophages from fresh bone marrow by the J-2 virus. In these experiments, fresh bone marrow, from weanling C3H/HeJ mice, was infected with the various viruses and then placed into standard medium without the addition of specific growth factors. Cultures infected with v-raf- or v-myc-carrying viruses, or with helper virus alone, died after a few days in culture, whereas J-2-infected cultures reproducibly produced immortalized mature macrophage cell lines which were clonal and expressed high levels of v-myc and v-raf, but did not require or produce colony-stimulating factor-1 (CSF-1), which is normally essential for growth of these cells. Moreover, J-2 macrophage lines were functional in terms of producing esterase and lysozyme, were phagocytic, and, like normal peritoneal macrophages, became tumoricidal after treatment with interferon gamma. Differentiation-associated markers of normal macrophages, such as Ia antigens, were also inducible with gamma-interferon. (2) Transforming potential of 3611-murine sarcoma virus (MSV) and J-2 viruses in fetal liver cultures in interleukin-3 (IL-3). In similar experiments, we tested the effects of raf-, myc-, and raf + myc-carrying retroviruses, as well as murine retroviruses carrying other v-onc genes on the immortalization, differentiation, and growth factor requirements of primary hematopoietic cells from NFS/N fetal liver cultures in the presence and absence of the growth factor, IL-3. With time, IL-3-supplemented fetal liver cultures normally differentiate into mast cells, which then senesce and die. In the absence of specifically added growth factors, these cultures die rapidly. Again, in the absence of any added growth factors, only the J-2 virus was capable of immortalizing cells, and these cells were again predominantly of the macrophage type in their morphologic and biologic properties. In addition, J-2 infection also rarely resulted in clonal, growth factor-independent myeloid stem cells and pre-B cells. In contrast, infections with retroviruses carrying v-raf, v-Ha-ras, v-mos, and v-abl gave rise to immortalized mast cells which were, with the exception of v-abl virus-infected cells, still dependent upon IL-3. v-abl-infected mast cells and J-2 virus-infected macrophages, stem cells, and B-cells were not growth factor-independent as a consequence of

autocrine production of growth factors as assessed by Northern blot analysis and testing conditioned medium from these cell lines for specific growth factors.

These results suggest that v-raf and v-myc affect different components of growth regulation, as, for example, competence (v-myc) and cell cycle progression (v-raf). We conclude from the finding of distinct target cell preferences for raf and myc oncogenes that cells differ with respect to the rate-limiting pathway (for example, competence versus progression) through which their growth is normally controlled.

2. Transformation of B-lineage cells in vitro using conditions that favor B-cell growth. Murine bone marrow cells infected with replication-defective retroviruses containing v-raf alone or v-myc alone yielded pre-B-cell lines at low frequencies, whereas a retroviral construct containing v-raf- and v-myc-transformed cells with more than a tenfold higher efficiency. The raf/myc transformants included clonally related populations of mature B-cells and mature macrophages, suggesting a common bilineage progenitor as the target for transformation. The genealogy of these transformants demonstrated that mature myeloid cells were derived from cells with apparent B-lineage commitment including functional Ig gene rearrangements. This system should facilitate studies of developmental relationships in hematopoietic differentiation and analyses of onc gene interactions in lineage determination.

3. raf/myc-infected erythroid cells are restricted in their ability to terminally differentiate. A comparison was made of the in vitro transforming ability of v-raf, v-myc and v-raf/v-myc. We have described elsewhere that v-raf and v-myc alone transform lymphocytes poorly in agarose, but in combination these two oncogenes act synergistically to induce a 14-fold increase in colony numbers. Here we show that in methylcellulose, v-raf efficiently produces colonies of hemoglobin-synthesizing erythroid cells, while v-raf/v-myc-infected erythroid cells were inhibited from terminally differentiating, but retained the ability to replicate extensively. No cooperation was observed between v-raf and v-myc in erythroid colony formation.

Publications:

Cleveland JL, Huleihel M, Storm SM, Bressler P, Siebenlist U, Eisenman R, Rapp UR. Role of myc in tumor induction, growth factor abrogation, and control of c-myc expression. In: Alt FW, Harlow E, Zitt EB, eds. Current communications in molecular biology. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1987;199-205.

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Rapp UR, Cleveland JL, Storm S. Oncogenes: clinical relevance. In: Neth R, Gallo RC, Greaves MF, Kabisch H, eds. Modern trends in human leukemia VII haemotology and blood transfusion, vol 31. Berlin: Springer-Verlag, 1987;450-9.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05491-03 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders)*

Feedback Regulation of c-myc Transcription by myc Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John L. Cleveland	Senior Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Jacob Troppmair	Special Volunteer	LVC	NCI

COOPERATING UNITS *(if any)* Laboratory of Immunoregulation, NIAID, NIH, Bethesda, MD (U. Siebenlist, P. Bressler); Bionetics Research, Inc., Frederick, MD (J. Ihle); Fred Hutchinson Cancer Research Center, Seattle, WA (R. Eisenman); Program Resources, Inc., Frederick, MD (P. Lloyd, M. Dean)

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NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
1.2	1.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Infection of mouse cells from a variety of lineages with retroviruses expressing high levels of avian v-myc or mouse c-myc was found to be invariably associated with a lack of endogenous c-myc expression. To distinguish between v-myc-induced shutdown versus a cell-programmed down-regulation of c-myc expression, we have analyzed this phenomenon in detail in NIH 3T3 mouse fibroblast cells. In these fibroblasts exogenous v-myc was expressed at levels tenfold greater than those of c-myc present in uninfected cells. Suppression of steady state levels of c-myc mRNA occurred, at least in part, at the level of transcription from c-myc promoters P1 and P2, and involved v-myc protein since cells infected with constructs containing frameshifts and deletions in v-myc had normal levels of c-myc mRNA and protein. Suppression of c-myc expression was also observed in fibroblasts transfected with an N-myc expression vector and in fibroblasts infected with a c-myc retrovirus. Suppression of c-myc expression was not associated with any gross changes in chromatin structure and could be reversed by treating infected cells with anisomycin or by stimulating growth factor-deprived cells with serum. These findings establish that v-/c-myc proteins are involved either directly or indirectly in a regulatory circuit which represses c-myc proto-oncogene transcription. Feedback regulation of c-myc transcription may also be relevant in establishing the lineage-specific expression of myc family proto-oncogenes. Reduced steady state levels of c-myc mRNA were also observed in NIH 3T3 cells infected with 12S and 13S E1A recombinant retroviruses, suggesting that E1A protein can alleviate the requirement for myc for cell growth and may also share transcriptional target genes.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John L. Cleveland	Senior Staff Fellow	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
Jacob Troppmair	Special Volunteer	LVC	NCI

Objectives:

myc family genes have been speculated to function as transcriptional regulatory proteins because of their localization to the nuclear matrix, DNA binding properties, and their weak homology to the adenovirus transcription regulator E1A protein. To establish that myc proteins function in this manner, one needs to identify likely target genes. In the course of studies demonstrating that high levels of exogenous v-myc abrogates specific growth factor requirements, we made the observation that exogenous myc expressed from recombinant retroviruses negatively influences expression of normal c-myc mRNA. We have focused on this phenomenon as a model for unraveling the regulatory function of myc family proteins. The specific aims of this project are to (1) establish an in vitro model system where we reproducibly observe that exogenous myc negatively regulates expression of normal myc genes; (2) determine whether this regulation operates at the level of chromatin structure, transcription, or post-transcription; (3) test if the negative regulation of c-myc transcription is reversible by removing pools of exogenous myc by treatment with inhibitors of protein synthesis or can be circumvented by stimulating cells with growth factors; (4) determine which domains of myc proteins function in transcriptional down-regulation of c-myc; (5) identify other genes which may be transcriptionally regulated, positively or negatively, by myc proteins; and (6) test the functional equivalence of myc and adenovirus E1A proteins in the transcriptional regulation of specific target genes.

Methods Employed:

Recombinant retroviruses containing various portions of the v-myc gene (from the avian MH2 or MC29 viruses) were constructed using standard recombinant DNA technology. High-titer stocks of murine leukemia virus (MuLV) pseudotypes of these defective retroviruses were prepared from transfected NIH 3T3 cells using established techniques. RNA and DNA blot analyses, nuclear run-on assays, and DNase I hypersensitivity assays of chromatin were all performed using standard protocols.

Major Findings:

The data described in this report show, for the first time, that one of the functions of myc is as a transcription regulator. We speculate that myc protein normally regulates its own transcription and that of other "proliferation-associated" genes in a tightly controlled manner, and once this autoregulatory mechanism of control is lost through activating events, cells

constitutively progress through the cell cycle. These experiments can be summarized as follows:

1. Feedback regulation of c-myc transcription by exogenous v-myc proteins in NIH 3T3 cells. To establish that v-myc directly influences c-myc expression, we have utilized the murine NIH 3T3 fibroblast cell line, which has been extensively characterized in its expression of c-myc. RNA blot analysis of mass cultures of NIH 3T3 fibroblasts infected with the v-myc or v-myc + v-raf viruses did not have detectable levels of endogenous c-myc mRNA, as compared to uninfected fibroblasts. In contrast, mass cultures infected with 3611-murine sarcoma virus (MSV) containing only v-raf had levels of c-myc identical to those in uninfected cells. We also examined c-myc expression in single-cell clones from uninfected cells and from acutely infected cultures. Suppression of c-myc expression was not due to an inherent variability in c-myc expression as uninfected NIH 3T3 cell clones, whereas v-myc virus-infected single-cell clones showed no detectable c-myc mRNA. To further demonstrate that the suppression of endogenous c-myc in v-myc retrovirus-infected cells was not due to other secondary events, we also constructed a v-myc-neo recombinant retrovirus. Northern blot hybridization of RNA and immunoprecipitation of myc proteins from G418-resistant single-cell clones demonstrated that all clones expressing the predicted viral RNAs and proteins lacked expression of endogenous c-myc RNA and protein.

To demonstrate that normal v-myc protein was required for suppression of c-myc expression, we constructed a series of v-myc retroviruses containing frameshifts and deletions in v-myc. Two frameshift mutants were constructed, J-5A, which has a frameshift in the 5′ sequences of v-myc and J-5B, which has a frameshift in gag that only removes the gag-v-myc fusion protein. Four v-myc deletion mutants were also constructed which lacked either the DNA binding or the acidic domains of v-myc protein. RNA and protein analyses of cells infected with these mutants demonstrated that these constructs expressed high levels of the predicted sized v-myc RNAs and proteins. These proteins were similar to normal v-myc in their stability and ability to localize to the nucleus. Analysis of the frameshift mutant J-5A demonstrated high levels of endogenous c-myc expression, demonstrating that functional v-myc protein was required for suppression. The gag portion of v-myc protein made by the J-5 virus was not required for suppression of endogenous c-myc since the frameshift mutant J-5B, which expresses v-myc protein only from a subgenomic mRNA, also suppresses endogenous c-myc expression. Analyses of the deletion constructs demonstrated that both the DNA binding and acidic domains of v-myc protein are required for suppression of c-myc expression. Regulation of c-myc mRNA levels has been documented to occur at both transcriptional and post-transcriptional levels. To test if suppression of c-myc expression in v-myc-infected fibroblasts occurred at the level of transcription, we utilized a nuclear run-on transcription assay using a single-stranded c-myc exon 1 probe that specifically detects sense c-myc transcripts. The results showed that moderate levels of sense exon 1 transcription occurred in control cells, but was markedly depressed or not detectable in J-5 virus-infected cell clones. The depressed activity of the endogenous c-myc locus in these cells was not a consequence of gross changes in the gene as assessed by Southern blot analyses. To test if the regulatory regions of c-myc were altered in v-myc-infected

fibroblasts, we performed DNase I hypersensitivity assays. Control and v-myc-infected cells had identical patterns of DNase I hypersensitivity sites and they did not differ significantly in their intensity.

2. Suppression of c-myc transcription in v-myc-infected NIH 3T3 cells is reversible. Our findings that suppression of c-myc expression was at the level of transcription, yet did not include gross changes in chromatin structure, suggested that suppression of c-myc transcription in v-myc-infected fibroblasts might be reversible if we could remove intracellular pools of v-myc protein. Treatment of a single-cell clone of v-myc-infected cells with anisomycin alleviated the suppression of c-myc so that by 2 hours post-treatment, c-myc mRNA was detectable. The levels of c-myc mRNA that accumulated in v-myc cells were, however, approximately 100-fold lower than those that accumulated in uninfected cells. Since treatment with a protein synthesis inhibitor released suppression of c-myc, we also tested whether stimulation with growth factors could circumvent the down-regulation of c-myc in v-myc-infected cells. c-myc and c-fos expression was induced in v-myc-infected cells with kinetics similar to those of serum-treated, uninfected cells. The infected cells, however, differed in the amounts of c-myc mRNA which accumulated, and by 48 hours post-treatment, c-myc mRNA was no longer detectable in these cells.

3. Exogenous c-myc and N-myc suppress expression of endogenous c-myc in NIH 3T3 cells. It was possible that the observed suppression of c-myc transcription in v-myc-infected fibroblasts was due specifically to the mutations present in v-myc protein relative to c-myc. Therefore, we also tested whether a c-myc retrovirus which expresses murine c-myc exons 2 and 3 coding sequences and a selectable neo gene was able to suppress endogenous c-myc expression. G418-resistant single cell clones showed high levels of exogenous viral c-myc, but no detectable endogenous c-myc RNA, demonstrating that the point mutations in avian v-myc relative to murine c-myc protein are not required for repression of endogenous c-myc transcription. The v-myc deletion mutants which were unable to suppress c-myc transcription encompass two domains which are highly conserved among myc family members. Therefore, we also tested whether expression of high levels of exogenous N-myc were capable of suppressing endogenous c-myc in NIH 3T3 fibroblasts. NIH 3T3 cells were co-transfected with plasmids pSV2neo and pmp 34.1 which contain the entire human N-myc gene whose expression is driven by a Moloney MuLV enhancer in the 5′ flank of the gene. Single-cell G418-resistant cells expressed high levels of N-myc and low or nondetectable levels of c-myc mRNA. Therefore, high levels of exogenous N-myc also appear to suppress steady state levels of endogenous c-myc mRNA.

4. Exogenous 12S and 13S adenovirus E1A proteins suppress c-myc mRNA levels. The acidic domain deleted in J-5 virus-derived v-myc mutants has considerable homology with a similar domain in the adenovirus E1A gene. To test whether exogenous E1A nuclear oncoproteins were also able to influence expression of endogenous c-myc, we infected NIH cells with recombinant retroviruses which express the selectable neo gene and either the 12S or 13S E1A proteins. Blot analysis of single-cell infected clones demonstrated that expression of c-myc was suppressed in cells containing exogenous 12S and 13S E1A, with the more dramatic repression occurring in the 12S E1A-containing cells. Therefore, high levels of exogenous E1A also appear to negatively influence c-myc RNA levels.

In conclusion, our studies demonstrate that myc family proteins are directly or indirectly involved in the negative regulation of c-myc proto-oncogene transcription. This regulation is novel in that it occurs at the level of transcription initiation from the two c-myc promoters, and it is reversible and is not associated with any distinct changes in chromatin structure in the regulatory region of the gene. Our observations that exogenous N-myc also suppresses expression of endogenous c-myc additionally suggests that myc family proteins are involved in cross-regulation of other myc family members, a fact that may have relevance for establishing their lineage-specific patterns of expression. Finally, our findings that exogenous E1A functions similarly to myc in suppression of endogenous c-myc suggests that these two proteins may share transcriptional target genes and may function similarly in transcriptional regulation.

Publications:

Cleveland JL, Huleihel M, Bressler P, Siebenlist U, Akiyama L, Eiseman R, Rapp UR. Negative regulation of c-myc transcription involving myc family proteins. Oncogene Res (In Press).

Cleveland JL, Huleihel M, Storm S, Bressler P, Siebenlist U, Eisenman R, Rapp UR. Role of myc in tumor induction, growth factor abrogation, and control of c-myc expression. In: Alt F, Ziff E, eds. Current communications in molecular biology. New York: Cold Spring Harbor Laboratory, 1987;199-205.

Cleveland JL, Morse HC III, Rapp UR. myc oncogenes and tumor induction. ISI atlas of science: Biochemistry (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05527-02 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of HIV Mutants Defective in gag Gene Processing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Single-cell clones of human immunodeficiency virus-1 (HIV-1)-infected HuT 78 cells have been obtained which produce virus with no mature gag proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analyses of proteins associated with this virus show only large amounts of the HIV-1 gag precursor, Pr55. Electron microscopy reveals aberrant "immature" virus particles which contain an electron-lucent core surrounded by a semielectron-dense, incomplete ring of ribonucleoproteins. Purified and lysed virus preparations lack an intact protease; the addition of partially purified protease isolated from a "wild-type" virus results in the cleavage of Pr55 to proteins that co-migrate and cross-react antigenically with the mature HIV-1 gag proteins, p26 and p17. This in vitro assay for HIV protease using its natural substrate, Pr55, is being used to identify HIV protease-specific inhibitors that may have therapeutic applications in treating HIV-infected patients.

The proteolytic cleavage products of the gag precursor of a related virus, simian immunodeficiency virus (SIV/Mne), have been purified and their amino acid sequences partially determined. The SIV/Mne gag precursor (Pr60gag) is cleaved to proteins with the order p16-p28-p2-p1-p6. p16 is the 5′ gag protein, and p8 was identified as the nucleic acid binding protein. Peptide bonds cleaved during proteolytic processing of the SIV gag precursor are similar to bonds cleaved during processing of HIV-1 gag precursors, suggesting that the SIV and HIV viral proteases have similar cleavage site specificities.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

To characterize the molecular basis of an HIV gag gene processing defect. To develop a specific in vitro assay for HIV protease that can be used to identify potential HIV protease inhibitors for eventual clinical trials. To study the kinetics of the cleavage of the HIV gag precursor (Pr55) in order to delineate the maturation steps of HIV, and to compare HIV gag processing to that seen in the related simian immunodeficiency viruses (SIVs).

Methods Employed:

The HuT 78 lymphocyte cell line was cloned on a feeder layer of primary sheep choroid plexus cells in microtiter plates. Antigens and antibodies were detected by radioimmunoassays and by Western immunoblot techniques. HIV (FRE-3) virus is being molecularly cloned by screening a library constructed in the lambda phage, EMBL3. HIV protease is being purified by gel-filtration chromatography and by high pressure liquid chromatography (HPLC). SIV gag proteins were purified by HPLC.

Major Findings:

1. Characterization of SIV gag proteins and their processing from the precursor. SIV/Mne was isolated from a pig-tailed macaque (M. nemestrina) with lymphoma housed at the University of Washington Regional Primate Research Center. In order to better understand the relationship of SIV/Mne to HIV-1, we have purified and determined the partial amino acid sequences of six structural proteins (p1, p2, p6, p8, p16, and p28) from SIV/Mne and compared these amino acid sequences to the translated nucleotide sequences of SIV/mac and HIV-1 and HIV-2. A total of 125 residues of SIV/Mne amino acid sequence were compared to the predicted amino acid sequences of the gag precursors of SIVs and HIVs. In the compared regions, 92% of the SIV/Mne amino acids were identical to predicted residues of SIV/mac; 83% were identical to predicted residues of HIV-2; and 41% were identical to predicted residues of HIV-1.

These data reveal that six SIV/Mne proteins are proteolytic cleavage products of the gag precursor ($Pr60^{gag}$) and that their order in the structure of $Pr60^{gag}$ is p16-p28-p2-p8-p1-p6. Rabbit antisera prepared against the purified p28 and p16 were shown to cross-react with proteins of 60k, 54k and 47k present in the viral preparation and believed to be SIV/Mne $Pr60^{gag}$ and intermediate cleavage products, respectively. SIV/Mne p16 was shown to contain covalently bound myristic acid and p8 was identified as a nucleic acid binding protein.

The high degree of amino acid sequence homology between SIVs and HIV-2 around proven proteolytic cleavage sites in SIV $Pr60^{gag}$ suggests that proteolytic processing of the HIV-2 gag precursor is probably very similar to processing of the SIV gag precursor. Peptide bonds cleaved during proteolytic processing of the SIV gag precursor are similar to bonds cleaved during processing of HIV-1 gag precursors, suggesting that the SIV and HIV viral proteases have similar cleavage site specificities.

2. Biological cloning of an HIV-1 mutant that is defective in processing of the gag precursor. An HIV-1 isolate, obtained by cocultivating the peripheral blood lymphocytes from an HIV antibody-positive patient on the HuT 78 cell line was shown to be poorly infectious. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins associated with this virus (designated HIV-1 [FRE-3]) showed that it contained large amounts of a 55k protein. Electron microscopy (EM) of HIV (FRE-3)-infected HuT 78 cells revealed a mixed population of lymphocytes; some were producing only extracellular particles with a normal mature HIV morphology and the remainder were releasing only aberrant particles with an "immature" morphology. Single-cell clones were obtained by growing these infected HuT 78 cells in microtiter plates containing a feeder monolayer of primary sheep choroid plexus cells.

Retroviruses produced by these clones were characterized for the presence of reverse transcriptase and envelope glycoproteins, and for the morphology and infectivity of virus particles. Some of the clones produced infectious "wild-type" HIV-1 (reverse transcriptase positive, mature gag proteins visualized on SDS-PAGE), which by EM resembled mature virus particles. Other single-cell clones (for example, CL E5D) were releasing viruses, which by EM contain an electron-lucent core surrounded by an incomplete dense ring of ribonucleoproteins, and thus resemble immature extracellular virus particles. SDS-PAGE and Western immunoblot analyses of the proteins present in the immature virus particles reveal a 55k protein that has antigenic determinants that are recognized by rabbit polyclonal antisera raised against purified HIV p17 and p24 proteins. None of the mature HIV gag proteins (p24, p17, or p7) are detectable. HuT 78 cells (CL E5D) producing the noninfectious viruses that contain only the 55k gag precursor cannot be superinfected by "wild-type" HIV-1 or by SIV/Mne. The morphology of HIV-1 is thus apparently associated with maturation of the gag precursor in the virus particle.

3. Development of an in vitro assay for cleavage of the HIV-1 gag precursor. HIV-1 clone E5D virus was grown, banded on sucrose density gradients, disrupted, and its proteins analyzed on SDS-PAGE gels before or after incubation at 37°C for 6 hours in the presence of 10 mM Tris-HCl, pH 7.7, 10% glycerol, 0.2% NP40, and 2 mM DTT. These studies showed that CL E5D did not contain detectable gag precursor proteolytic activity. When HIV protease, partially purified by Sephadex G-100 chromatography, was added to disrupted CL E5D virus preparations, cleavage of the Pr55 gag precursor occurred and yielded proteins that co-migrate and reacted antigenically with sera prepared to the mature HIV gag proteins p24 and p17. These results suggest that the virus released by CL E5D lacks a functional protease and that the addition of exogenous protease cleaves the gag precursor.

These HIV viruses represent the first description of a gag processing defect in lentiviruses. The in vitro assay will be useful to test for protease inhibitors that might have therapeutic potential in the treatment of patients infected with the AIDS virus.

Publications:

Henderson LE, Benveniste RE, Sowder R, Copeland TD, Schultz AM, Oroszlan S. Molecular characterization of gag proteins from simian immunodeficiency virus (SIV_{Mne}). J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05528-02 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Bovine Leukemia Virus Regulatory Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (L. Martarano)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.4	0.4	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The expression of the retrovirus, bovine leukemia virus (BLV), is highly restricted in vivo and in vitro. The virus has been molecularly cloned and its various genes and control sequences have been isolated and tested by reintroduction into mammalian cells. The logic underlying the regulatory system is emerging from such analysis; BLV encodes a transcriptional activator protein of 38 kilo Daltons (KDa) termed tat, which interacts with enhancer sequences upstream of the viral promoter. This protein acts to increase the rate of transcription of BLV. The tat gene encodes a second protein (in a different reading frame) termed rex. This 18-KDa phosphoprotein was found to be essential for the accumulation of viral mRNAs encoding BLV structural genes but was not required for the synthesis of the mRNA encoding the regulatory proteins tat and rex. The mechanism of rex-mediated regulation of RNA processing was identified by independently expressing tat and rex in mammalian cells transfected with BLV proviral mutants. It was found that rex acts at the level of mRNA 3′ end processing reactions and requires the specific processing signals provided by the 3′ BLV long terminal repeat. The molecular dynamics of this reaction are being examined in in vitro reactions using HeLa nuclear extracts and RNA substrates generated by bacteriophage RNA polymerases. Both tat and rex are being expressed in E. coli.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David Derse — Senior Staff Fellow — LVC — NCI

Objectives:

1. Define the role and mechanism of regulation of the second regulatory protein of bovine leukemia virus (BLV), $p18^{rex}$.

2. Construct bacterial expression plasmids to produce $p38^{tat}$ and $p18^{rex}$ in *E. coli* for *in vitro* studies of proteins.

3. Produce polyclonal rabbit antisera to oligopeptides deduced from $p38^{tat}$ and $p18^{rex}$ sequences to study regulatory proteins.

4. Examine the mechanism of $p18^{rex}$ in *in vitro* RNA processing reactions.

Methods Employed:

The following techniques were employed: (1) cloning and construction of prokaryotic and eukaryotic expression plasmids, (2) transfections of mammalian cells with recombinant plasmids, (3) analysis of gene expression by Northern and Southern blotting or enzyme assay, and (4) *in vitro* synthesis of RNA and *in vitro* analysis of processing reactions using nuclear extracts.

Major Findings:

1. *$p38^{tat}$ is a transcriptional activator*. It was previously shown in this lab that $p38^{tat}$ is a transcriptional *trans*-acting regulator of BLV expression. Those studies were extended and confirmed by constructing new eukaryotic expression plasmids. These new plasmids do not require splicing or 3′ end processing signals derived from BLV and can produce either $p38^{tat}$ or $p18^{rex}$ exclusively, even though they are encoded by overlapping genes. It was thus confirmed that $p38^{tat}$ is necessary and sufficient to activate BLV transcription initiation.

2. *$p18^{rex}$ regulates BLV mRNA processing*. Cotransfection of cells with $p18^{rex}$ expression plasmids in combination with BLV proviruses deficient in regulatory protein synthesis revealed that $p18^{rex}$ is essential for the accumulation of certain viral RNAs. In the absence of $p18^{rex}$ expression, BLV structural genes were not expressed, whereas the mRNA for the regulatory proteins was synthesized. This defect could be complemented in *trans* by $p18^{rex}$. Proviruses with large deletions or additions revealed that sequences at the extreme 3′ terminus of the virus were necessary for the regulatory effects of $p18^{rex}$. A test plasmid was constructed containing a rabbit β-globin gene controlled by a Rous sarcoma virus promoter and whose expression was dependent on 3′ end processing and polyadenylation signals provided by a BLV 3′ long terminal repeat (LTR). Accumulation of β-globin mRNA was observed in cells transfected with this

plasmid. Moreover, β-globin mRNA synthesis was increased 10 to 20 times from this plasmid in response to p18^{rex} expression. Thus, p18^{rex} appears to be required for efficient utilization of the 3′ end processing signals present in the viral LTR. A 100-bp deletion was introduced into the LTR of this β-globin test plasmid, in a region between the poly (A) addition signal and poly (A) addition site. While this deletion does not affect known processing signals, it prevented β-globin mRNA accumulation and response to p18^{rex}. It thus appears that p18^{rex} acts in trans with sequences in the 3′ LTR to regulate viral mRNA 3′ end processing. This novel mechanism of regulating gene expression has not been observed with other retroviruses except human T lymphotrophic virus-I (HTLV-I) and is reminiscent of the regulation described for adenovirus and certain immunoglobulins. To more precisely define the mechanism of p18^{rex} and its potential intracellular interactions, in vitro tests were performed. The BLV LTR was cloned into plasmids that allow in vitro synthesis of RNA transcribed by bacteriophage RNA polymerase. The in vitro processing of this RNA was examined by gel electrophoresis after incubation with HeLa cell nuclear extracts. To complement these studies, p18^{rex} protein is being synthesized in large amounts in bacteria and antisera to p18^{rex} has been generated.

Publications:

Derse D. Bovine leukemia virus transcription is controlled by a virus encoded trans-acting factor and cis-acting response elements. J Virol 1987;61:2462-71.

Derse D. Trans-acting regulation of bovine leukemia virus mRNA processing. J Virol 1988;62:1115-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05529-02 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic and Molecular Organization of the MHC in the Domestic Cat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Naoya Yuhki	Visiting Fellow	LVC	NCI
	Janice Martenson	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	1.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The feline major histocompatibility complex (MHC) was characterized to examine the immune system and the gene evolution in the domestic cat. The sequences of eight MHC class I cDNA clones showed that at least two different allelic forms of transplantation antigen were identified and that the molecular structure is similar to that of humans. Three unique features of the transplantation antigen were observed in the domestic cat: (1) a cysteine residue of the cytoplasmic domain was lacking, (2) a cluster of amino acid substitutions was observed in a highly constant region of the α2 domain, and (3) no amino acid substitutions were observed in a highly variable region of the α2 domain. These evidences suggest that (1) feline transplantation antigen does not interact with a cytoplasmic component by disulfide bonding, (2) one transplantation antigen has a different framework from that of human and mouse, and (3) the capacity of feline transplantation antigens to bind various foreign antigens may be somewhat less than those of human and mouse. Southern blot analysis using 3′ untranslated (UT) regions of class I cDNA clones showed that the majority of class I genes have a similar 3′ UT region and one class I gene has a unique 3′ UT region, which suggest that the majority of class I genes evolved from a common ancestor gene in a manner of gene duplication, but that one unique class I gene evolved from a different ancestor gene or by a recent drastic nucleotide change of the 3′ UT region. The former 3′ UT region probe hybridized well with the DNAs of the species from the domestic cat lineage and a part of the panthera lineage and did not hybridize well with the DNAs of the species from the ocelot lineage or the panthera group. This evidence suggests that the species of the cat family are able to be classified into those three groups in terms of the evolution of class I genes and that the ancestor gene of the majority of class I genes of the domestic cat appeared after the separation of those three groups.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Naoya Yuhki	Visiting Fellow	LVC	NCI
Janice Martenson	Microbiologist	LVC	NCI

Objectives:

The major histocompatibility complex (MHC) encodes two classes of genes whose products regulate the immune responses against both humoral and cellular foreign antigens. In addition to their immunological importance, the MHC genes are proposed as an excellent model for studying gene evolution since this complex has approximately 20 to 30 class I genes and 7 to 14 class II genes.

The domestic cat is a good model for studying viral infections, the immune system, and gene evolution. Many viruses are endemic to this animal, such as feline leukemia virus, feline T-lymphotropic lentivirus, and feline infectious peritonitis virus. The MHC of the domestic cat has been poorly characterized to date because of numerous difficulties in the preparation of allotypic antisera. One simple explanation of these two evidences is that the domestic cat has a limited extent of polymorphism of either class I or class II molecules and fails to induce humoral and cellular immune responses against viral and alloantigens. To examine this possibility, as the first step, we isolated class I cDNA clones of the domestic cat and performed sequence analysis.

Methods Employed:

The following techniques were employed: (1) RNA and DNA blotting, (2) molecular cloning, and (3) DNA sequencing using the dideoxy sequencing method.

Major Findings:

1. Two different transplantation antigen-coding cDNA clones and the similarity to that of human. Eight class I cDNA clones were isolated from a library of the 3201 T-cell lymphoma cell line of the domestic cat and sequenced. Three different cDNA clones were identified. Out of these clones, one clone (pFLA2) had a unique 3′ untranslated (UT) region. Using this region as a probe, all DNAs from different cats showed one or two bands, and every organ examined so far contained this gene's transcripts, suggesting that this cDNA encodes a transplantation antigen. Another clone (pFLA24) had a different 3′ UT region which hybridized with many bands in cat DNAs and detected transcripts in every organ. The deduced amino acid sequence of pFLA24 showed that this molecule conserved the consensus of transplantation antigen to human and mouse at many points. These evidences suggest that this clone also encodes a transplantation antigen. The third clone (pFLA62) had the same nucleotide sequence as that of pFLA24 except for a seven nucleotide insertion into the transmembrane coding region, which causes frameshift mutations of the amino acid sequences.

Nonetheless, this molecule contained four positively charged amino acids after hydrophobic amino acid sequences and had a stop codon in the middle of the cytoplasmic region of the normal transplantation antigen. Since this truncated class I molecule is typical of the Qa/Tla antigen of human and mouse, we speculated that this cDNA clone encodes the Qa/Tla antigen, although the possibility that alternative splicing of the transcript of the transplantation antigen causes the different transcript still remains.

Comparison of transplantation antigens among human, mouse, and cat showed that the feline transplantation antigen has a high degree of homology to human at the nucleotide (>80%) and amino acid (>70%) sequence levels. In addition, the positions of four cysteine in the α2 and α3 domains and one possible N-glycosylation site (N-X-S/T) in the α1 domain were conserved between human and cat. The conservation of the length of each domain except a three amino acid deletion in the cat was also observed between human and cat. These evidences suggest that feline transplantation antigen has a similar structure to that of human.

2. The feline transplantation antigen has three unique features. Transplantation antigens of human and mouse have one conserved cysteine in the cytoplasmic region which is located at the distal portion of the carboxyl-terminal end (-CKV.) in the human and mouse or at the middle of the cytoplasmic region in human. However, one feline transplantation antigen (pFLA2) does not have cysteine and another one (pFLA24) has a cysteine just after the transmembrane region. These evidences suggest that the feline transplantation antigen does not interact with a cellular component by disulfide bonding.

Comparison between two feline transplantation antigens (pFLA2 and pFLA24) showed that 34 amino acid substitutions existed out of 289 deduced amino acid sequences (11.7%) and 13 (38.2%) are located in the α1 domain. Eleven in the α1 domain are located in a hypervariable region of human, mouse, and porcine transplantation antigens. However, the nested five amino acid substitutions were located on a highly conserved region of the α2 domain. This region is well conserved in human, mouse, and porcine transplantation antigens and may participate in making a framework of a transplantation antigen molecule. This nested substitution was caused by pFLA2 since the deduced amino acid sequence of this region in pFLA24 was matched to that of human. Thus, one feline transplantation antigen has a different framework than other transplantation antigens.

The last important feature of the feline transplantation antigen is that no amino acid substitutions were found in the middle of the α2 domain and only one conserved amino acid substitution (valine -> leucine) was found in the border region of the α2 and α3 domains. These regions are identified as hypervariable regions in human, mouse, and pig. Comparison of any two transplantation antigens in these species showed amino acid substitutions in these regions without exception. This evidence suggests that these two feline transplantation antigens have limited polymorphism in these hypervariable regions.

3. Evolution of MHC class I genes. The number of class I genes of the domestic cat was roughly estimated at 20 by the number of bands obtained using a Southern blot method. Out of these genes, one transplantation antigen coding

gene has a unique 3′ UT region and the majority of other class I genes have similar 3′ UT regions. This evidence suggests that the majority of class I genes evolved from a single ancestor gene in a manner of gene amplification and that a unique gene evolved from a different ancestor gene or by a recent drastic nucleotide change of the 3′ UT region.

In BamHI- or EcoRV-digested DNAs from various species of the cat family, 7 to 11 average bands of hybridization were found using the coding region of feline class I cDNA as a probe. Accordingly, MHC class I genes constitute a multigene family with similar numbers in different species of the cat family. However, the 3′ UT region of feline cDNA detected the bands in DNAs from the species of domestic cat lineage, and panthera lineage with the exception of the panthera group, but not of the ocelot lineage and panthera group. These evidences suggest that the species, which have similar 3′ UT regions to that of the domestic cat, are closely related in terms of the evolution of MHC class I genes and a common ancestor gene appeared after separation of these species from other groups.

4. Others. (a) Organization of feline MHC: To determine the organization of feline MHC, 60 class I cosmid clones were isolated and are going to be characterized. Preliminary experiments showed that approximately 30% of the clones contained two class I genes and this value was the middle between that of human (15%) and mouse (50%). The study using pulse field gel electrophoresis is also in progress and shows that the entire group of class I genes belongs to a 800-kb single NotI DNA fragment. (b) T-cell receptor genes of the domestic cat: To expand the study of immunogenetics of the domestic cat, we isolated cDNAs of α, β, and γT-cell receptor genes of the domestic cat using human cDNAs as probes and sequence analyses of these clones are underway.

Publications:

Yuhki N, O'Brien SJ. Molecular characterization and genetic mapping of class I and class II MHC genes of the domestic cat. Immunogenetics (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05531-02 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular and Functional Characterization of raf Oncogene-Related Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Walter Kolch	Visiting Fellow	LVC	NCI
	John L. Cleveland	Senior Staff Fellow	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Thomas Beck	Biotechnology Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS *(if any)*

Physiologisch-Chemisches Institut der Universitaet Marburg, Marburg, Federal Republic of Germany (D. Gallwitz)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	1.5	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The raf oncogene family is evolutionarily old and well conserved throughout species. To address aspects of raf protein function which are difficult to study in mammalian cells we turned to the yeast system because of its amenability to genetic analysis. Using raf-specific probes we were able to molecularly clone a putative raf-related gene from S. cervisiae. The nucleotide sequence is currently being determined. As this and other projects required raf family-specific, but not species-restricted probes, various portions of human c-raf-1 and v-raf were expressed in E. coli. A 29-kd v-raf kinase-specific protein was produced in high yields and was used to raise a polyvalent antiserum, which detects v-raf and c-raf proteins in a variety of species. As an alternative approach to investigate regulation of raf kinase activity and ligand/substrate specificity we designed recombinant retroviruses carrying c-raf/protein kinase C hybrid DNAs. Thereby, one can take advantage of known regulators and substrates of either one kinase to follow up on their role in signal transduction and mutual interaction.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Walter Kolch	Visiting Fellow	LVC	NCI
John L. Cleveland	Senior Staff Fellow	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
Thomas Beck	Biotechnology Fellow	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

The goals of this study are (1) to identify and molecularly clone raf oncogene-related DNA sequences; (2) to determine their nucleotide sequences; (3) to test these genes for transforming ability in vitro and in vivo; (4) to assay their expression in transformed and normal cells; (5) to identify raf-regulating ligands, as well as substrates for raf kinase activity; and (6) to determine whether cell responses to raf activity include changes in gene expression. The latter two aspects are also to be covered in the yeast system focusing on (a) complementation experiments with existing yeast mutants and (b) mutation analysis of yeast raf genes.

Methods Employed:

Screening strategies were based on filter hybridizations with various raf-specific nucleic acid as well as a polyvalent antibody probe. Standard recombinant DNA technology is used for cloning, characterization of subclones and DNA sequencing. Gene expression is monitored by Northern blotting and Western blotting. For production of raf proteins in E. coli, inducible protein expression vectors were used.

Major Findings:

1. The isolation and characterization of new raf-specific cDNAs from human cDNA libraries is included in Project Z01CP05417-04 LVC.

2. A raf-related gene in the yeast S. cervisiae has been detected. In yeast several lines of evidence implicate serine-specific protein kinases in regulation of cell growth. As raf functions downstream of membrane-associated signal transducers in mammalian cells and proximal mechanisms governing DNA replication appear to be evolutionarily conserved we attempted to clone the yeast raf cognate gene. Based on previous results from Southern blot hybridizations a size-selected yeast library was made in phage lambda gt10. Screening of this library with raf-specific probes yielded two groups of clones: one group representing the epsilon subunit of DNA polymerase III was detected by an oligonucleotide complementary to the ATP-binding site of the raf kinase; the other group likely corresponds to a raf-related gene as suggested by comparing hybridzation patterns on Southern and Northern blots using c-raf or these clones as the probe. The nucleotide sequence analysis is currently underway.

In a different approach, a lambda gt11 yeast protein expression library was screened with a polyvalent antiserum raised against the raf kinase domain, which will be described below. Two pools of antibody-reactive clones have been isolated for further characterization.

3. In order to obtain reagents useful for the functional characterization of raf proteins we constructed inducible expression vectors to produce different versions of raf proteins in E. coli.

The carboxyl terminal portion of v-raf was expressed at high levels fused to the promoter-leader region of the E. coli tryptophan operon. Two differently sized proteins, 29 and 26 kD, were made, the smaller version being a proteolytically processed form as judged by differential reactivity with antipeptide sera. The larger size protein was used to raise a polyvalent antiserum suitable for studying v-raf and c-raf proteins in a variety of species ranging from mouse to man.

Full-length and truncated versions of the human c-raf-1 cDNA have been expressed under the control of the lambda P_L promoter. c-raf proteins of 73 kd, 57 kd and 39 kd were produced upon induction. p73 differs from normal c-raf-1 by deletion of the two first N-terminal amino acids and their replacement by 16 amino acids encoded by the vector. The p57 and p39 represent N-terminal deletions which leave the transforming protein kinase intact. These proteins could be readily purified from E. coli lysates by immunoprecipitation.

4. Given the overall structural similarity shared between the raf and protein kinase C (PKC) family, the role of raf and PKC in serine/threonine kinase-mediated signal transduction is being explored in regard to ligand/target specificity, regulation of kinase activity and mutual interaction. These questions are being addressed by switching regulatory and catalytic domains in chimeric constructs. The hybrid DNAs as well as full-length and truncated versions corresponding to the kinase domains are incorporated in a Moloney murine leukemia virus (MoMLV)-based neomycin selectable retroviral vector. Neither full-length raf nor full-length PKC are transforming in this context.

Publications:

Kolch, W, Bonner TI, Rapp UR. Expression of human c-raf-1 oncogene proteins in E. coli. Biochem Biophys Res Commun (In Press).

Kolch W, Schultz A, Oppermann H, Rapp UR. Preparation of raf oncogene precipitating antisera with raf protein produced in E. coli. Biochim Biophys Acta 1988;949:233-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05532-02 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of raf Family Protein Kinases on Cell Physiology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Thomas W. Beck	Biotechnology Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	Walter Kolch	Visiting Fellow	LVC	NCI
	John L. Cleveland	Senior Staff Fellow	LVC	NCI
	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI
	Robert Bassin	Senior Investigator	LTIB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.4	1.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Since the identification of v-raf as the oncogene of the acutely transforming retrovirus, 3611-murine sarcoma virus, significant progress has been made in the molecular and functional characterization of raf proteins and their effects on cell physiology. (1) Amino terminally truncated versions of c-raf-1 and A-raf-1 are transforming in vitro and in vivo. (2) raf proteins are cytoplasmically located protein kinases related to the src gene superfamily and truncated versions possess serine-threonine-specific protein kinase activity. Moreover, c-raf and A-raf show homology to protein kinase C, not only in the C-terminal kinase domain, but also in the N-terminal putative regulatory domain. (3) Certain raf-transformed fibroblasts release transforming growth factor(s) (TGF), express TGF-alpha mRNA in certain cases, and are inhibited in collagen synthesis. (4) Functional assays utilizing NIH 3T3 cells that are growth arrested by micro-injection of ras monoclonal antibody or transformation of flat revertants of Kirsten sarcoma virus-transformed fibroblasts suggest that raf family oncogenes act independent of ras, either through a signal transduction pathway not involving ras or one in which raf has a position downstream of ras. (5) Consistent with a downstream position in mitogen signal transduction, raf protein kinase was found to be activated by treatment of cells with growth factors such as platelet-derived growth factor, epidermal growth factor or 12-O-tetra-decanoyl-phorbol-13-acetate, as well as by intracellular mitogens, including src, middle T or fms. Activation of the raf signal transduction pathway ultimately leads to stimulation of Ap-1-dependent transcription.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC NCI
Thomas W. Beck	Biotechnology Fellow	LVC NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC NCI
Walter Kolch	Visiting Fellow	LVC NCI
John L. Cleveland	Senior Staff Fellow	LVC NCI
Mahmoud Huleihel	Visiting Fellow	LVC NCI
Robert Nalewaik	Microbiologist	LVC NCI
Robert Bassin	Senior Investigator	LTIB NCI

Objectives:

The objectives of these studies are (1) to characterize the raf family proteins with respect to their positions in signal transduction and to identify the pathway(s) in which they operate, (2) to determine how these proteins are regulated in normal and transformed cells (by phosphorylation, or the possibility of down regulation by proteolysis), (3) to identify putative ligands which may control raf protein kinase activity, (4) to determine the specific cellular targets of "activated" and normal raf, and (5) to identify raf-regulated genes.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and subclone raf cDNA species into Moloney murine leukemia virus (strain Leuk). Specific cDNA clones were inserted into expression vectors and high levels of synthesis of raf polypeptides in E. coli demonstrated by protein gel and immunoblot analyses. Protein analyses of raf in normal and transformed cells were carried out by metabolic labeling, immunoprecipitation and gel analyses, and raf-associated kinase activity was demonstrated using our established protocol.

Major Findings:

The following properties of raf proteins in transformed cells have been elucidated from the analysis of v-raf, the transforming oncogene of 3611-murine sarcoma virus (MSV) long terminal repeat (LTR)-raf, which was obtained by LTR trans-fection of NIH 3T3 cells and which contains an activated c-raf-1 locus via promoter insertion and in vitro construct viruses, A-raf-MSV and c-raf-MSV.

1. Truncated versions of c-raf-1 and A-raf-1 cDNAs, when incorporated into a retrovirus expression vector, cause transformation of cells in culture and induce fibrosarcomas and erythroid hyperplasias in newborn mice similar to those induced by 3611-MSV.

2. One of the hallmarks of oncogenic transformation of fibroblastic cells in culture is the induction of the release of transforming growth factors (TGFs) as measured by a block in epidermal growth factor (EGF) receptor binding and

conditioning of the growth medium. 3611-MSV-transformed rat fibroblasts, as well as A-raf-MSV-transformed cells, release TGFs, as do other cells transformed by src superfamily oncogenes, such as v-fes, v-abl, and v-mos, and also the members of the ras oncogene family. Recently, we have shown that TGF-alpha mRNA is induced in raf-transformed rat epithelial cells, but not in untransformed cells or raf-transformed NIH 3T3 cells.

3. The gag-raf/mil fusion proteins of the 3611-MSV and MH2 viruses purified by immunoaffinity chromatography have been demonstrated to possess serine/threonine (ser/thr)-specific protein kinase activity detectable in autophosphorylation assays, as well as by phosphorylation of exogenous substrates in vitro. Interestingly, the p48 raf protein produced by the LTR-activated c-raf locus in F12 cells shows autophosphorylating activity in vitro, whereas the normal-sized p74 cellular c-raf protein appears essentially inactive in this assay. This finding is consistent with the hypothesis that oncogenic activation of c-raf involves N-terminal truncation, which releases the molecule from negative regulation of its kinase domain.

4. c-raf and A-raf proteins are localized primarily in the cytoplasm of human and mouse cells based on cell fractionation and immunoprecipitation, as well as on immunofluorescence studies. These findings are consistent with the presence of amino acid sequences which lack extensive hydrophobic regions characteristic of transmembrane domains of membrane proteins.

5. Comparison of the predicted amino acid sequences of c-raf-1 and A-raf-1 to the multiple species of protein kinase C revealed sequence homology not only in the C-terminal kinase domain (as with other protein kinases, such as cAMP-dependent kinase, cGMP-dependent kinase and src superfamily kinases), but more interestingly, striking homology (>30%) in the N-terminal putative regulatory domain. This 46-amino acid region encompasses the cysteine-rich putative metal/nucleic acid-binding region (Berg et al., Science 232: 485, 1986). Moreover, both c-raf as well as A-raf, show potentially significant homology to the cysteine-rich region in the extracellular ligand-binding domains of the EGF and insulin receptors, suggesting that (a) this region represents a ligand-binding domain, (b) raf protein kinase activity may be regulated by effector molecules related to the effectors of the above receptor-kinases or kinase C, and (c) raf functions in signal transduction.

6. The tissue expression patterns suggest that c-raf functions in a common signal transduction pathway(s), whereas A-raf functions in a pathway(s) normally restricted to a limited number of cell types.

7. A functional assay has been developed in which NIH 3T3 cells become growth arrested (as measured by ^{3}H-thymidine incorporation) by microinjection of ras antibody. This assay can be used to provide insight into signal transduction pathways utilized by the oncogenes. Cells infected with oncogene-containing viruses can be microinjected with ras antibody and assayed for DNA synthesis as a measure of the virus' ability to overcome the arrested growth due to the ras antibody block. In this assay, A-raf-MSV and 3611-MSV overcome the antibody block, whereas other oncogenes (e.g., v-sis, v-fms, and v-src) are unable to overcome the antibody block. Another functional assay utilizes flat revertants

from Kirsten sarcoma virus-transformed cells (v-ras transformed) and these cells were found to be resistant to transformation by v-ras-containing viruses (Kirsten, Harvey and Balb MSV) and some viruses containing the oncogenes v-fes and v-src. However, we have found that these cells are susceptible to transformation by A-raf-MSV and 3611-MSV. These results suggest that raf family oncogenes act independent of ras either through a signal transduction pathway not involving ras or one in which raf has a position downstream of ras. To further test this preliminary pathway map we have now isolated raf revertant cell lines.

8. We have examined the phosphorylation and the serine/threonine-specific kinase activity of the proto-oncogene product, c-raf, in response to oncogenic transformation or growth factor treatment of mouse 3T3 cells. Expression of the membrane-bound oncogene products encoded by v-fms, v-src, v-sis, polyoma virus middle T antigen, and Ha-ras increased the apparent molecular weight and phosphorylation state of the c-raf protein, while expression of the nuclear oncogene and proto-oncogene products encoded by v-fos and c-myc did not. Changes in electrophoretic mobility and phosphorylation occurred rapidly in response to treatment of cells with platelet-derived growth factor (PDGF), acidic fibroblast growth factor, EGF, and 12-O-tetradecanoyl-phorbol-13-acetate, but not insulin. The phosphorylation of the c-raf protein occurred primarily on serine and threonine residues. However, a subpopulation of c-raf molecules was phosphorylated on tyrosine residues in cells transformed by v-src or stimulated with PDGF. Transformation by v-src, or treatment with PDGF or phorbol 12-myristate 13-acetate, activated the c-raf-associated serine/threonine kinase activity as measured in immune-complex kinase assays. Our results suggest that the c-raf protein can be activated by tyrosine and/or serine and threonine phosphorylation as a result of direct or indirect action of membrane-bound oncogene products and growth factor receptors. c-raf activation may thus serve to transduce signals from the membrane to the cytoplasm and perhaps on to the nucleus.

Publications:

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Horton WR, Cleveland JL, Rapp UR, Kohno K, Yamada Y, Moiyashita T, Hassell JR. The regulation of genes encoding extracellular matrix proteins: in vitro studies and relationships to teratogenic mechanisms. In: Kimmel GL, Kochar EM, eds. In vitro techniques in developmental toxicology: use in defining mechanism and risk parameter. Boca Ratan: CRC Press (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05533-02 LVC

PERIOD COVERED
October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
raf Domains Required for Transformation and Regulation of Kinase Activity

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Others:	Mahmoud Huleihel	Visiting Fellow	LVC	NCI
	Walter Kolch	Visiting Fellow	LVC	NCI
	Thomas Beck	Biotechnology Fellow	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI
	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (F.-M. Duh)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The murine cellular homolog of the 3611-murine sacroma virus (MSV) oncogene was isolated as a cDNA clone and characterized. The gene is highly conserved; only 10 of the 178 nucleotide exchanges led to amino acid substitutions which are evenly spaced. The longest cDNA clone has a 260 bp leader which contains two translational start coclous (ATG) codons preceding the c-raf translational start. It is not likely that removal of the ATG codons alone results in oncogenic activation as expression of the full-length c-raf coding region by a retroviral expression vector did not result in cell transformation. However, a construct that encodes a fusion protein of the gag sequences of 3611-MSV and full-length c-raf transforms NIH 3T3 cells with high efficiency, suggesting that some feature in the amino-terminal end is involved in (auto)regulation of the c-raf kinase. A likely candidate for this is a serine/threonine kinase substrate site (Arg-Arg-Ala-Ser) which is located around position 40. Changing either both arginine residues or the alanine residue by site-directed mutagenesis did not result in oncogenic activation of the raf gene, indicating that phosphorylation of this site might be involved in activation. Mutation of another possible regulatory region of the raf protein, the cysteine-zinc-finger domain, has not resulted in oncogenic activation either. However, some of these mutations revert the transforming activity of gag-c-raf fusions. The minimal transforming sequence of the raf protein has been determined to encompass residues 340 to 635 of the 656 residues of c-raf. This constitutes conserved region 3 which was identified by comparing A-raf and c-raf. This region contains the kinase domain which shows homology to other kinases, among them protein kinase C (PKC). Creating a retroviral vector in which the v-raf sequences were replaced with the equivalent sequences of the rat PKC II gene did not give rise to an acutely transforming virus. NIH 3T3 cells carrying these sequences show higher than normal PKC activity and have an increased growth rate.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Mahmoud Huleihel	Visiting Fellow	LVC	NCI
Walter Kolch	Visiting Fellow	LVC	NCI
Thomas Beck	Biotechnology Fellow	LVC	NCI
Robert Nalewaik	Microbiologist	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI

Objectives:

The gene products of the various raf genes are cytoplasmic serine/threonine protein kinases. Similar to other protein kinases and growth factor receptor proteins, the raf polypeptide contains two major domains: the amino-terminal part is involved in ligand binding, while the carboxyl-terminus is the actual kinase moiety with the ATP-binding site. To define the functional structures of the normal and the transforming raf proteins, we are using several experimental approaches. DNA sequence comparison of normal and biologically activated murine c-raf gene indicates mutations which might be involved in oncogene activation. At the same time we are subjecting the raf genes to extensive mutagenesis to pinpoint regions and amino acids necessary for the function of the proteins. The function is analyzed in several systems: retroviral and eukaryotic plasmid vectors are used to reintroduce the mutated genes into eukaryotic cells and the constructs are scored for kinase and transforming activities. Normal and mutant raf proteins produced in bacteria will be assayed for kinase studies used to identify the as yet unknown substrates and ligands of the raf proteins analogous to those experiments performed with various cell surface receptors. Furthermore, large-scale preparations of wild-type and mutant proteins will be used for microinjection into cell lines transformed by various oncogenes to further define the place of raf in the oncogenic cascade and for the generation of antisera with different specificities.

Methods Employed:

Molecular cloning and DNA sequence analyses were performed following standard protocols. Site-directed mutagenesis was carried out on single-stranded plasmid DNA using synthetic oligonucleotides. For this purpose we have cloned v-raf, mouse and human c-raf, and human A-raf into KS plasmids. These vectors are especially useful for this project as they contain an M13 origin of replication for the production of single-stranded DNA for site-directed mutagenesis, and T7 and T3 promoters for in vitro transcription of genes inserted into an extensive polylinker. Protein studies, including immuno-precipitation kinase assay and Western blotting, have been described previously.

Major Findings:

1. Characterization of the murine cellular c-raf gene. The DNA sequence of a c-raf cDNA clone derived from the mouse cell line, FD, was established. The clone is 2907 base pairs long, thus representing an almost full-length copy of the major transcript which is 3 kb in length. The clone consists of 260 base pairs of 5′ leader sequence, 1947 of coding sequence, and 424 of 3′ non-translated region. Comparison of the human and mouse c-raf sequences indicates a very strong selective pressure on the gene as only 9 amino acid exchanges result from 178 nucleotide exchanges found between the 5′ halves of the two genes. The 3′ untranslated sequence contains three blocks of sequences which are conserved between man and mouse. These blocks are interrupted by sequences that differ in length in the mouse and the human genes. The homology between the conserved sequences is relatively high (80 to 83%), suggesting that they are still under selective pressure. Comparison between mouse c-raf and v-raf shows that 12 nucleotide exchanges have occurred during the process of oncogene activation. Eight of these exchanges are located in the coding sequence and have resulted in four amino acid exchanges. None of the amino acid exchanges are conservative. The high ratio of non-silent to total mutations, and the kind of amino acid changes observed, indicate that the selection on v-raf is different from that on c-raf. None of the exchanges between mouse v- and c-raf coincide with those observed for the mouse and human genes, nor are they found for v-mil, the avian counterpart of v-raf.

The 5′ leader contains one open reading frame (ORF) which starts at the very 5′ end of the cDNA clone. A translational start coclous (ATG) located within this ORF would initiate a translation of 47-residue peptide. A second translation start site found upstream of the c-raf ATG is immediately followed by a termination codon. Other proto-oncogenes have also been shown to contain translational start sites in their 5′ leader sequences. This feature is thought to be involved in regulation of protein expression at the translational level. We will investigate whether this peptide is synthesized and possibly plays a role in the regulation of raf gene expression.

2. Analysis of functional domains of raf proteins by site-directed mutagenesis. raf proteins belong to the oncogene family of protein kinases and show a distant but clear homology to protein kinase C (PKC). To verify these relationships and learn which positions of the proteins are functionally important for transforming and normal activities, we have initiated a site-directed mutagenesis program. The first target was the putative ATP-binding domain which consists of the sequence Gly-X-Gly-X_2-Gly-X_3-Lys-X_2-Lys, and which shows a high degree of conservation in most kinases. The first lysine in this sequence is the most highly conserved amino acid and has been shown to bind ATP. Changing this position in the v-raf gene to a tryptophan codon abolished transforming activity. In contrast, inserting glutamic acid or glutamine instead of the second lysine had only modulating effects on the transforming activity. The Lys to Glu exchange resulted in a somewhat less pronounced transformed focus, while the Lys to Gln mutation caused even more rapid growth and less surface adhesion of the cells carrying this gene. Mutations in the so-called zinc finger motif, Cys-X_2-Cys-X_2-Phe-X_6-Cys-X_2-Cys, did not result in oncogenic activation. The mutations that were introduced changed both downstream cysteine residues to serine, either singly or at the

same time. In addition, the phenylalanine was changed to isoleucine. While none of the retroviral vectors carrying these mutations in a full-length c-raf gene induced cell transformation, the double-cys mutation resulted in loss of transforming potential when introduced in the normally transforming gag-c-raf fusion background.

A third region currently studied is the serine/threonine kinase substrate site, Arg-Arg-Ala-Ser, found around position 40 in the c-raf protein. Several lines of evidence suggest that this region is of importance to the regulation of raf kinase activity. PKC has a pseudo-substrate sequence located at its amino-terminal end; oligopeptides carrying this pseudo-substrate were found to inhibit PKC activity. We have found that full-length c-raf protein, when over-expressed, does not induce cell transformation. However, when expressed as a gag-c-raf fusion, the protein induces cell transformation, suggesting that the configuration of the amino-terminal end of the raf protein is important to the regulation of its kinase activity. To test whether this involves the substrate site, we mutated two Arg-residues to Ile-Glu and separately the Ser residue to Ala. Neither of these mutations on the full-length c-raf gene brought about its oncogenic activation in NIH 3T3 cells. We are currently investigating whether the mutations resulted in loss of the inducibility of the c-raf autophosphorylation activity after treatment with platelet-derived growth factor. Similar studies are performed with the zinc finger mutants.

3. Activation of raf genes by truncation. v-raf constitutes a gag-raf fusion which contains 384 amino acids of gag and 323 of murine c-raf-1 protein. We have delineated the minimal transforming sequence of this protein further by deletion and site-directed mutation analysis. The carboxyl-terminal end of the minimal transforming region was determined by insertion of stop codons by site-directed mutagenesis at codons -16, -20, and -30. Terminating the protein 30 residues prematurely resulted in loss of transforming activity, while the stop codon at -16 did not affect it. The gene with a stop codon at -20 had an intermediate phenotype characterized by a low level of raf-type transformation. Removing 34 codons from the 3′ end of the gag part and 12 codons from the 5′end of the raf sequences did not result in loss of transforming activity, while extending the deletion for two more codons into raf did. Similarly, deleting codons 2 to 312 of the c-raf gene resulted in oncogenic activation, while a gene with a deletion from codon 2 to 314 did not cause transformation. In addition to demonstrating that the raf kinase domain, by itself, can transform cells, this experiment also demonstrated that the level of translation from the c-raf translational start codon is sufficient to produce transforming levels of protein.

4. Studies of the oncogenic potential of PKC. The raf gene family shows a relationship to the PKC genes, not only by nucleic acid and amino acid homology of regions of genes and proteins, but also in overall structure of the proteins. To test whether PKC is also a proto-oncogene that can be activated, we constructed a v-PKC-carrying 3611-murine sarcoma virus-derived retroviral vector which also contains the neomycin resistance gene. In this construct the v-raf sequences were substituted by the kinase domain of PKC. Cells expressing a gag-PKC fusion protein showed elevated levels of PKC kinase activity and grew faster; they were, however, not transformed.

5. Production of raf proteins. The availability of purified protein has proven instrumental for the understanding of many systems as it allows one to perform complementation assays not only in vitro, but also in vivo through microinjection. Alternatively, expressing the protein of interest in a controlled fashion in a heterologous system also facilitates various assays like ligand- and substrate-binding assays.

We have now inserted the human A- and c-raf genes into vectors of the T7 expression system and into a pUC-derived expression vector. We have succeeded in expressing full-length and slightly truncated versions of both genes at up to 10% of total protein levels. The proteins still behave like predicted from our eukaryotic studies in that the full-length proteins show no autophosphorylation activity, while the slightly truncated versions do. We are currently testing the effect that our mutations have on this activity.

Publications:

Heidecker G, Cleveland JL, Beck T, Huleihel M, Kolch W, Storm SM, Rapp UR. Role of raf and myc oncogenes in signal transduction. In: Colburn N, ed. Genes and signal transduction in multistage carcinogenesis. New York: Marcel Dekker (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05580-01 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human Genetic Loci Which Influence Susceptibility to HIV Infection and Pathology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI;	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	James Evermann	IPA Fellow	LVC	NCI
	James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
	William A. Blattner	Chief, Viral Epidemiology Section	EEB	NCI

COOPERATING UNITS *(if any)* Ludwig Inst. of Cancer Res., Montreal, Canada (W. Cavenee); LCS, ALC, NIH (D. Goldman); City Clinic Annex, San Francisco, CA (G. Rutherford, P.M. O'Malley); Dept. Microbiol. & Immunol., Univ. Miami Sch. of Med., Miami, FL (W.P. Parks); AIDSP, NIAID, NIH (H.M. Ginzburg); PRI, Fred.,MD (C.Winkler,M.Dean)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.9	1.6	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The epidemic of human acquired immunodeficiency syndrome (AIDS) has resulted in a massive research effort to understand the virology, epidemiology, and molecular biology of human immunodeficiency virus (HIV), the etiologic agent. A striking feature of the epidemiology is the observation that only about 1-5% of the exposed individuals actually have clinical AIDS. Although the temporal data must await several years before a definitive conclusion can be reached, it is probable that many of the exposed individuals will never develop AIDS. There are three simple explanations for heterogeneous response to virus exposure based upon model systems from other disease outbreaks. These include (1) functional genetic variability in the virus population; (2) cofactor requirement for a stochastic event such as a second virus, a somatic mutational event, blastogenic transformation of latently infected cells, etc.; and (3) genetic polymorphism in host populations for alleles which delineate viral infection and effect. The focus of this project is the search for human genes in category three. We shall employ the human restriction fragment length polymorphism markers dispersed throughout the human genome to detect distortion in population genetic equilibrium of disease-resistance/sensitivity loci. The combination of rapid advances in AIDS research, human gene mapping, population genetics, and epidemiology makes the timing appropriate for this analysis. We anticipate the use of approximately 400 gene probes and 5,000 patients from different disease categories in about 12 collaborative cohorts. At this writing we have collected 392 probes and 730 patients from 10 AIDS cohorts. Their genetic characterization is in progress.

PHS 5040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
James Evermann	IPA Fellow	LVC	NCI
James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
William A. Blattner	Chief, Viral Epidemiology Section	EEB	NCI

Objectives:

To search for human genetic factors which impact on susceptibility to infection, replication, pathology, and/or immune response to the HIV and other human viral pathogens.

Methods Employed:

The following techniques were employed: (1) resolution of human restriction fragment length polymorphism (RFLP) using human DNA clones derived by the human genetics community; (2) immortalization of B-lymphocytes from fresh blood by transformation with Epstein-Barr virus (EBV); (3) immunological assays (ELISA, R.I.P.) for detection of HIV in fresh lymphocytes and serum; (4) standard mathematical methods to detect distortion in genetic equilibria in natural populations; and (5) electrophoretic resolution of polymorphic protein markers resolved using allozymes or two-dimensional electrophoresis (2DE).

Major Findings: Progress 1 May 1988

1. Strategy. By analogy to murine and feline retroviral diseases, there are clearly scores of genetic loci which can influence (positively or negatively) disease susceptibility. Human genetic loci which impact on AIDS sensitivity must also exist. This project represents an attempt to use a combination of state-of-the-art human genetic technology, population genetic principles, and epidemiology to identify those genes in man. The strategy follows: The human gene map has grown in the last decade to include over 3,500 genes. Over 2,500 are defined by molecular clones and over 1,000 of these genes are polymorphic for RFLPs. In addition, nearly 50 polymorphic loci for proteins (resolved by isozyme and 2DE gels) have been reported. Thus, it is possible to take the human gene map and to identify abundantly polymorphic loci at 10- to 20-centiMorgan (cM) units along every chromosome (from 1 to X).

The project involves collection of blood from AIDS cohorts in several locales with large numbers of patients in each disease category (e.g., antibody positive for $\geq$3 years and asymptomatic) plus matched control populations. Patient B-cells will be transformed with EBV and expanded for DNA extraction. Distortion of three population genetic parameters of polymorphic loci (allelic frequency, Hardy-Weinberg equilibrium, linkage equilibria of paired loci) would

be interpreted as a signal for the occurrence of genes which impact on a patient's occurrence in a particular disease category. The gene markers would include polymorphic DNA segments, allozyme, and 2DE variants. Included in this panel would be clones of candidate loci such as the T-cell receptor, HLA, IgG, CD4, IL2R, etc. We would take advantage of the thorough serological work-ups on human AIDS patients by also noting exposure, symptoms, and presence of virus for other diseases, such as cytomegalovirus, HTLV-I, hepatitis-B virus, and herpesvirus. The wealth of epidemiology background would be considered throughout in defining new disease categories for gene identification.

2. Collection of human samples and establishment of lymphoid cell lines. Collaborations have been made with clinicians or epidemiologists at ten medical centers in the U.S.A. and Africa to provide the laboratory with patient samples from the establishment of lymphoblastoid cell lines (LCL) for DNA extraction and RFLP analysis. A total of 734 patient samples have been received from five of these centers and 334 LCL have been established. DNA has been extracted from 159 of the LCL. We have received 320 samples from homosexuals (n=100) and I.V. drug users (n=220) from Washington, D.C. or New York City; 32 hemophiliacs from Pennsylvania; 11 children with AIDS-related complex (ARC) or AIDS from Miami, Florida; 368 homosexuals were recruited from the San Francisco hepatitis vaccine trial cohort. We expect to receive an additional 1,000-1,500 samples in the next 12 months.

In addition to establishing an LCL repository, red blood cell plasma, and peripheral blood lymphocyte specimens are also collected from most patients. Supernatants from LCL's of 200 LCL supernatants tested contained antibodies that bind purified HIV gp120 in radioimmunoassay and are reactive to viral protein in Western blots.

3. Inventory and data analysis. The project is supported by two IBM AT's and a MacIntosh computer and developed software. Inventories of lymphoblastoid cell lines and DNA probes are managed on DBase III programs. The cell line program tracks samples received, progress of the cultures and the storage of samples. The plasmid data base contains information on each RFLP, including chromosomal location, structure of the construct, and the size and frequency of the alleles detected by each probe. In addition, a diagram of each chromosome has been constructed showing the location of each probe on both a physical and a linkage map. A program to manage the collection of data is being written using the DBase III data base program. This program will be used to record the genotype of each patient generated with the RFLP probes and will allow this information to be interacted with patient information data bases generated by the collaborators. In addition, our terminal is linked to the VAX computer system at the Frederick Cancer Research Facility and to the Human Gene Mapping Library at Yale. The Yale library maintains an updated data base of all known human cloned genes and RFLP probes.

4. Collection of human RFLP probes. To date we have collected 392 clones which detect human DNA polymorphisms. Each chromosome is represented by at least five probes, and the collection covers over 90% of the human genome at a resolution of 10 cM. An additional 260 clones have been requested or are available from the American Type Culture Collection. The majority of these clones (290) have been grown in bulk and are stored in duplicate as both a

glycerol stock of transformed bacteria and as DNA. We have tested 90 of these clones, confirming their restriction map, recognition of reported human RFLP, and molecular purity.

5. Detection of new polymorphisms. We have discovered novel, informative RFLPs for several candidate genes which we feel will be useful to our study. These include the gene for the CD4 molecule; the proto-oncogenes jun, ovc-2 and c-raf-1 (also linked to Von Hippell-Lindau Disease); the interleukin-1 (IL-1) growth factor gene; the retinoic acid receptor gene; the T-cell receptor zeta gene; and two probes tightly linked to the cystic fibrosis (CF) gene. Two of these polymorphisms are of the variable number of tandem repeat (VNTR) class (IL-1 and TCR-zeta). VNTRs show extremely high levels of heterozygosity, and are very useful genetic markers. DNA samples have been extracted from each member of nine, three-generation families originally collected by Dr. Ray White at the University of Utah Medical School. These pedigrees will be used to confirm the segregation of newly discovered markers.

6. Linkage disequilibrium in the CF locus. Several RFLP probes tightly linked to the cystic fibrosis gene show significant levels of linkage disequilibrium both with the disease and with each other. As a model system for the use of linkage disequilibrium in human populations, we have examined the degree of disequilibrium between newly discovered RFLPs in this locus in collaboration with Dr. Francis Collins, University of Michigan. Preliminary results show that a group of polymorphisms spanning 200 kb surrounding the pJ3.11 probe show very high levels of disequilibrium in a group of unrelated Caucasians. This result suggests that large regions of DNA displaying significant disequilibrium are likely to exist in the human genome.

Publications:

O'Brien SJ, Evermann JF. The interface of epidemiology and genetic diversity in free-ranging animal populations. Trends Ecol Evol (In Press).

Table I
AIDS Patient Cohorts Involved in Project

Risk Group	Location	Sample	Number Projected	Number Received	No. LCL Established	No. of DNA Extracted	Collaborators
Male Homosexual	New York/ Wash., D.C.	Peripheral blood	--	100	93	86	Goedert, Bigger, and Blattner (NCI)
I.V. Drug Users	New York/ New Jersey/ Wash., D.C.	Cryopreserved PBL's	--	220	204	69	Goedert, Bigger, and Blattner (NCI)
Children of HIV(+) Mothers; Family Members	Miami	Peripheral Blood	200	11	9	2	Parks (Univ. of Miami)
Male Hemophiliacs	U.S.A. and Canada	Cryopreserved PBL's	1200	32	28	2	Goedert, Bigger, and Blattner (NCI)
Families at Risk for Hepatitis B	Taiwan	Cryopreserved PBL's	2500	0	--	--	Beasley, Hwang (Univ. of Texas)
Heterosexuals HIV Cases and Controls	Tanzania	Cryopreserved PBL's	500	0	--	--	Maselle (Muhimbili Medical Center)
Heterosexuals HIV Cases and Controls	Kenya	Cryopreserved PBL's	500	0	--	--	Koech (Kenya Medical Medical Center)
Prostitutes	West Africa	Cryopreserved PBL's	500	0	--	--	Potts (Family Health International)
Male Homosexual (MAC Study)	U.S.A.		400	368	--	--	Ginsberg (NIAID)
HIV(+) Homosexuals, Asymtomatic	San Francisco	Peripheral Blood	500	3	3	--	Rutherford, O'Malley, Lifson (San Francisco City Clinic)
		TOTAL	6300	734	332	159	

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05581-01 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Kinase Oncogenes in Growth Factor Abrogation and c-myc Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John L. Cleveland	Senior Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (M. Dean, P. Lloyd); Bionetics Research Inc., Frederick, MD (J. Ihle, B. Isfort); University of California at San Diego, La Jolla, CA (J. Wang)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unraduced type. Do not exceed the space provided.)*

The role of specific oncogenes in growth factor signal transduction and regulation of c-myc expression was examined in interleukin-3 (IL-3)-dependent murine myeloid FDC-P1 cells. c-myc mRNA levels are tightly controlled by IL-3 and introduction of exogenous v-myc or murine c-myc either partially or fully abrogates the requirements of these cells for this growth factor. The intermediates involved in signal transduction following binding of IL-3 to its receptor are unknown. However, a role for tyrosine kinases in the signal cascade has come from experiments demonstrating a rapid tyrosine phosphorylation of a discrete set of proteins following addition of IL-3. To test whether tyrosine kinase oncogenes or other classes of oncogenes, such as raf serine/threonine kinases and ras GTP-binding regulators, function in IL-3 signal transduction and regulation of c-myc, we introduced constitutively expressed forms of these oncogenes into FDC-P1 cells. The data from these experiments show a direct linkage between activated tyrosine kinase oncogenes and induction of c-myc in IL-3 signal transduction and growth factor abrogation. First, tyrosine kinase oncogenes acutely abrogate requirements for IL-3 at very high frequencies and, therefore, factor abrogation in these cells is not likely to require secondary events. In contrast, introduction of raf family kinases only slightly increased (tenfold) frequencies of abrogation. Second, using three different conditional tyrosine kinase abl mutants we have shown that IL-3 factor abrogation is temperature-sensitive and requires the presence of functional abl protein. Third, all FDC-P1 cells rendered factor-independent by introduction of tyrosine kinase oncogenes constitutively express c-myc in the absence of IL-3. Finally, conditional expression of functional abl protein correlated with the ability of this oncogene to abrogate IL-3 dependence and trans-activate expression of c-myc.

PHS 6040 (Rev. 1/84) GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John L. Cleveland	Senior Staff Fellow	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI

Objectives:

The general objective of this study is to identify the second messengers involved in growth factor signal transduction and regulation of c-myc in interleukin-3 (IL-3)-dependent murine myeloid cells and in NIH 3T3 fibroblastic cells. Specifically, we will determine (1) candidate genes involved in growth factor signal transduction by introducing a variety of oncogenes into these cells and testing their ability to abrogate growth factor requirements, (2) whether regulation of c-myc is affected by these exogenous oncogenes and what the significance of this regulation is, (3) how expression of c-myc is regulated in IL-3 signal transduction and in cells abrogated of growth factor requirements by introduction of exogenous oncogenes and what targets on the c-myc gene are responsive to this regulation, and (4) the cascade of second messengers acting downstream in both ligand/receptor signal transduction and in signalling from oncogenes which abrogate factor dependence.

Methods Employed:

Recombinant retroviruses containing various oncogenes were constructed using standard recombinant DNA technology. High-titer stocks of these viruses pseudotyped with various helper viruses were prepared from transfected NIH 3T3 cells using established techniques. RNA and DNA blot analyses, S1 nuclease and RNase A mapping of transcripts, and nuclear run-on assays are performed using standard protocols.

Major Findings:

The data described in this report demonstrate, for the first time, that tyrosine kinase oncogenes can trans-activate expression of the c-myc proto-oncogene. Combined with our previous findings showing that addition of IL-3 rapidly induces both tyrosine phosphorylation and c-myc expression, the experiments described here demonstrate a direct physiologic link between these two events in IL-3 signal transduction. The major findings are as follows:

I. Introduction of tyrosine kinase oncogenes (including v-abl, v-src, v-fms, and trk) acutely abrogate IL-3 dependence of FDC-P1 cells at very high frequencies (4-5 logs higher than with control neo viruses), suggesting that factor abrogation is not likely to require other secondary events. Using conditional temperature-sensitive (ts) abl retroviruses we demonstrated that, at least for this tyrosine kinase oncogene, factor abrogation requires the presence of functional abl protein. In contrast, raf family serine/threonine kinase oncogenes were inefficient in abrogating IL-3 requirements (only a tenfold increase in frequency) of FDC-P1 cells, but did relieve the

requirements of NIH 3T3 cells of their serum growth factor requirements, suggesting different signalling pathways in the two different cell types.

2. FDC-P1 cells which have been abrogated of their IL-3 requirements by introduction of tyrosine kinase oncogenes all constitutively express c-myc in the absence of IL-3, whereas in normal FDC-P1 cells, c-myc expression strictly requires the presence of this ligand. Therefore, although tyrosine kinase oncogenes likely perform several functions that are jointly involved in growth factor abrogation, one essential activity which they share for IL-3 abrogation is their ability to induce c-myc. The constitutive expression of c-myc in these cells was not due to any gross alterations or proviral insertions, as determined by Southern blot analyses, nor was it due to alternative promoter usage or to mutations in c-myc exon 1, which has been shown to harbor sites required for an attenuation regulation of c-myc transcription.

3. Using viruses expressing wild-type and ts versions of the abl tyrosine protein kinase, we have demonstrated first that constitutive expression of c-myc requires the presence of functional abl protein and second, using temperature shift experiments, that abl tyrosine protein kinase trans-activates expression of c-myc mRNA.

Publications:

Cleveland JL, Dean M, Wang JY, Hedge A-M, Ihle JN, Rapp UR. Abrogation of IL-3 dependence of myeloid FDC-P1 cells by tyrosine kinase oncogenes is associated with induction of c-myc. In: Melchers F, Potter M, eds. Current topics in microbiology and immunology. New York: Springer-Verlag (In Press).

Dean M, Cleveland J, Kim H-Y, Campisi J, Levine RA, Ihle J, Rapp U. Deregulation of the c-myc and N-myc genes in transformed cells. In: Melchers F, Potter M, eds. Current topics in microbiology and immunology. New York: Springer-Verlag (In Press).

Dean M, Cleveland JL, Rapp UR, Ihle JN. Role of myc in the abrogation of IL-3 dependence of myeloid FDC-P1 cells. Oncogene Res 1987;1:279-96.

Ihle JN, Weinstein Y, Cleveland JL, Dean MC, Reddy EP, Rapp UR. Interleukin-3 regulation of normal and transformed hematopoietic stem cell growth. In: Waymouth C. ed. Proceedings of the fourth decennial review conference of the Tissue Culture Association. Molecular mechanisms in the regulation of cell behavior. New York: Alan R Liss, 1987;5:91-100.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05582-01 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth Modulation and Analysis of Chemically Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Stephen M. Storm	Biologist	LVC	NCI
	John L. Cleveland	Senior Staff Fellow	LVC	NCI
	Jakob Troppmair	Special Volunteer	LVC	NCI

COOPERATING UNITS (if any)

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH (B. Moss); Program Resources, Inc., Frederick, MD (S. Giardina)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	0.9	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have established a mouse model system for the rapid induction of lung adenocarcinomas and lymphomas in order to investigate lung carcinogenesis in vivo, and to examine potential regimens for growth modulation of these tumors. A transplacental injection of 1-ethyl-1-nitrosourea at day 16 of gestation, followed by promotion with butylated hydroxytoluene beginning 5 weeks after birth, results in 90% of the animals developing tumors within 5 to 14 weeks of age. Both tumor types contain transforming DNA as defined by NIH 3T3 cell transformation assays. We are currently investigating the nature of this transforming DNA through several means. Although raf does not appear to be the transforming gene in NIH 3T3 cell assays, high levels of normal-sized c-raf-1 are expressed in both tumors and cell lines as determined by Northern and Western blotting, consistent with a role for raf in the development and/or maintenance of these tumors.

raf protein vaccinations administered at 3, 4, and 5 weeks of age apparently eliminate the promoted phase of tumor growth. Since the constitutive presence of oncogene proteins as anti-tumor antigens may provide even more effective protection, we have developed raf- and myc-carrying vaccinia virus vectors for use as vaccines. These constructs are being tested for their tumor growth modulating ability separately and in conjunction with each other, as raf and myc have been shown to act synergistically in transformation in several cases. In addition, we intend to generate vaccinia viruses expressing other oncoproteins that may play a role in the generation or maintenance of these tumors for use as vaccines.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Stephen M. Storm	Biologist	LVC	NCI
John L. Cleveland	Senior Staff Fellow	LVC	NCI
Jakob Troppmair	Special Volunteer	LVC	NCI

Objectives:

To employ an animal model system which we have developed for the induction of lung carcinomas relevant to human lung cancer in order to define regimens for the prevention and/or reversal of neoplasms involving raf and other oncogenes. To carry out the molecular analysis of tumors in order to identify potential neoplastic mutations, enabling us to devise more effective treatment procedures.

Methods Employed:

For induction of lymphomas and lung adenocarcinomas in mice, pregnant females were injected transplacentally with ethylnitrosourea (ENU). To accelerate tumor development in the offspring, weanling age F_1 mice were promoted with weekly injections of butylated hydroxytoluene (BHT). Vaccination of newborn mice with oncogene protein followed procedures previously developed for vaccination of high leukemia strains of mice with retroviral structural proteins. Oncogene-expressing recombinant vaccinia viruses were generated by standard procedures for in vivo recombination between vaccinia virus and plasmids. raf- and myc-expressing vaccinia viruses were administered to carcinogen-treated animals via tail scratch. Molecular analysis of tumors was per standard procedures for DNA (Southern), RNA (Northern), and protein (Western) analyses. In addition, tumor-derived DNA and RNA are being screened for potential mutations via RNase protection assays and the polymerase chain reaction (PCR).

Major Findings:

Tumor induction experiments, in which varying amounts of carcinogen (ENU) and promoter (BHT) were administered, determined dosages for maximal tumor induction. This is of importance in testing the efficacy of various treatment regimens as the protective effect of purified raf protein vaccination was seen only in animals subjected to a schedule of very rapid tumor induction. Generation of monoclonal antibodies against the purified 30-kilodalton v-raf protein used in earlier vaccination experiments showed that it is indeed immunogenic in these animals. To test the effectiveness of constitutively expressed oncoprotein as a vaccine in our system, raf- and myc-expressing vaccinia viruses were constructed and administered to carcinogen-treated animals. These experiments are currently underway. In addition, we plan to test vaccinia viruses expressing other oncogenes for their ability to affect tumor growth modulation in their host. Transformed cell lines from both T-cell

lymphomas and lung adenocarcinomas were generated by transfection of tumor DNA into NIH 3T3 cells and also by the culturing of primary tumors. Tumors and tumor-derived cell lines show no evidence of rearranged oncogenes at the level of Northern and Southern blotting for approximately 15 proto-oncogenes tested to date. Many of the proto-oncogenes tested so far are expressed in higher levels in tumor than in control tissue; however, none of them show a uniform high level in all tumors as does c-raf-1. We are in the process of examining expression levels and looking for rearrangements of other proto-oncogenes. One chemically induced T-cell lymphoma showed evidence of a K-ras mutation at codon 12, but none of the other lymphomas (12), lung adenocarcinomas (17), or cell lines (10) tested were positive for a mutation at this site as determined by RNase protection assays, suggesting that this may have been a secondary event. These tumors and cell lines are now also being tested for other potential mutations through differential hybridization of oligonucleotides to DNA amplified by the polymerase chain reaction. Since all of these tumors were generated in the same way, we feel that identification of the transforming sequence(s) will allow us to tailor an even more effective vaccination protocol and give further insight into the role raf may be playing in these tumors.

Publications:

Giardina SL, Storm SM, Longo DL, Mathieson BJ, Rapp UR, Varesio L. Characterization of a murine monoclonal antibody that detects a C-terminal fragment of the raf oncogene product. J Immunol (In Press).

Rapp UR, Cleveland JL, Storm SM, Beck TW, Huleihel M. Transformation by raf and myc oncogenes. In: Aaronson SA, Bishop JM, Sugimura T, Terada M, eds. Oncogenes and cancer. Proceedings of the seventeenth international symposium of the Princess Takamatsu Cancer Research Fund. Tokyo: Japan/VNU Scientific Press, 1987;55-74.

Rapp UR, Storm SM, Cleveland JL. Oncogenes: clinical relevance. In: Neth R, Gallo RC, Greaves MF, Kabisch H, eds. Modern trends in human leukemia VII: new results in clinical and biological research including pediatric oncology, vol 31. Berlin/Heidelberg: Springer-Verlag, 1987;450-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05583-01 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cis- and Trans-Acting Regulation of EIAV Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS (if any)

Program Resources Inc., Frederick, MD (P. Dorn)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	0.4	0.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This laboratory previously showed that expression of the lentivirus equine infectious anemia virus (EIAV) is controlled in part by activation of the virus promoter by factors unique to EIAV-infected cell lines. This work has been extended to the identification of the precise cis-acting, transcriptional regulatory elements in the viral long terminal repeat (LTR) and localization of the putative EIAV trans-acting factor (tat) gene. The location of transcriptional activator response (tar) elements was revealed by deletion analysis; the EIAV LTR was digested from either the 5′ or 3′ end with nucleases and the residual LTR fragment was inserted into test plasmids containing the chloramphenicol acetyl transferase (CAT) gene. These plasmids were introduced into EIAV-infected and uninfected cell lines and promoter activity was monitored by CAT assays. These studies showed that the EIAV tar element is located within a 53-bp region located between -31 and +22 nucleotides with respect to the RNA start site (+1). This unusual location for a tar element is shared with the lentiviruses, HIV-1 and HIV-2. A tat gene was identified in EIAV by inserting various regions of the viral genome into eukaryotic expression plasmids and transfecting these, in combination with EIAV LTR-directed CAT gene plasmids, into uninfected cell lines. An active region was identified that was located downstream of the pol gene and overlapping the 5′ end of env. Northern blot analysis revealed that the EIAV tat gene acts to increase steady state levels of RNAs controlled by the LTR. To better understand the splice pattern, structure and sequence of the tat message and other multiply spliced virus mRNAs, a cDNA library has been constructed representing mRNAs present in EIAV-infected cell lines. These clones are now being sequenced.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David Derse — Senior Staff Fellow — LVC NCI

Objectives:

1. Definition of cis-acting elements in the equine infectious anemia virus (EIAV) long terminal repeat (LTR) that control gene expression by deletion analyses and chloramphenicol acetyl transferase (CAT) assays.

2. Insertion of EIAV subgenomic fragments in eukaryotic expression vectors to determine whether EIAV encodes a trans-acting factor (tat) gene.

3. Determine mechanism of tat-mediated control of gene expression by analyzing effects on RNA and protein synthesis.

4. Construction of a cDNA library from EIAV-infected cells to examine the structure and sequence of EIAV tat mRNA as well as the pattern of EIAV RNA processing.

Methods Employed:

The following methods were employed: (1) recombinant plasmid construction, (2) transfection of mammalian cells, (3) CAT assays, (4) Northern and Southern blotting, (5) cDNA library construction, and (6) nucleotide sequence analysis.

Major Findings:

1. The EIAV LTR contains a novel transcriptional activator response (tar) element. Sequences were deleted from the 5′ and 3′ ends of the EIAV LTR and the remainder was tested for promoter activity in EIAV-infected and uninfected cells by the CAT assay. It was observed that sequences upstream of the TATA box (or to 31 nucleotides upstream of the RNA start site) were not required for promoter activation in virus-infected cells. The basal levels of promoter activity were decreased by about one-half as a result of these upstream deletions. While dispensable for EIAV-mediated activation, these upstream sequences may be important for promoter regulation in vivo. Deletion of sequences downstream of the RNA start site had no effect on promoter activity up to nucleotide +22. Further deletion of sequences to +4 abolished promoter activity and response to EIAV activation. Thus, the EIAV tar elements are located within a 53-bp LTR region between -31 and +22 nucleotides (RNA start site is +1).

2. Location of an active EIAV tat exon. In order to identify a tat gene for EIAV, specific subgenomic fragments were inserted into a eukaryotic expression plasmid developed in this lab for this purpose. The various expression plasmids were transfected in combination with EIAV LTR-directed CAT plasmids into uninfected cell lines. High level CAT expression would indicate

trans-activation by the expression plasmid. These studies indicated that EIAV does encode a tat gene and that an active coding exon is located immediately downstream of the pol gene and overlaps the 5' end of the env gene. At present it is impossible to precisely deduce the tat amino acid sequence since there are several short open reading frames in this region.

3. EIAV tat activates transcription of the EIAV LTR. Cotransfection of cells with EIAV tat expression plasmids and CAT plasmids directed by EIAV LTR fragments revealed that the 53-bp tar region, identified in EIAV-infected cells, is responsive to tat. This trans-activation was specific for the EIAV LTR, since an HIV-1 LTR CAT-plasmid was unresponsive to EIAV tat. Comparison of steady state levels of RNA directed by the EIAV LTR, revealed that tat-mediated activation results in increased steady state RNA levels. The increases in RNA paralleled increases seen in protein synthesis (CAT activity) indicating that tat functions to increase the transcription or stability of the mRNA.

4. Pattern of EIAV RNA processing and sequence of tat RNA. A cDNA library has been constructed from poly(A)$^+$ RNA purified from an EIAV-infected cell line. The library was constructed in bacteriophage lambda ZAP (Stratagene) and screened with an EIAV LTR probe. Fifty positive clones have been plaque purified and subcloned into plasmid vectors for dideoxynucleotide sequence analyses.

Publications:

Derse D, Dorn PL, Stephens RM, Rice NR, Casey JW. Characterization of equine infectious anemia virus long terminal repeat. J Virol 1987;61:743-7.

Dorn PL, Derse D. Cis- and trans-acting regulation of gene expression of equine infectious anemia virus. J Virol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05584-01 LVC

PERIOD COVERED

October 1, 1987 to September 30, 1988

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genomic Organization in Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hector N. Seuanez	Visiting Scientist	LVC	NCI
Others:	Mary Eichelberger	Microbiologist	LVC	NCI
	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (K. Richards)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.8	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nonhuman primates are frequently used in several areas of biomedical research such as neurobiology, reproductive physiology, infectious diseases, immunology and cancer research. Despite their frequent utilization, however, several primate species require a genetic characterization for the establishment of adequate comparisons with the human and for their standardization as reliable animal models.

The study of the genome organization of the nonhuman primates has been approached by karyological studies of genera in which fragmentary data are available. These genera, belonging to the neotropical family of Callitrichid monkeys (marmosets) are widespread in the wild and frequently captive bred in colonies, though their genetic characterization is presently incomplete. Moreover, very limited data are presently available on gene assignment in the nonhuman primates. Comparative gene charts are available for only 12 species in which the number of mapped genes ranges from a minimum of 25 to a maximum of 65. This contrasts strikingly with the human in which the known number of structural loci and anonymous gene sequences amounts to some 3500 markers. For this reason, a hybrid cell panel has been constructed for the neotropical species Ateles paniscus (2n=34) using a rodent receptor cell line and a donor primate fibroblast cell line. Approximately 70 hybrid cell lines have been cloned in selective medium and analyzed by electrophoresis for 25-30 gene products. A preliminary analysis of these results has allowed for the identification of presumptive syntenic groups in this species.

GPO 914-918

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Hector N. Seuanez	Visiting Scientist	LVC	NCI
Mary Eichelberger	Microbiologist	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

The specific objectives of this project are: (1) establishment of fibroblast cell lines from approximately 15 species of New World monkeys; (2) comparison of chromosomal banding patterns among these monkey species; (3) gene assignment in Ateles paniscus by somatic cell hybridization; (4) comparison of linkage group conservation of Xenopus with mammals, birds, and fish; (5) generation of a fundamental genetic foundation for the study of molecular embryogenesis in Xenopus; (6) attempt to identify heteromorphic sex chromosomes and to address the question of whether or not dosage compensation occurs in amphibians; and (7) Xenopus laevis is tetraploid. The organization and syntenic relationships of duplicate loci in a tetraploid will be examined.

Methods Employed:

The following techniques are being utilized: (1) primary fibroblasts are obtained by cultivating skin biopsies in tissue culture medium, (2) somatic cell fusion and tissue culture propagation of cell hybrids, (3) cytogenic analysis of metaphase chromosomes, (4) protein starch gel electrophoresis, and (5) Southern blot and DNA filter hybridization.

Major Findings:

1. Chromosome banding comparisons of Cebuella pygmaea, three Leontopithecus morphotypes and Callithrix jacchus penicillata.

2. Establishment of presumptive phylogenetic relationships within the callitrichid family. The genus Callithrix is intermediate between Cebuella and Leontopithecus as demonstrated by a parsimonial reconstruction of chromosome rearrangements within the family.

3. Karotypic standardization of the neotropical species, Ateles paniscus.

4. Preliminary analysis of approximately 70 hybrid cell lines (Ateles paniscus X RAG) has shown presumptive syntenic associations. These associations need subsequent confirmation in a more selective panel, which is why they should be presently considered as tentative and subject to modifications. Up to the present, the following syntenic associations have been identified: PGD-ME1-AK1, PEPC-ACP1-GOT2-DIA4-SOD2-SOD1, PEPB-NP-MPI-HEXA, ACP2-LDH1, and G6PD-HPRT. These results differ from the human gene associations, taking into consideration that the diploid chromosome number of man (46) is higher than in

Ateles paniscus (2n=34). However, some human syntenic associations are apparently conserved as in the case of ACP1-MDH1 (human 2p), GOT2-DIA4 (human 16), MPI-HEXA (human 15), ACP2-LDH1 (human 11), and G6PD-HPRT (human X). Moreover, four more loci (IDH1, ACY, GUSB, and GPI) have been found to be asyntenic with each other and with any other marker, while a possible ESD-ADA association has been detected.

5. Viable Xenopus-rodent cell hybrids result from cell fusion experiments. Somatic cell fusion was carried out between Xenopus erythrocytes and fibroblast cells of four different mutant rodent cell lines: RAG (mouse, $HPRT^-$); LM (mouse, TK^-); E36 (hamster, $HPRT^-$); and BHK (hamster, TK^-). Viable hybrids result from the RAG and BHK fusions. Approximately 20 such hybrids were expanded in tissue culture and cryogenically frozen.

6. Bromodeoxyuridine substitution generates Xenopus replication G-bands. Adopting a cytogenic procedure developed for human chromosomes, Xenopus chromosomes have been uniquely identified in several cell hybrids. The future preparation of a standardized G-banded karyotype for Xenopus will allow gene assignment to individual chromosomes.

7. Protein electrophoresis suggests that most Xenopus genes are not expressed in the cell hybrids. Analyses of 15 allozymes in 12 BHK hybrids yielded only two positive frog allozymes in three hybrids. The preliminary interpretation is that frog genes are not usually expressed in the rodent cellular environment. Confirmation of this conclusion must await additional karyotypic and isozymic analyses.

8. Mapping of homologous Xenopus cDNA clones by Southern blotting. A fair number (approximately 50) complementary DNA clones from Xenopus have been obtained in different laboratories around the world. Efforts are currently underway to contact collaborators who will make these clones available for mapping purposes. Genomic DNAs have been prepared from Xenopus liver, rodent parental cell lines, and hybrids and await preliminary hybridization analyses.

Publications:

O'Brien SJ, Seuanez HN, Womack JE. Mammalian genome organization: an evolutionary view. In: Campbell A, ed. Annual reviews of genetics. Palo Alto: Annual Review (In Press).

ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1987 to September 30, 1988

The Biological Carcinogenesis Branch (BCB) plans, develops, directs and manages a national extramural program of basic and applied research concerned with the role of biological agents as possible etiological factors or co-factors in cancer and on the control of these agents and their diseases; establishes program priorities, and evaluates program effectiveness; provides a broad spectrum of information, advice and consultation to individual scientists and institutional science management officials relative to NIH and NCI funding and scientific review policies and procedures, preparation of grant applications and choice of funding instruments; provides NCI management with recommendations as to funding needs, priorities and strategies for the support of relevant research areas consistent with the current state of development of individual research activities and the promise of new initiatives; plans, develops and manages research resources necessary for the conduct of the coordinated research program; develops and maintains computerized data management systems; plans, organizes and conducts meetings and workshops to further the program objectives; and maintains contact with the relevant scientific community to identify and evaluate new research trends relating to its program responsibilities.

The objectives of the research program are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), first independent research support and transition (FIRST) awards (R29), conference grants (R13), cooperative agreements (U01), contracts (N01), small business innovative research (SBIR) grants (R43/44), SBIR contracts (N43/44), academic research enhancement awards (AREA) (R15), outstanding investigator grant (OIG) awards (R35), and the method to extend research in time (MERIT) awards (R37). Currently, the Branch administers 423 research grants with an annual budget of approximately 78 million dollars. Administratively, the Branch is divided into seven functional entities which are shown in Figure I. The Branch scientific components are based primarily on the major subdivision of viruses by their type of nucleic acid core. Research programs on viruses with a DNA core which are involved in the induction of malignant transformation are included in the DNA Virus Studies components. The component designated DNA I deals with research on the two main groups of large DNA viruses, the herpesviruses and adenoviruses. The DNA II component supports research on the small DNA viruses, the polyoma, simian virus 40 (SV40), and papillomaviruses. Similarly, research dealing with RNA core viruses are covered by the RNA Virus Studies components. The component designated RNA I involves research concerning murine, feline, bovine, non-human primate, and human viruses. The RNA II component incorporates research involving avian tumor viruses, picornaviruses, hepatitis B virus, and other microbial agents. The Research Resources component arranges for the storage and distribution of research materials, helps oversee the various resource contracts, and maintains computerized information systems covering the distribution of resources. The AIDS Virus Studies

component is a new branch element recently established to deal with the increasing concern about this public health problem. Originally, acquired immunodeficiency syndrome (AIDS) activities in the program were treated as special initiatives administered by the Branch Office and subsequently were dealt with as part of the human retrovirology portfolio. However, the high visibility of these efforts and the need for an accountable individual to expedite reports and responses to senior echelons dictated that a program director be assigned exclusively to these research oversight activities. Finally, the Office of the Branch Chief oversees and coordinates all of these activities, establishes program objectives and priorities, evaluates accomplishments, and interacts with the Division and Institute leadership.

The BCB research resources payback system has been described in previous reports. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor, in turn, credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. During this period, five resource contracts were functioning in the payback mode. These include one for production of viral reagents, one for animal resources, two for specialized testing services, and one for storage and distribution of frozen biological reagents. The payback system seems to be performing as expected. The demand for high quality biological reagents, not readily available from commercial sources, has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use by investigators of costly resource reagents, with a subsequent reduced level of effort in several resource contracts or the termination of now unnecessary activities.

Table I focuses on mechanisms of support of extramural research and related activities in biological carcinogenesis. The total BCB grant and contract budget in FY88 is estimated to be about 80 million dollars. It should be noted that the Branch now administers 21 program project grants at a level of 19.7 million dollars, 18 outstanding investigator awards at the level of 12.6 million dollars, and 16 merit awards at a level of 3.3 million dollars. Table II provides an estimate of the grant and contract support, respectively, in each of the seven Branch components and thus illustrates, in quantitative terms, the main areas of scientific endeavor. As can be seen from the table, the four primary long-established Branch research components are well balanced in terms of their number of grants and funding level. The Branch currently administers 423 grants and 8 contracts. Table III summarizes the research activities initiated by the Biological Carcinogenesis Branch since 1982. The table demonstrates the broad spectrum of research activities, funded through the traditional grant and cooperative agreement mechanisms, undertaken to stimulate activity in newly emerging areas of scientific opportunity.

During FY88, the Branch was again active in sponsoring a variety of research initiatives which reflected emerging areas of research opportunity. There were two requests for grant applications (RFAs) funded, two RFAs issued, and two Branch-sponsored workshops during the past fiscal year. In addition, the competitive continuation of a resource contract providing a cell culture identification service was completed.

As a result of previous Branch workshops held in 1986, two RFAs received concept approval by the Division of Cancer Etiology (DCE) Board of Scientific Counselors during FY87 and were funded during this fiscal year. The first was entitled "Studies on Papillomavirus-Host Interactions" and was based on a workshop which met on February 18-19, 1986. The sum of $750,000 was approved to stimulate basic research on papillomavirus-host interactions at both the cellular and immune response levels. The second initiative was entitled "Studies of Functional Anti-Sense RNA in Oncogenic Viral Systems" and was based on a discussion group held March 28, 1986. The sum of $500,000 was approved to fund studies on the mechanism of action of anti-sense RNA and on methods for the construction of vectors used to express anti-sense sequences in oncogenic virus model systems. Eight new traditional research projects were funded as a result of the responses received to these two RFAs. Additionally, a Branch workshop held in 1987, co-sponsored with the National Institute of Allergy and Infectious Diseases (NIAID), resulted in the issuance of an RFA entitled "Animal Models for Human Papillomavirus-Associated Neoplastic Diseases." The sum of $1,000,000 was approved by the DCE Board of Scientific Counselors to fund meritorious applications in response to this RFA, whose goal was the promotion of studies on the host immune mechanisms involved in the regression of human papillomavirus (HPV)-associated lesions and on the molecular mechanisms which may lead to the progression of these lesions to carcinoma. Established or new animal models were felt to be the best experimental system to gather the needed data. This RFA was issued in May 1988, with a receipt date of September 1988, and anticipated funding in FY89.

On October 5, 1987, the AIDS Virus Studies component and the RNA Virus Studies I component sponsored a workshop entitled "AIDS: Progress and Future Directions for Vaccine Development." The workshop focused on defining the state-of-the-art in the field of AIDS vaccine development and concluded with a comprehensive discussion of human immunodeficiency virus (HIV) pathogenesis and strategies for intervention. The workshop participants indicated that there was an urgent need for valid animal models for investigating the pathogenesis of HIV infections and their subsequent oncogenic sequelae. The participants indicated that the technology is available to prepare a variety of antigens for use as prototype vaccines, but because of the lack of basic understanding of the mechanism of viral pathogenesis and the lack of information on the proper preparation, amount, and route of virus challenge, there is little basis for an informed decision on the most promising antigen(s) for future vaccine studies. The recommendation from the workshop participants was that an RFA should be issued by the NCI which would focus on the development of animal models for investigations of HIV infection and pathogenesis. At its February 1988 meeting the DCE Board of Scientific Counselors approved the issuance of an RFA, entitled "Retrovirus Animal Models and HIV Pathogenesis." The goals of this RFA were to 1) encourage applications and studies emphasizing the development of animal models using HIV, the simian immunodeficiency virus or other appropriate retroviruses, which mimic long-term HIV infections including viremia, latency, and disease progression to immune dysfunction and possible neoplastic sequelae; and 2) use these animal models for investigations emphasizing virus-host interactions to better define and understand viral-induced pathogenic and immune functions/alterations. Applications in response to this RFA were reviewed in the Fall of 1988, with funding expected in FY89.

On April 25-26, 1988, the DNA I component co-sponsored with the Bacteriology and Virology Branch, NIAID, a workshop entitled "Mechanisms of Pathogenic Diversity of Epstein-Barr Virus." The workshop participants summarized the recent progress

in defining Epstein-Barr virus (EBV) gene function, EBV gene regulation and the host response to EBV infection. The participants identified a number of gaps in our knowledge of EBV pathogenesis and oncogenesis on which future research should focus. The participants indicated that the EBV research field would benefit from having available well-characterized monoclonal antibodies to the structural and regulatory gene products already known, with the capability of having additional reagents as new genes were identified. They noted that a reference reagent laboratory which could compare new antisera with the classical immunofluorescent antibodies would facilitate EBV research. Until his recent death, the laboratory of Dr. Werner Henle had served some of these purposes. After additional programmatic evaluation, concepts based on these suggestions may be developed for presentation at the October 1988 meeting of the DCE Board of Scientific Counselors.

During FY87 an RFP (request for contract proposals) was issued for the competitive continuation of a contract for the inter- and intraspecies identification of cell cultures. One approach to experimental cancer is the study of cultured tumor cells of animal and human origin. Many experimental techniques in virology, immunology, cell biology, and biochemistry require the precise duplication of cells or mixtures of cells, with the result that cell identification services are of critical importance. The extensive use and informal cross-supply of cell cultures among investigators has resulted in a major problem of erroneous or mislabeled cell lines. The availability of validly identified cell lines is of extreme importance to cancer research programs since research projects utilizing bogus cell lines are a waste of time and research funds. After peer review of the proposals received in response to this RFP, a contract was awarded in FY88 to the successful offeror, Children's Hospital of Michigan.

Past research sponsored by the Branch has yielded much fundamental information on biological carcinogenesis by studying human and animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may, themselves, be directly responsible for some malignant transformations in man. Investigations of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur either through the direct effects of viral transforming genes; through the influence of viral enhancing elements (promoters or long terminal repeats) on cellular oncogenes; or through the interaction of viruses with a variety of environmental factors, such as hormones, chemicals, radiation, and the like. In the research program, attention is given to studies defining the interaction of viruses and cells in both animal and human cancers. The work stresses efforts to identify discrete regions of viral nucleic acid and cell chromosomes which are responsible for malignancy, to understand the molecular pathways of viral replication, to identify virus products which may trigger the transformation of a cell to malignancy, and to understand host responses to viruses which ultimately may prevent cancer.

Recent studies have shown that a number of rapidly transforming RNA viruses have derived their ability to cause cancer in animals, and perhaps in humans, from genes found in normal cells. These genetic sequences are unrelated to those necessary for normal viral replication and have been found to produce proteins which are, in many cases, necessary for the initiation of the transformed state.

Since the gene products result in transformation, the genes have been termed "oncogenes." Furthermore, a new class of recessive oncogenes has recently been discovered in which the absence of the oncogene product, rather than its presence, is responsible for transformation. Oncogenes have been found to possess the following characteristics: they are essential for certain types of transformation; they are derived from cellular genetic sequences; they have a limited number of specific targets; they act by means of their translational protein products; and they are probably limited in number (approximately 35 have been found). Currently, ongoing research seeks to continue the search for oncogenes in both animal and human tumor systems and to characterize these genes; to study human oncogenes and their relationship(s) to viral oncogenes in terms of nucleic acid homology and translational (oncogene) products; to purify and characterize the translational gene products of these genes; to use these purified products in delineating the mechanism(s) of transformation; and to define the function(s) and mechanisms of regulation of the cellular homologs (c-oncs) of viral transforming genes. Research highlights of the past year are presented here and, in greater detail, in the various section reports which follow.

An in vitro assay system for retroviral integration has been developed. The assay has shown that linear DNA serves as a precursor to the integrated form of the virus, and the absence of a requirement for a nucleotide energy source argues that circularization does not occur in the reaction. Other properties of the reaction have also been determined. The availability of such an in vitro system should facilitate the isolation, characterization and purification of components required for retroviral integration, and the assay could be used to screen for agents such as antiviral drugs which block the integration of retroviral DNA.

A similar development involves studies with an SV40 replication system which demonstrated the important role of topoisomerase enzymes in DNA replication. Topoisomerases are enzymes which allow either unwinding of parental DNA to accommodate the progression of the replication fork (type I) or allow separation of concatenated or intertwined DNA strands (type II). Functional information about these enzymes is of clinical interest since one class of anti-cancer drugs appears to inhibit these enzymes. The new SV40 studies have shown that the type I topoisomerase supplies the main "swiveling" action necessary for most of the replication of the SV40 mini-chromosome, but that a type II topoisomerase is necessary for the last 5% of the replication to allow separation of the daughter DNA strands. These SV40 studies made extensive use of anti-tumor topoisomerase inhibitors. It is known that these inhibitors interfere with the breakage/reunion cycle of these enzymes, causing crosslinking of protein (presumably topoisomerases) to replicating DNA. The SV40 studies have also shown the type II inhibitors prevent the productive binding of the type II topoisomerases to the DNA as well as interfering with topoisomerase action once it has begun. Individual drugs vary widely in their relative ability to act on breakage/reunion or binding mechanisms. This new information will be useful in the design of future drugs of this class. The in vitro SV40 DNA replication system also provides a good method for initial screening of such drugs.

The development of a vaccine against the human T-cell lymphotropic virus-1 (HTLV-1) has been pursued in a manner similar to that previously described for a commercially available feline leukemia virus vaccine. The HTLV-1 persistently infected cell line MT-2 was shown to produce high levels of HTLV-1 antigens. Vaccine material was prepared as cell- and virus-free preparations. Rabbits were immunized with the candidate HTLV-1 vaccine emulsified in complete Freund's

adjuvant. The anti-HTLV-1 titer of rabbit sera increased markedly upon repeated immunization. Western blot analysis, using purified HTLV-1 as an antigen, demonstrated that the vaccine evoked antibodies directed to several HTLV-1-specific polypeptides, including envelope and core components of the HTLV-1 virion. Furthermore, the vaccine-induced antibodies inhibited syncytia formation in the MT-2(HTLV-1):XC syncytia inhibition assay; the antibodies fixed complement and effected lysis of MT-2 target cells. The HTLV-1 candidate vaccine was subsequently administered to two pigtailed macaques. The antisera from the macaques recognized the spectrum of HTLV-1-specific polypeptides in purified HTLV-1 preparations, including the core and envelope components of the virion. These studies demonstrate the feasibility of vaccination approaches for the prevention of human retrovirus infections.

A murine model with a short latency period for the study of neurotropic retroviral infection was developed. This model is relevant to studies on the maternal transmission of AIDS. Microinjection of a neurotropic strain of a murine leukemia virus of Lake Casitas wild mice (Cas-Br-E) into mid-gestation mouse embryos caused paralysis and death within 25 days after birth. To evaluate whether antiviral drugs could cross the placental barrier and influence the course of the disease, the drug 3'-azido-3-deoxythymidine (AZT) was administered through the drinking water to pregnant mice. AZT treatment markedly retarded the onset and course of the virus-induced central nervous system disease, permitting the infant animals to survive beyond four months of age. Preliminary data suggested that AZT suppressed the replication of the virus in the fetus and limited its spread to the central nervous system. These results are evidence for effective antiviral treatment during gestation and the perinatal period.

Other novel approaches and reagents for viral therapy and treatment have recently been developed. The CD4 lymphocyte receptor gene was isolated and expressed in an in vitro mammalian system in which a recombinant, soluble form of CD4 (sCD4) is secreted into tissue culture supernatants. sCD4 retains the structural and biological properties of native CD4 on the cell surface, binds to the HIV envelope gp120 and therefore inhibits the binding of virus to CD4+ lymphocytes. This effect on binding results in a striking inhibition of virus infectivity in vitro. Although significant variance exists among different HIV isolates, all appear to use CD4 as a virus receptor. Thus, sCD4 might prove to be a useful inhibitor of HIV virus infections. This type of reagent should also allow a more precise understanding of the structural basis for the association of CD4 with the HIV envelope glycoprotein and with the surface of antigen-presenting cells.

In addition to Kaposi's sarcoma, AIDS is also associated with an increased incidence of other neoplasia, such as malignant non-Hodgkin's lymphomas (AIDS-associated-NHL), Hodgkin's disease, and T-cell chronic lymphocytic leukemias. AIDS-associated Burkitt's lymphomas (AIDS-BL) are characterized by specific chromosomal translocations involving the c-myc oncogene locus on chromosome 8 and one of the immunoglobulin (Ig) loci located on chromosomes 2, 14 or 22, suggesting a role for c-myc activation in the pathogenesis of AIDS-associated-NHL.

The breakpoints occurred within or near the Ig heavy chain switch region on chromosome 14 and within the c-myc locus on chromosome 8. The nature of these breaks suggests that they occur at the time of Ig heavy chain isotype switching, relatively late during B-cell development. EBV infection and c-myc activation appear sufficient for the malignant conversion of human B-cells in vitro. This

suggests that lymphomagenesis in AIDS is a multistep process in which the oligoclonal B-cell expansions observed in AIDS related complex (ARC)-associated lymphadenopathy may represent a "pre-malignant" condition for the development of AIDS-associated-NHL. The state of HIV-induced immunosuppression and continuous EBV reactivations during ARC appear to favor the expansion of multiple EBV-transformed B-cell clones which increases the probability of the occurrence of additional genetic alterations such as the translocation of the c-myc gene. These translocations can result in the malignant transformation of B-cells.

The development of hepatocellular adenomas and carcinomas in a transgenic lineage of mice that contains the entire envelope open reading frame of hepatitis B virus (HBV) was recently reported. This lineage spontaneously develops ground glass cells and hepatocellular injury due to the storage of cytotoxic amounts of nonsecretable long filaments of hepatitis B surface antigen (HBsAG) within the endoplasmic reticulum. This lineage also exhibits a chronic hyperplastic response to the stimulus of ongoing liver cell necrosis. Virtually all mice of this lineage develop hepatocellular neoplasms after 12-14 months of age. Prior to the development of these cancers, serum alpha-fetoprotein elevations that can exceed levels 100 times normal limits are often found. Analysis of restriction digests of genomic DNA from these tumors reveals no evidence of gross rearrangement of the integrated HBV transgene or of cellular flanking sequences, suggesting that transformation may be due to chance mutations occurring elsewhere in the mouse genome in the setting of chronic hepatocellular regeneration. If this is correct, in this model, HBV sequences serve to initiate a series of events that eventually lead to neoplasia. Although they are necessary, the HBV sequences are clearly not sufficient for malignant transformation as they might be if HBV contained an acutely transforming oncogene. This new transgenic model for liver cancer provides the opportunity for additional research to elucidate the mechanism(s) responsible for this lesion and its biological characteristics, and an evaluation of the relevance of the model to human primary hepatocellular carcinoma.

Several interesting studies involving oncogenes have recently been reported. The genome of the previously uncharacterized avian sarcoma virus CT10 has recently been cloned and sequenced. No hybridization was observed with a battery of 19 probes for currently known viral oncogenes. This suggested that CT10 contained a novel oncogene. This viral oncogene was found to encode a 47 kD polyprotein in which the carboxyl half is derived from a cellular proto-oncogene, c-crk (CT10 regulator of kinase). Additionally, the viral oncogene contains two blocks of homology, each consisting of about 50 amino acids, to the N-terminal, non-catalytic domain of $p60^{c\text{-}src}$. The structure of the oncogene protein has a striking similarity to a part of bovine brain phospholipase C (PLC). Since the protein product of this newly discovered oncogene has no homology to the catalytic domain of any known protein kinases, and infection with the parent virus caused increases in the intracellular phosphotyrosine levels, the v-crk gene product must be regulating the activity of endogenous protein kinases. Thus, crk is not only a new oncogene, but it appears to have a novel mode of action as well.

A temperature-sensitive mutant (ts1 S13) of the S13 virus which contains the v-sea oncogene, was isolated and characterized. The temperature-sensitive lesion of the ts1 mutant was found to affect the tyrosine kinase activity, but not the plasma membrane localization of the ts1 S13 v-sea gene product. Erythroblasts transformed by the ts1 mutant could be induced to synchronously differentiate at the non-permissive temperature into erythrocytes in an erythropoietin-dependent

fashion. An analysis of erythrocyte-specific gene expression in the ts1 S13 erythroblasts revealed that the transformed, self-renewing erythroblasts obtained at the permissive temperature already expressed all of the erythrocyte genes tested for, although at low levels. When differentiation was induced, expression of erythrocyte-specific genes was found not to be coordinately regulated, but to involve complex regulatory mechanisms which appeared to be specific for the individual genes. These findings provide not only a model system for studying the complex mechanisms of gene expression during erythroid differentiation, but also a fruitful system for exploring mechanisms of viral oncogenesis.

The human c-jun proto-oncogene has recently been localized to the short arm of chromosome 1 at 1p31-32. The most interesting properties of this new oncogene are the structural similarities of its protein product to those of the DNA-binding end of the yeast GCN4 protein, a transcriptional regulator of amino acid synthesis, and the activator protein-1 (AP-1) of HeLa cells. The latter two proteins have well-established functions in regulating the transcription of genes, a process which transforms genetic information into a template from which proteins can be synthesized. While transcriptional regulation is relatively common in yeast and mammalian cells, there has been only suggestive evidence to date that it could occur in the viral transformation of cells. The evidence that the cellular progenitor of the v-jun oncogene has a protein product identical to the AP-1 protein provides the strongest basis to date for the hypothesis that transcriptional factors can function directly as transforming proteins. This observation should stimulate efforts to find similar functions in other oncogenes and proto-oncogenes, and to determine if other genes encoding transcriptional factors are proto-oncogenes. The significance of these findings is the establishment of transcriptional control elements as direct mediators of oncogenic change.

Although many slowly transforming retroviruses do not encode onc gene proteins, they cause tumors in vivo. However, none have been found to effect the growth of cultured cells in vitro. Recently, it was shown that Moloney murine leukemia virus (M-MuLV), a replication competent retrovirus which lacks an oncogene, can immortalize mouse B-cells. Prior infection with Abelson murine leukemia virus or pretreatment with lipopolysaccharide (LPS) was sufficient to allow M-MuLV to immortalize the cells. This newly developed in vitro system offers an approach to studying viral genes of slowly transforming retroviruses that are associated with cell immortalization and cancer induction.

In Epstein-Barr virus-associated tumors, studies of the structure of the terminal region of the viral DNA in epithelial and lymphoid tumor cells and of the immunoglobulin gene arrangements in lymphoid cells have enabled investigators to assess whether EBV has infected cells prior to proliferation of transformed cells. These studies are based on the fact that EBV DNA termini have variable numbers of a 500 basepair direct tandem repeat which are characteristic of a particular clonal cell line arising from a single EBV-infected cell, but differing between cell lines arising from other EBV infections. The results of studies of the clonality of tumor cells have provided evidence for the etiologic association of EBV with differentiated and undifferentiated nasopharyngeal carcinoma (NPC), certain B-cell lymphomas and parotid gland tumors. Additional analysis of EBV termini in tissues from both differentiated and undifferentiated NPC demonstrated that the differentiated tumors had fewer copies of viral DNA than did undifferentiated NPCs. The lower level of EBV genomes may explain the previous failure to detect viral DNA and viral gene products in the differentiated NPC tumors. By

contrast, predominantly replicative linear forms of viral DNA were found in the tumors from the base of the tongue, suggesting that EBV infection occurred after development of this type of malignancy.

Adenovirus 2 (Ad2) transformed hamster and rat cells are susceptible to lysis by natural killer (NK) cells from the host of origin and are non-tumorigenic in immunocompetent hamsters and rats, respectively. Ad2-transformed hamster and rat cells were also highly susceptible to lysis by nude-rat NK cells and, as would be expected, were non-oncogenic in these animals. Surprisingly, these NK-susceptible, virus-transformed cells were highly tumorigenic in athymic (nude) mice which presumably have an intact NK-cell response. The results of the studies with the nude rats suggested that thymus-dependent, cytotoxic T-lymphocyte-mediated, host cellular immune responses (which are dependent on class-1 MHC expression) were not essential for rejection of xenogeneic cells transformed by non-oncogenic Ad2. The data suggest that immunologically nonspecific host cellular immune responses, such as those mediated by NK cells, were sufficient for rejection of Ad2-transformed cells. However, the results in the nude mice indicated that biologically important differences existed in the NK cell-mediated defenses mounted by nude mice and nude rats against transformed cells which may account for different patterns of tumor induction. Thus, the basis for the cellular immune response to adenovirus transformed cells is host dependent, and analysis of a number of virus-host systems is underway in order to fully understand the basis for tumor cell rejection.

Studies on metastasis have established a close correlation between tumorigenicity and the interaction of tumor cells with the extracellular matrix. Metastatic potential, invasiveness and tumorigenicity are usually associated with affinity for type IV collagen versus type I collagen and with high concentrations of the glycoprotein laminin on the cell surface. Cells transformed with the highly oncogenic adenovirus strain, Ad12, were compared for these properties with cells transformed by the non-oncogenic strain, Ad2, and with recombinant viruses deriving the E1A and E1B genes from one of these two adenovirus types. It was demonstrated that the preferential binding to type IV collagen was related to the expression of the Ad12 E1A gene. The expression of the Ad12 E1B gene resulted in cells being able to bind three times more exogenously added laminin than cells expressing the Ad2 E1B gene. Thus, the interaction of adenovirus-transformed cells with collagen is controlled by the serotype origin of the E1A gene, whereas the ability of cells to bind laminin is controlled by the serotype origin of the E1B gene.

Human papillomavirus type 16 (HPV 16) is closely associated with cervical cancer and is believed to play a role in its development. Specific genes and proteins have been identified which may be responsible for HPV-associated carcinogenesis. They were found by examining the RNA transcription of integrated HPV 16 DNA in cervical carcinoma tissues and in established cell lines derived from such tissues. The most abundant transcripts in these cells were from the HPV 16 genes E6 and E7. The presence of these transcripts in cervical carcinomas and transformed cell lines (e.g., CaSki cells which have been in culture for years, but have maintained the transcription of these viral genes) suggests that the E7 and E6 gene products of HPV 16 may be transforming proteins that are needed for the maintenance, and probably the initiation, of transformation. Both the E6 and E7 proteins have also been detected in cellular extracts from both established cell lines and carcinomas. The identification of these two proteins may lead to the development of specific diagnostic tests to help predict the clinical course of

precancerous human lesions. However, the predominance of E6 and E7 transcription in cervical cancers may be a late event in the oncogenic process. When the spatial distribution of HPV transcription was examined in serial sections from precancerous dysplastic lesions (HPV 16 specific) and condylomas (genital warts that are HPV 6 specific), the E6 and E7 mRNAs were found at moderate levels predominantly in the middle to upper (more differentiated) strata of cells. Another pair of early viral messages, the E4 and E5 gene transcripts were, by far, the most abundant mRNAs in these same epidermal cell layers. The E4 and E5 viral genes appear to be important in the natural life cycle of HPVs, since they appear in both HPV 16- and HPV 6- (a virus with low oncogenic potential) associated lesions and have not been linked to oncogenic progression. Viral mRNAs were also detected in the basal cell layers, suggesting that this rapidly dividing layer of cells (which may be more prone to transformation) can be infected by HPV. These data were obtained by in situ hybridization using new, highly sensitive RNA probes for viral mRNAs. Thus, the event(s) that led to the predominance of E6 and E7 mRNAs in cervical cancers may not have yet occurred, even in the high grade precancerous tissues that were examined.

The progression of papillomavirus-associated precancerous lesions to carcinoma usually has a very long latency (5 to 20 years). It is believed that other cofactors are necessary to promote the development of tumors. Recent studies have suggested that activated proto-oncogenes could represent such co-factors. A junction fragment of HPV 16 integrated into the genome of a lung carcinoma has been isolated. This fragment contains the E6/E7 open reading frame (ORF) and adjacent cellular DNA sequences. Transfection of 3T3 cells with the whole junction fragment produced transformants at high frequencies; however, transfections with either the viral or the cellular sequences alone did not cause transformation. The transformed cells produced tumors in mice and expressed both the HPV and cellular sequences. The cellular sequences are currently being characterized. In another related study, the chromosomal locations of integrated HPV DNA sequences have been determined in several established cervical tumor cell lines and in one primary cervical carcinoma. In two established cell lines, HeLa and C4-I, HPV 18 DNA is integrated on chromosome 8 near the location of the c-myc proto-oncogene. Steady state levels of c-myc mRNA are elevated in these cell lines. In a primary genital carcinoma that was examined, HPV 16 DNA was found integrated into two chromosomes, 3 and 20, in regions that contain the c-raf-1 and c-src-1 proto-oncogenes, respectively. Thus, in at least some genital tumors, cis-activation of cellular oncogenes may be involved in malignant transformation of HPV-infected cervical cells.

Thus, the BCB has supported a variety of studies on both RNA and DNA viruses. These studies have demonstrated novel mechanisms by which some of these agents cause oncogenic transformation and/or cancer. In addition, a number of new scientific initiatives have been developed. Although the seminal questions of how viral oncogenes transform cells and how cellular oncogenes may be related to human cancer have yet to be answered, the research activities carried out by the BCB are providing the fundamental information necessary for their ultimate resolution.

FIGURE I

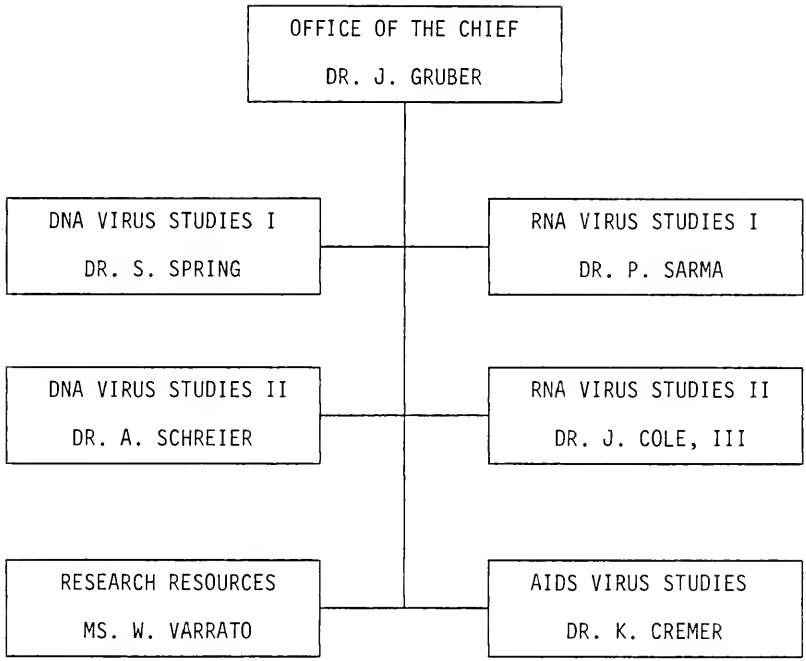
OFFICE OF THE CHIEF
DR. J. GRUBER
DNA VIRUS STUDIES I
DR. S. SPRING
RNA VIRUS STUDIES I
DR. P. SARMA
DNA VIRUS STUDIES II
DR. A. SCHREIER
RNA VIRUS STUDIES II
DR. J. COLE, III
RESEARCH RESOURCES
MS. W. VARRATO
AIDS VIRUS STUDIES
DR. K. CREMER

TABLE I

BIOLOGICAL CARCINOGENESIS BRANCH
EXTRAMURAL PROJECTS ACTIVE FY 1988
(dollars in thousands)
(estimated)

	GRANTS/CO-OPS/CONTRACTS	
	NUMBER	DOLLARS
Research Grants		
Traditional Project Grants (R01)	319	40,744
Conference Grants (R13)	15	47
Academic Research Enhancement Awards (R15)	1	0
First Independent Research Support and Transition (FIRST) Awards (R29)	18	1,605
Outstanding Investigator Grants (R35)	18	12,626
Method to Extend Research in Time (MERIT) Awards (R37)	16	3,283
Small Business Innovative Research (SBIR) Awards (R43)	2	0
Program Project Grants (P01)	21	19,689
Cooperative Agreements (U01)	13	193
Research Contracts (SBIR)	3	1,273
Research Resources Contracts (N01)	5	1,014
TOTAL	431	80,474

TABLE II

BIOLOGICAL CARCINOGENESIS BRANCH

Contracts and Grants Active During FY 1988

	FY 88 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
DNA Virus Studies I	0	0	88	17.21
DNA Virus Studies II	3	1.27	104	20.27
RNA Virus Studies I	0	0	110	17.11
RNA Virus Studies II	0	0	101	20.49
AIDS Virus Studies	0	0	17	3.11
Office of the Branch Chief	0	0	3	0
Research Resources	5	1.01	0	0
TOTAL	8	2.28	423	78.19

TABLE III

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-88

Title	Date Workshop	Date BSC Review	------AWARDS 1ST YEAR--- FY	No.	Total Dollars
NIH-NCI-DCT-CTRP-82-13 (COOP) Studies of Acquired Immune-Deficiency Syndrome (KS & Opportunistic Infections)	-	May 82	83	5	$ 962,575
NIH-NCI-DCCP-82-18 (RFA) Hepatitis B Virus and Primary Hepatocellular Carcinoma	May 82	Sep 82	84	8	$1,073,037
NIH-NCI-DCCP-BCB-83-3 (COOP) Infectious Etiology of AIDS and Kaposi's Sarcoma	-	Feb 83	84	11	$1,537,613
NIH-NCI-DCE-BCB-84-19 (COOP) Studies on Bovine Leukemia	May 83	Mar 84	85	4	$ 380,758
NIH-NCI-DCE-BCB-84-27 (COOP) Studies on Human T-cell Leukemia & Lymphoma Virus Types I & II	Apr 84	May 84	85	7	$ 690,272
NIH-NCI-DCE-85-10 (RFA) The Role of Human Papillo-mavirus in the Etiology of Cervical Cancer	Jun 84	Oct 84	86	7	$ 763,074
NIH-NCI-DCE-85-20 (RFA) Basic Studies on the Development and Assessment of Retroviral Vaccines	Dec 84	Feb 85	86	4	$ 594,667
NIH-NCI-DCE-85-21 (RFA) Studies on Novel Human Exogenous and Endogenous Retroviruses	Mar 85	May 85	86	4	$ 547,709

TABLE III (cont.)

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-88

Title	Date Workshop	Date BSC Review	------AWARDS 1ST YEAR--- FY	No.	Total Dollars
NIH-NCI-DCE-86-07 (RFA) The Transformation Mechanisms of Human Polyomaviruses	Mar 85	Oct 85	87	6	$ 771,480
NIH-NCI-DCE-87-19 (RFA) Studies on Papillomavirus-Host Interactions	Feb 86	Oct 86	88	5	$ 827,972*
NIH-NCI-DCE-87-18 (RFA) Studies of Functional Anti-Sense RNA in Oncogenic Viral Systems	Mar 86	Oct 86	88	3	$ 346,210*
NIH-NCI-DCE-88-13 (RFA) Animal Models for Human Papillomavirus-Associated Neoplastic Diseases	Sep 87	Feb 88	89		
NIH-NCI-DCE-88-14 (RFA) Retrovirus Animal Models and HIV Pathogenesis	Oct 87	Feb 88	89		

* Estimated Funding

OFFICE OF THE BRANCH CHIEF

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. CHOPPIN, Purnell W. American Society for Virology, Inc. 1 R13 AI/CA 24708-01	International Congress of Virology - Canada - ASV Bloc Travel
2. VARMUS, Harold E. Gordon Research Conference 1 R13 CA 45494-01	Gordon Conference on Animal Cells and Viruses
3. YOHN, David S. International Association for Comparative Research on Leukemia and Related Diseases (Columbus, OH) 1 R13 CA 45890-01	XIII International Symposium on Comparative Leukemia Research

SUMMARY REPORT

DNA VIRUS STUDIES I

The DNA Virus Studies I component of the Branch involves research on two groups of large DNA viruses, the herpes- and adenoviruses. In this component, extramural research is supported primarily by the grant mechanism. There are 88 research grants with an estimated total funding level of 17.21 million dollars. These include the traditional research grants, program project grants, conference grants, MERIT awards and outstanding investigator grants. The major research emphasis lies in studies of the mechanism(s) of viral transformation, which include genome structure, function and expression (67%); and virus-cell interaction (33%). In terms of the viruses being studied, 29% involve herpes simplex virus (HSV), 25% involve Epstein-Barr virus (EBV), 8% involve cytomegalovirus (CMV), 9% involve other herpesviruses, and 29% involve adenoviruses.

Investigators supported by this program are attempting to elucidate the mechanism(s) of transformation of herpes- and adenoviruses by a variety of approaches, including localization of transformation function(s) to specific sequences of the viral genome, elucidation of the function(s) of individual viral genes, and determination of the mechanism(s) of regulation of synthesis of viral gene products.

Most members of the herpesvirus family can transform cells in vitro and all of them can establish latent infections in man and animals. Moreover, many of the herpesviruses have been suspected of having a role in tumor induction in man, either directly or as cofactors. Additionally, because all herpesvirus infections result in life-long latent infections, reactivation of these viruses during immunosuppressive cancer therapy is a cause of morbidity and mortality among cancer patients. Basic molecular studies of herpesviruses have sought to define the region(s) of the viral genome involved in transformation, the regulation of the synthesis of virus proteins, and the function(s) of the various virus proteins. Studies with a more biological orientation have investigated virus-host interactions in order to define the process of viral pathogenesis and the mechanism(s) of latency and reactivation.

Epstein-Barr virus is a lymphotropic herpesvirus which has been associated with several disease entities including infectious mononucleosis, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). More recent evidence suggests an etiological role for EBV in B-cell lymphomas in immunosuppressed individuals and in parotid gland tumors. EBV is remarkably efficient in its ability to infect human B-lymphocytes and immediately induce their sustained proliferation. The virus-infected lymphocytes can be cloned and grown as continuous cell lines. The cells are tumorigenic in the brains of nude mice. Despite the continued presence of the complete virus genome, the infection is largely latent with only a few virus genes being expressed.

Because no cell lines fully permissive for in vitro virus replication are available, investigators have been primarily limited to studying human lymphoblastoid lines derived by in vivo or in vitro transformation of B-lymphocytes by EBV or by studying Burkitt's lymphoma-derived cell lines. The majority of the transcripts expressed in these cells are associated with latency. Viral-encoded peptides

were initially identified using immunofluorescence techniques and human antisera. The antigens thus recognized were, in fact, complexes of several polypeptides. Recombinant DNA technology, monoclonal antibodies and Western blots have allowed investigators to start to identify the various components of these complexes. Thus, the EBNA complex (EBV associated nuclear antigen) has now been determined to consist of at least seven components; and each of the early antigen complexes, designated EA(D) and EA(R), have also been shown to consist of several components. Studies of the messenger RNA (mRNA) transcripts have also allowed the identification of other viral gene products. The transcripts and open reading frames (ORFs) have often been identified prior to the definition of the viral function they encode. Such transcripts are designated by the restriction enzyme cleavage fragment and the direction of transcriptional reading. Thus, the BHRF-1 is the BamHI-H rightward frame 1. Recent progress in the definition of EBV encoded proteins is described below.

The restricted early antigen, EA(R), complex was first identified using immunofluorescence technology. More recent studies have defined the viral polypeptides within this antigen complex as being transcripts of the BamHI-O (ORF2) and BamHI-H fragments encoding polypeptides of 85 and 17 kilodaltons (kDa), respectively. Sequence analysis of the 85 kDa polypeptide suggests that it corresponds to the 93 kDa subunit of the EBV-encoded ribonucleotide reductase (36,54).

Additional studies of the 17 kDa BamHI-H transcriptional product suggested that it is a membrane protein. It also has significant homology with both the anchor region of the polyoma middle T-antigen and with the predicted protein product of the bcl-2 mRNA which is activated by the 14/18 translocation characteristic of follicular lymphomas. This latter homology is extensive and colinear, suggesting a common evolution and function of the bcl-2 oncogene and 17 kDa polypeptide (22,36,54,74,77).

The availability of monoclonal antibodies to each of the EA(R) components may permit additional studies of the diagnostic and prognostic value for EBV-associated malignancies of responses to individual EBV polypeptides. In previous studies using immunofluorescence technology and multivalent antisera, patients with Burkitt's lymphoma exhibited high antibody titers against the EA(R) complex and low titers against the EA(D) complex. A reverse pattern was observed with patients with nasopharyngeal carcinoma. Preliminary studies with antisera against the EA(D) complex indicated that these antisera also had titer against the 17kDa polypeptide. Thus, more detailed studies of serial specimens from patients using monoclonal antibodies may provide additional insights on disease-state specific antibody responses to individual EA(D) and EA(R) antigen (36,54).

The constituents of the EBNA polypeptide complex are also being defined. Previous studies have described EBNA-1 as a 78 kDa polypeptide which binds to metaphase chromosomes and is responsible for maintaining the plasmid form of EBV DNA in eukaryotic cell lines. EBNA-2 has been shown to be a 87 kDa protein. The presence of EBNA-2 deletions in non-transforming strains of EBV suggests that EBNA-2 plays an important role in EBV transformation. Recent studies have defined five other components of the EBNA complex. EBNA-3 is, itself, a complex of three polypeptides--EBNA-3A, EBNA-3B and EBNA-3C--which are encoded by the BERF1, BERF2b and BERF4 reading frames. The polypeptides are 145, 165 and 155 kDa, respectively. EBNA-4 consists of a size-related group of polypeptides between 28 and 62 kDa (36, DNA II component, reference 99).

The EBNA-5 transcript is encoded by the BamHI-W region direct repeats and encodes a set of polypeptides probably expressed from a family of transcripts containing a variable number of W1-W2 repeat exons. The basic transcript encodes a polypeptide of 62 kDa with most EBV-infected cell lines preferentially expressing only one or two size classes of EBNA-5 (which occur in increments of 7 kDa). While all human lymphoblastoid cell lines express EBNA-5, the EBV-infected marmoset cell line, B95-8, and a number of EBV-positive Burkitt's lymphoma cell lines do not express any detectable EBNA-5. Thus, expression of the EBNA-5 may reflect differences in EBV gene expression in different host cell types (74).

An additional EBNA protein designated EBNA-LP is encoded by a portion of the EBV genome adjacent to the EBNA 2 coding region. EBNA-LP is a 40 kDa protein which is translated from an unusual mRNA which encodes two proteins from two non-overlapping reading frames. Of the five cell lines tested for EBNA-LP, it was expressed only in three lines, suggesting that EBNA-LP expression may be host cell restricted (36).

EBV has been consistently detected in specimens from undifferentiated nasopharyngeal carcinomas (NPC), but not in specimens from patients with differentiated NPC. Patients with differentiated squamous cell nasopharyngeal carcinomas do not have the characteristic EBV serological profiles associated with undifferentiated NPC. However, Southern blot analysis demonstrated the presence of EBV DNA in five samples of differentiated NPC. An analysis of the structure of the viral DNA in NPC and in Burkitt's lymphoma was undertaken to determine whether EBV infection of these cells occurred before or after tumor cell proliferation. This study is based on the fact that EBV DNA termini have variable numbers of a 500 basepair direct tandem repeat sequence. The terminal restriction endonuclease fragments and the fused terminal fragments from the intracellular plasmid form of EBV DNA isolated from different clonal lineages are heterogeneous in size and vary by increments of 500 basepairs. Terminal fragments derived from a clone of cells in which the progenitor cell was infected with EBV prior to the cell's malignant proliferation would be expected to be homogeneous in terms of the number of terminal repeats. Analysis of EBV termini in NPC- and BL-derived cell lines demonstrated that all tumor specimens contained a single fragment representing the fused termini of EBV. The presence of the viral DNA in the circular plasmid forms rather than in the linear viral form also indicated that the virus was in a latent rather than a replicative state. The detection of a homogenous population of DNA termini suggests that NPC, like BL, is a monoclonal expansion of a single EBV-infected progenitor cell. This finding indicates that the cellular proliferation occurred after EBV infection and supports an etiological role for EBV in this malignancy.

Further analysis of these tumor cells provided insight about the serological differences between patients with the differentiated and undifferentiated forms of NPC. Some specimens from undifferentiated NPC contained a set of fragments representing the linear form of the viral DNA. Early replicative mRNAs could also be identified in RNA obtained from these specimens. These data indicate that reactivation of viral replication does occur in the tumor tissue and may promote the high IgA titers characteristic of undifferentiated NPC. Additionally, the differentiated NPC tumors appeared to have fewer copies of viral DNA than did undifferentiated NPCs. This observation of lower genome copy number and level of gene expression may explain the previous failure to detect viral DNA and viral gene products in patients with differentiated NPC tumors. Thus, it appears

that the more highly differentiated state of the cells in differentiated NPC may be more restrictive for virus replication (53,58).

Similar studies of EBV DNA termini were carried out with carcinomas of the parotid gland and base of the tongue. These studies indicated that parotid gland tumors were monoclonal proliferations and suggested an etiological role for EBV in these tumors. By contrast, predominantly replicative linear forms of viral DNA were found in the tumors from the base of the tongue, suggesting that EBV infection occurred after development of this type of malignancy (53,58).

Studies of B-cell lineages in some B-cell lymphomas also indicated that EBV infected the progenitor cell prior to its proliferation and metastasis. Analysis in this case was more extensive and included not only the number of EBV termini, but also the length of the termini and the clonal state of immunoglobulin gene rearrangements and expression (4).

Two key interactions of a virus with the host cell are the adsorption of the virus to the cell surface and the interaction of the host immune cells with the infected cell surface. In its interaction with host cells and the host immune system, EBV exhibits several close associations with the complement system which appear to be related to its pathogenesis. First, EBV uses CR2, a complement receptor, to attach to and infect B-lymphocytes; second, the gp350 envelope glycoprotein of EBV, which is involved in virus attachment to cells, shares amino acid sequence homology with C3dg; third, lymphoblastoid cells bearing the EBV genome in a latent state activate the alternative complement pathway; and fourth, gp350, intact EBV and EBV-producing cells also activate the alternative complement pathway. Finally, it has been demonstrated that EBV can act as a cofactor for factor-I-mediated cleavage of complement components, C3b, iC3b, C4b and iC4b. EBV also accelerates the decay of the alternative pathway C3 convertase. While, the full consequences of these activities for EBV pathogenesis are still being defined, the importance of the complement system in disease pathogenesis is clear from studies of individuals with congenital complement deficiencies who exhibit characteristic disease complexes related to the absence of particular components of the complement system (11,36,48).

In the case of EBV, at least two virus proteins, EBNA-2 and the latency membrane protein (LMP) have been associated with viral transformation. Investigators have sought viral transformation proteins for herpes simplex virus, but, to date, no such proteins have been identified. Moreover, in contrast to EBV where the entire viral genome can be found in BL and NPC cells, only small fragments of the herpesvirus genome are detected in low frequency in transformed cell lines. Several recent findings summarized below provide insight into the possible mechanisms of transformation by herpes simplex virus.

Two DNA fragments from herpes simplex type 2 (HSV-2), BglII-C and BglII-N, have been demonstrated to transform cells in vitro. The BglII-N fragment does not code for a viral protein, whereas the BglII-C fragment encodes the virus ribonucleotide reductase protein. However, no role for this protein has been demonstrated in viral transformation. The BglII-N fragment has been shown to cause one-step focal transformation of BALB 3T3, NIH 3T3 and primary Wistar rat embryo cells. The BglII-C fragment was shown to induce multistep transformation of non-established Syrian hamster embryo cells. No homology was detected between the two fragments. It is possible that they each cause transformation by different,

but related mechanisms. The BglII-N fragment has been shown to form a stem-and-loop structure and to cause an increase in alterations in the HGPRT gene when transfected into NIH 3T3 cells. It also has been demonstrated to cause a two-fold amplification of human papillomavirus (HPV) type-18 sequences when used to infect HPV-18 containing cervical carcinoma cell lines. These two observations are consistent with the hypothesis that HSV-2 may act as a cocarcinogen rather than causing cancer directly (16,34,44).

Two observations relating to the BglII-C fragment suggest that it has a different mechanism of action. A plasmid isolated from a rat cell line morphologically transformed by the BglII-N fragment was shown to transform cells at high frequency. Sequence analysis of this plasmid demonstrated that it retained a portion of the input BglII-N sequences, but contained mainly rat cell sequences. These rat sequences hybridize to the BglII-C fragment, thus suggesting a rat homologue of the BglII-C HSV-2 sequences. These homologous cellular sequences could serve as targets for HSV-2 transformation. The structure of the BglII-C region suggests that portions of it may also form a stem-and-loop structure which could allow it to induce cellular gene alterations, by a second mechanism. The BglII-C fragment has been additionally divided into a left hand fragment which caused cellular immortalization without tumorigenesis and a right hand sub-fragment which induced tumorigenic transformation of immortalized cell lines. Additional studies are aimed at defining the exact mechanism of HSV-2 transformation (34,44,87).

Another important issue in herpes simplex virus (HSV) pathogenesis is the reactivation of latent virus. HSV reactivation has clinical importance in immunosuppressed individuals such as cancer patients, where it is a significant cause of morbidity. In order to understand the viral functions involved in reactivation, investigators have studied the factors which regulate viral gene expression during the HSV replication process. The goal of such studies are prevention of viral reactivation in immunocompromised individuals and generation of vaccine strains of virus unable to attain the latent state, but capable of eliciting a protective host response.

The expression of HSV-1 genes is a tightly and coordinately regulated process. The control of expression is primarily at the level of transcription. The earliest (alpha) viral genes expressed in the infected cell have regulatory functions, and the continued expression of at least the ICP4 (infected cell polypeptide) gene is needed for subsequent normal viral expression. Prior to DNA replication, the beta genes are expressed. Many of these genes are involved in viral DNA replication. Concomitant with DNA replication is the expression of two late classes of genes, the beta-gamma and gamma genes. Many of these late genes are virion structural proteins. The function of many other viral genes is still unknown.

While the overall pattern of the regulatory cascade is known, more detailed analysis has demonstrated that complex interactions between the alpha genes and other genes are important in the precise regulation of transcription. Additionally, the regulatory control elements on the viral DNA and the role of host factors are still being defined. In addition to a minimal transcription unit that regulates their basal expression, alpha genes have at least five cis-acting elements which respond to a variety of cellular and viral transactivating factors. Using monoclonal antibodies to inhibit binding of the ICP4 protein to DNA fragments of alpha-regulated genes (ICP0, ICP4 and ICP27), an ICP4 binding

nucleotide sequence in the promoter regulatory domains of these genes was identified. A second binding site in the ICP4 promoter domain for the autoregulatory activity of ICP4 was also demonstrated. Preparation of deletion mutants of ICP4 indicated that the amino terminal 60% of the ICP4 molecule is necessary for the stimulation of beta genes and for the autoregulatory functions of ICP4. The alpha genes themselves are induced by a structural component of the virus, Vmw65, which is also designated as alpha-TIF (alpha gene trans-activating factor). Vmw65 action is mediated through a consensus sequence TAATGARATTC and appears to involve interaction with a host protein. Thus, both cis- and trans-acting factors are involved in alpha gene induction. While ICP4 appears to be the major regulatory alpha protein and has an autoregulatory function, recent studies have demonstrated that another alpha gene, ICP0, can positively regulate ICP4 expression. In contrast, the beta gene, ICP8, is partially responsible for the negative regulation of ICP4. Additionally, comparison of the upstream regulatory regions of two beta genes, ICP8 (the major DNA binding protein) and viral thymidine kinase detected significant similarities between the regulator signals of both of these beta genes. While the complexities of HSV gene regulation are apparent, the knowledge gained about HSV gene regulation will have applicability not only to gene regulation in other herpesviruses, but also to the regulation of cellular processes in general (23,24,39,64,65,66,68,71,82).

Because of their large genome, herpesviruses could potentially serve as vectors for the expression of proteins of therapeutic value or of proteins for induction of the immune response. Investigators are studying both herpes simplex virus type 1 and the varicella zoster (VZV) Oka vaccine strain for possible use as vectors. The advantage of the Oka strain of VZV is that it has already been shown to be safe and immunogenic in immunocompromised children. Thus, adding exogenous genes from other herpesviruses such as EBV would allow the preparation of a bivalent vaccine for VZV and EBV which would contain only the appropriate portion of the EBV genome needed for synthesis of EBV immunogenic proteins. The advantage of herpes simplex virus is that the regulatory sequence of viral proteins has been carefully defined so that the expression of foreign genes could be regulated in the recombinant vectors (36,64,65,66).

In contrast to the situation with herpesviruses, the transforming region of adenoviruses is well defined. Extensive analysis of the adenovirus gene products has demonstrated that the E1 genome region of the virus contains the genetic information necessary for transformation. Two subregions, E1A and E1B, each have important roles in this process. The E1A and E1B regions also play important roles in the replicative cycle of the virus where they serve as regulators of virus gene and host cell gene transcription. To more specifically localize the domains of the E1 proteins required for the transformation and transcriptional regulatory functions, investigators have constructed a variety of point, deletion, and missense mutations in the E1 region. They have then assayed the functions of these mutations either as transfecting plasmids or as reconstructed viral mutants.

The mechanism for transcriptional regulation by E1A was further delineated by several observations. Both viral and host cell transcription are dependent on an array of transcriptional factors (TF). The demonstration that expression of adenovirus E1A results in an increase in activity of a host cell transcription factor TFIIIC may explain E1A transactivation of polymerase III-transcribed genes. Additional studies will involve purification and characterization of the

various components of TFIIIC. Studies of mutants with defects in the E1B promoter TATA box demonstrated that the TATA box was the E1B element responsive to E1A transactivation. Thus, part of the transcriptional regulation by E1A may involve interaction of E1A regulated factors with the TATA box of polymerase II transcribed genes (2,3).

Infection of primary baby rat kidney cells with an adenovirus variant encoding only the 12S gene of the E1A region of adenovirus 5 (Ad5) results in the production of a growth factor that stimulates primary epithelial cells. These epithelial cells which normally die rapidly in culture, not only survive, but proliferate for several weeks in the presence of conditioned medium isolated from Ad5-infected cells. The role of this factor in lytic infection and transformed cells is under investigation. Since adenoviruses normally infect differentiated epithelial cells which are growth arrested, the ability to induce the production of growth factors would enable infected epithelial cells to enter a proliferate phase and thus support viral DNA replication (43).

The reasons for the great difference in in vivo tumorigenicity of individual adenovirus serotypes remains to be elucidated. The transforming ability of adenoviruses has been localized to the E1 region. The initial observation that the E1A genes of the highly oncogenic adenovirus type 12 down-regulate the expression of class 1 MHC antigens led investigators to assume that the removal of the critical recognition signal for cytotoxic T-lymphocytes was the explanation for the higher oncogenicity of adenovirus 12 compared to adenoviruses 2 and 5. More recent studies investigating two other classes of immune cells, natural killer (NK) cells and lymphokine-activated killer (LAK) cells, have suggested that resistance to lysis by NK and LAK cells may also be important in determining the ability of virus-transformed cells to induce tumors. Additional studies have suggested that the nonspecific lysis by NK cells may be host-dependent.

Adenovirus 2 (Ad2) transformed hamster and rat cells are susceptible to lysis by NK cells from the host of origin and are non-tumorigenic in immunocompetent hamsters and rats, respectively. As would be expected if NK cells were sufficient to lyse the Ad2 transformed hamster and rat cells, these Ad2-transformed cells were highly susceptible to lysis by nude-rat NK cells and were non-tumorigenic in nude rats. These results with nude rats demonstrated that thymus-dependent, cytotoxic T-lymphocyte-mediated, host cellular immune responses were not essential for rejection of xenogeneic cells transformed by non-oncogenic Ad2. These NK-susceptible, virus-transformed cells are, however, highly tumorigenic in athymic (nude) mice--animals which apparently possess an intact NK-cell response. In vitro analysis of lysis of xenogeneic rat or hamster Ad2-transformed cells by nude-mouse NK cell showed that NK cell lysis of Ad2-transformed cells was defective both in athymic and euthymic mice, suggesting that the mouse NK cells could not effectively lyse adenovirus-transformed cells. The data suggest that nonspecific cellular immune responses, such as those mediated by NK cells, may be host-dependent. Further studies using virus mutants indicated that the induction of transformed cell susceptibility to NK cell lysis is a function encoded by the E1A gene of the virus. Thus, in order to completely understand the basis for rejection of virus transformed cells, several different model systems may need to be studied (10).

Studies on metastasis have established a close correlation between tumorigenicity and the interaction of tumor cells with the extracellular matrix. Metastatic potential, invasiveness and tumorigenicity are usually associated with affinity

for type IV collagen versus type I collagen and with high concentrations of the glycoprotein laminin on the cell surface. Highly tumorigenic Ad12-transformed cells attached preferentially to type IV collagen, whereas non-tumorigenic Ad5 transformants preferred type I collagen. Additionally, tumorigenic cells expressing the entire Ad12 E1 region had higher concentrations of cell surface laminin than weakly tumorigenic cells expressing only the Ad12 E1A genes. The availability of cells transformed with recombinant adenoviruses deriving the E1A and E1B genes from Ad5 or Ad12 permitted studies to further define the regions of the viral genome responsible for laminin expression and preference for type IV collagen. Cells deriving both genes from Ad12 were tumorigenic, adhered preferentially to type IV collagen and expressed cell surface laminin. Weakly tumorigenic cells, which express the E1A gene of Ad12 and E1B genes of Ad5 also attached preferentially to type IV collagen, but did not contain laminin on their surface. In the reciprocal combination of genes, the cells are non-tumorigenic, do not preferentially attach to type IV collagen, but have laminin on their surface. There is no difference in the amount of laminin secreted into culture by cells having either the Ad5 E1B gene or the Ad12 E1B gene. In vitro studies showed that cells which expressed the E1B gene of Ad12, regardless of the origin of the E1A gene could bind three times more exogenously added laminin. Thus, the interaction of adenovirus-transformed cells with collagen is controlled by the serotype origin of the E1A gene, whereas the level of cell surface laminin is controlled by the serotype origin of the E1B gene (61, DNA II component, reference 79).

Additional research on the reduction of major histocompatibility complex (MHC) class-1 gene expression in adenovirus 12-transformed cells has demonstrated that beta-2 microglobulin synthesis is not diminished in adenovirus 12-transformed cells. This finding was unexpected since the MHC class-1 and beta-2 microglobulin synthesis are thought to be coordinately regulated. In subsequent experiments, Ad5- and Ad12-transformed cells were infected with influenza A virus and tested for sensitivity to cytolysis by influenza A-specific cytotoxic T-cells. The Ad12-transformed influenza-infected cells were less susceptible to lysis than the Ad5-transformed influenza-infected cells. The amount of influenza antigen expressed on each cell line was the same, indicating that this was not a factor. When the Ad12 cells were treated with interferon to increase the expression of MHC class-1 molecules, both the Ad12 and Ad5 cells showed the same level of lysis by influenza-specific cytotoxic T-lymphocytes (CTL). Thus, it appears that the reduction of expression of MHC class-1 molecules is important in allowing Ad12-transformed cells to escape CTL recognition at either the afferent (CTL stimulation) or efferent (target cell recognition) level and may enhance the survival of Ad12-transformed cells in the host and lead to its enhanced oncogenicity when compared to Ad2-transformed cells (63).

At its October 1986 meeting, the DCE Board of Scientific Counselors approved the issuance of an RFA, sponsored by this Branch component, entitled "Studies of Functional Anti-Sense RNA in Oncogenic Viral Systems." The goals of this RFA were to stimulate applications which systematically studied the mechanism of action of anti-sense RNA and investigated the principles for construction of vectors to optimally express anti-sense sequences in oncogenic virus model systems. The availability of efficient vectors would facilitate basic research on the function of viral oncogenes and the mechanism(s) of transformation by different oncogenic viruses. The long-term goal would be the in vivo control of

neoplasia by such anti-sense vectors. Applications in response to this RFA were reviewed in November 1987 and three applications of high scientific merit and program relevance were funded in FY88.

On April 25-26, 1988, this Branch component co-sponsored with the Bacteriology and Virology Branch, NIAID, a workshop entitled "Mechanisms of Pathogenic Diversity of Epstein-Barr Virus." The workshop participants summarized the recent progress in defining EBV gene function, EBV gene regulation and the host response to EBV infection and EBV-infected cells. The participants identified a number of gaps in our knowledge of EBV pathogenesis and oncogenesis on which future research should focus. The participants indicated that the EBV research field would benefit from having readily available and well-characterized monoclonal antibodies to the structural and regulatory gene products already known, with the capability of having additional reagents as new genes were identified. They noted that a reference reagent laboratory which could compare new antisera with the classical immunofluorescent antibodies would facilitate EBV research. Until his recent death, the laboratory of Dr. Werner Henle had served some of these purposes. After additional programmatic evaluation, a concept or concepts based on these suggestions may be developed for presentation at the October 1988 meeting of the DCE Board of Scientific Counselors.

In summary, much progress has been made in elucidating the regions of the genomes of herpes- and adenoviruses involved in transformation in vitro. However, further studies must be done in order to elucidate the mechanisms by which these viruses cause oncogenesis in vivo. The final goal of such studies is the control and reversal of this process.

DNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. AURELIAN, Laure University of Maryland (Baltimore) 5 R01 CA 39691-03	Transformation by Restriction Fragments of HSV DNA
2. BERK, Arnold J. University of California (Los Angeles) 5 R37 CA 25235-10	Biosynthesis of Adenovirus Early RNAs
3. BERK, Arnold J. University of California (Los Angeles) 2 R01 CA 41062-04	Transcription Stimulation by Adenovirus E1A Protein
4. BROWN, Nathaniel A. North Shore University Hospital (Manhasset, New York) 5 R01 CA 35536-06	Clonal Virulence Features of the EBV Terminal Region
5. CALNEK, Bruce W. Cornell University (Ithaca) 5 R01 CA 06709-26	Studies on the Avian Leukosis Complex
6. CHANG, Robert S. University of California (Davis) 5 R01 CA 43051-02	Epstein-Barr Virus and Nasopharyngeal Carcinoma
7. CHINNADURAI, Govindaswamy St. Louis University 2 R01 CA 31719-07	Genetic Analysis of Adenovirus 2 Early Genes
8. CHINNADURAI, Govindaswamy St. Louis University 5 R01 CA 33616-09	Adenovirus LP Locus: Role in Oncogenic Transformation
9. CLOUGH, Wendy G. University of Southern California 5 R01 CA 23070-10	EBV DNA Synthesis in Transformed Lymphocytes
10. COOK, James L. National Jewish Hospital and Research Center 5 R01 CA 38796-03	Tumor Cell Resistance to Destruction by Effector Cells

11.	COOPER, Neil R. Scripps Clinic and Research Foundation 5 R01 CA 14692-16	Humoral Immunity to Viruses and Virus-Infected Cells
12.	COURTNEY, Richard J. Louisiana State University Medical Center (Shreveport) 2 R01 CA 42460-04	Studies of Purified Herpes Simplex Virus Glycoproteins
13.	DESROSIERS, Ronald C. Harvard University 2 R01 CA 31363-07	Molecular Basis for Herpesvirus Saimiri Oncogenicity
14.	EGGERDING, Faye A. University of California (Los Angeles) 5 R01 CA 25545-06	Regulation of Adenovirus 2 Transcription
15.	EISENBERG, Roselyn J. University of Pennsylvania 1 R13 CA 45764-01	Twelfth International Herpesvirus Workshop
16.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 26001-10	Herpesvirus Expression in Transformation and Latency
17.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 35568-05	Molecular Studies on Herpes- and Papillomavirus Proteins
18.	GAYNOR, Richard B. University of California (Los Angeles) 2 R01 CA 30981-07	Transcriptional Regulation by the Adenovirus E1A Protein
19.	GLASER, Ronald Ohio State University 5 R01 CA 29066-07	Epstein-Barr Virus DNA in Transfected Cells
20.	GOODING, Linda R. Emory University 1 R01 CA 48219-01	Mechanism of Adenovirus-Induced TNF Resistance
21.	GREEN, Maurice St. Louis University 5 R01 CA 29561-31	Biochemistry of Animal Virus Multiplication
22.	HARDWICK, Jan Marie Johns Hopkins University 5 R01 CA 43532-03	Epstein-Barr Virus: Regulation of Gene Functions

23.	HAYWARD, Gary S. Johns Hopkins University 5 RO1 CA 22130-11	Structure and Regulation of Human Herpesvirus Genomes
24.	HAYWARD, Gary S. Johns Hopkins University 5 RO1 CA 28473-08	Cellular Transformation by DNA of Human Herpesvirus
25.	HAYWARD, S. Diane Johns Hopkins University 2 R37 CA 30356-07	EBV Genome Expression: Localization of Specific Functions
26.	HAYWARD, S. Diane Johns Hopkins University 5 RO1 CA 42245-03	Regulation of Replication and Latency by EBV EBNA 1
27.	HIRSCH, Martin S. Massachusetts General Hospital 5 RO1 CA 12464-18	Immune Reactivity and Oncogenic Virus Infections
28.	HOLMES, Edward W, Jr. Duke University 1 RO1 CA 47631-01	Retroviral Anti-Sense RNA: Cellular and Viral Responses
29.	HORWITZ, Marshall S. Yeshiva University 5 RO1 CA 11512-19	Adenovirus DNA Synthesis and Polypeptide Assembly
30.	HUANG, Eng-Shang University of North Carolina (Chapel Hill) 2 RO1 CA 21773-09	Cytomegaloviruses and Human Malignancy
31.	HYMAN, Richard W. Pennsylvania State University Hershey Medical Center 5 RO1 CA 16498-14	Malignancy and DNA Homology among the Herpesviruses
32.	ISOM, Harriet C. Pennsylvania State University Hershey Medical Center 2 RO1 CA 23931-11	Regulation of Differentiation in Hepatocytes in Vitro
33.	IZANT, Jonathan G. Yale University 1 RO1 CA 47629-01	Enhancement and Modulation of Anti-Sense RNA Activity
34.	JARIWALLA, Raxit J. Linus Pauling Institute 5 RO1 CA 42467-02	Role of Transforming HSV-2 DNA Sequences

35. JONES, Clinton J.
University of Mississippi
Medical Center
1 R29 CA 47872-01

Mechanistic Approaches to HSV-2 Induced Transformation

36. KIEFF, Elliott D.
Brigham & Women's Hospital
(Boston)
5 R35 CA 47006-02

Molecular Biology of Epstein-Barr Virus Infection

37. KLEIN, George
Karolinska Institutet
2 R01 CA 28380-07

EBNA and Other Viral Products in EBV Transformed Cells

38. KLEIN, George
Karolinska Institutet
2 R01 CA 30264-07

Immune Effector Mechanisms in EBV-Carrying Patients

39. KNIPE, David M.
Harvard University
2 R37 CA 26345-09

Genetics of Herpesvirus Transformation

40. LEWIS, James B.
Oncogen
(Seattle)
7 R01 CA 29600-07

Functions of Adenovirus Proteins in Transformation

41. LEWIS, James B.
Oncogen
(Seattle)
7 R01 CA 39636-03

Adenoviral Oncogene Expression and Transformation

42. MARTIN, Terence E.
University of Chicago
1 R01 CA 48189-01

Effects of HSV on Nuclear Structure and mRNA Processing

43. MATHEWS, Michael B.
Cold Spring Harbor Laboratory
5 P01 CA 13106-17

Cold Spring Harbor Laboratory Cancer Research Center

44. MC DOUGALL, James K.
Fred Hutchinson Cancer
Research Center
5 R01 CA 29350-08

The Biology of Transformation by Herpesvirus

45. MEDVECZKY, Peter G.
University of Massachusetts
(Worcester)
5 R01 CA 43264-03

Growth Factors and Herpesvirus Saimiri Induced Lymphomas

46. MILLER, I. George, Jr.
Yale University
5 R01 CA 12055-17

Studies of Epstein-Barr Virus

47. MILLETTE, Robert L.
Portland State University
5 ROI CA 39067-03

In Vitro Transcription of Herpes Simplex Virus Genes

48. NEMEROW, Glen R.
Scripps Clinic and Research Foundation
2 ROI CA 36204-04

Infection of B Lymphocytes by Epstein-Barr Virus

49. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
5 ROI CA 31949-07

Marek's Disease Virus: Transformation and Oncogenesis

50. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
5 ROI CA 31950-05

Oncogenicity of Epstein-Barr Virus

51. NONOYAMA, Meihan
Showa University Research Institute for Biomedicine in Florida
5 ROI CA 36895-03

Latency of Epstein-Barr Virus DNA in Transformed Cells

52. PADMANABHAN, Radha K.
University of Kansas Medical Center
2 ROI CA 33099-04

Structure and Functional Analysis of Adenovirus Genomes

53. PAGANO, Joseph S.
University of North Carolina (Chapel Hill)
5 POI CA 19014-11

DNA Virus Genomes, Oncogenesis and Latency

54. PEARSON, Gary R.
Georgetown University
2 ROI CA 39617-05

Epstein-Barr Virus-Induced Membrane Antigens

55. PEARSON, Gary R.
Georgetown University
1 R13 CA 48657-01

Third International Symposium on Estein-Barr Virus and Associated Diseases

56. PEARSON, George D.
Oregon State University
5 ROI CA 17699-13

Replication of an Oncogenic Virus

57. PRUSOFF, William H.
Yale University
5 ROI CA 05262-28

Iododeoxyuridine, Iodo-DNA and Biological Activity

58. RAAB-TRAUB, Nancy J.
University of North Carolina
(Chapel Hill)
2 RO1 CA 32979-04

EBV Expression in
Nasopharyngeal Carcinoma

59. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
2 PO1 CA 27503-09

DNA Viruses and Neoplasia

60. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
5 RO1 CA 34479-06

Latency and Transformation
by Herpesviruses

61. RASKA, Karel, Jr.
Robert Wood Johnson
Medical School
(Piscataway, New Jersey)
5 RO1 CA 21196-11

Adenovirus T and Surface
Antigens and Tumorigenicity

62. REKOSH, David M.
State University of New York
(Buffalo)
5 RO1 CA 25674-09

Adenovirus Early Gene Function
and DNA Replication

63. RICCIARDI, Robert P.
Wistar Institute of Anatomy
and Biology
2 RO1 CA 29797-07

Organization and Expression of
Adenovirus Genes

64. ROIZMAN, Bernard
University of Chicago
5 R37 CA 08494-23

Mechanisms of Viral Infection
in Relation to Cancer

65. ROIZMAN, Bernard
University of Chicago
5 PO1 CA 19264-13

UCCRC: Viral Oncology Program

66. ROIZMAN, Bernard
University of Chicago
1 R35 CA 47451-01

Molecular Biology of Herpes
Simplex Viruses

67. ROUHANDEH, H.
Southern Illinois University
1 RO1 CA 38678-01

Transforming Sequences of Yaba
Virus DNA

68. SCHAFFER, Priscilla A.
Dana-Farber Cancer Institute
5 RO1 CA 20260-12

Immediate-Early Genes of HSV

69. SCHNEIDER, Robert J.
New York University
Medical Center
5 RO1 CA 42357-03

Translational Regulation of Adenovirus Gene Expression

70. SHENK, Thomas E.
Princeton University
5 R37 CA 38965-05

Structure and Function of DNA Tumor Virus Genomes

71. SILVERSTEIN, Saul J.
Columbia University (New York)
5 RO1 CA 17477-14

Molecular Biology of Herpesvirus

72. SIXBEY, John W.
St. Jude Children's
Research Hospital
2 RO1 CA 38877-04

Epstein-Barr Virus Expression in Normal Human Epithelium

73. SPEAR, Patricia G.
Northwestern University
(Chicago)
7 R37 CA 21776-12

Herpesvirus Gene Expression in Transformed Cells

74. SPECK, Samuel H.
Dana-Farber Cancer Institute
5 RO1 CA 43143-03

Viral Transcription in EBV Transformed Human B Cells

75. SPECTOR, Deborah H.
University of California
(San Diego)
5 RO1 CA 34729-06

Human CMV, Cell-Related DNA, Oncogenes and Kaposi's Sarcoma

76. ST. JEOR, Stephen C.
University of Nevada (Reno)
5 RO1 CA 28089-07

Herpesvirus Induced Malignancy

77. STROMINGER, Jack L.
Dana-Farber Cancer Institute
5 PO1 CA 21082-12

Molecular Basis of Viral Oncogenesis

78. SULLIVAN, John L.
University of Massachusetts
(Worcester)
5 RO1 CA 39653-03

Lymphotropic Herpesvirus of Cottontail Rabbits

79. THORLEY-LAWSON, David A.
Tufts University
5 RO1 CA 31893-07

Epstein-Barr Virus Membrane Antigen

80. TIBBETTS, Clark J.
Vanderbilt University
5 RO1 CA 34126-06

Adenovirus Genome Expression: Physical Mapping Studies

81.	VELICER, Leland F. Michigan State University (East Lansing) 5 RO1 CA 45479-02	Oncogenic Herpesvirus Secretory Glycoprotein Analysis
82.	WAGNER, Edward K. University of California (Irvine) 2 R37 CA 11861-19	Control of Viral RNA Synthesis in Herpesvirus Infection
83.	WAGNER, Edward K. University of California (Irvine) 1 R13 CA 47733-01	Thirteenth International Herpesvirus Workshop
84.	WILLIAMS, James F. Carnegie-Mellon University 2 RO1 CA 21375-11	Genetic Analysis of Adenoviruses
85.	WILLIAMS, James F. Carnegie-Mellon University 2 RO1 CA 32940-07	Type 12 Adenovirus Transformation-Defective Mutants
86.	WOLD, William S. St. Louis University 5 RO1 CA 24710-10	Adenovirus 2 Coded Early Glycoprotein
87.	WOLD, William S. St. Louis University 5 RO1 CA 33101-06	Regulatory Features of HSV Gene Expression
88.	YATES, John L. Roswell Park Memorial Institute (Buffalo) 5 RO1 CA 43122-03	The Functions of Epstein-Barr Virus Nuclear Antigen 1

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II component of the Branch involves the investigation of the two major classes of mammalian small DNA tumor viruses: papillomaviruses and polyomaviruses. In the component, there are 107 research grants and contracts with an estimated total funding of 21.54 million dollars. These include traditional research grants (R01), program project grants (P01), conference grants (R13), outstanding investigator grants (R35), merit awards (R37), and first independent research support and transition (FIRST) awards (R29). In addition, there are three phase II SBIR contracts. The major research emphasis of this area is the elucidation of the molecular events leading to the initiation and maintenance of cellular transformation by the small DNA tumor viruses and the determination of the role of these viruses in the etiology of human cancers. In terms of scientific areas, 24% of the grants deal with the structure and expression of viral genes, 24% deal with the biochemical properties and mechanisms of action of viral proteins, 24% deal with the expression and function of cellular genes that are involved in the transformation process, 14% deal with the potential etiological relationship of small DNA viruses to human cancers, and 10% deal with basic biochemical and physiological studies which use the small DNA tumor viruses as model systems. In terms of the viruses being studied, 32% of the grants involve the simian virus 40 (SV40); 22% concern human or animal papillomaviruses; 15% deal with viral or cellular oncogenes or related systems; 13% relate to mouse polyomavirus; 11% deal with other DNA or RNA viruses; and 7% relate to human polyomaviruses. Representative studies involving these classes of viruses are described below.

Among the advances in the DNA Virus Studies II program has been the continued progress in understanding the probable role of human papillomaviruses (HPVs) in the development of anogenital cancers, including cervical carcinoma. Previously, investigators had identified and classified HPV DNA from many premalignant, malignant, and metastatic lesions in humans. HPV types 16, 18, 31, 33, 35, 39 and 45 were usually associated with severe dysplasias and carcinoma in situ; whereas HPV types 6, 11, 42, 43 and 44 were associated with milder lesions such as genital warts and mild dysplasias. In addition, established human cervical cancer cell lines, such as HeLa and CaSki, were found to possess integrated HPV 18 and 16 DNA, respectively. These data strongly suggested a role for HPVs in human cancer. More recent clinical data has raised the possibility that HPV 18 is associated with a particularly virulent form of cervical cancer which has a rapid clinical progression. New transformation assays, DNA cloning methods and asymmetric mRNA probes have been used this year to continue the analysis at the molecular level of the transformation mechanism (48).

Specific HPV genes and proteins have been identified, through examination of the RNA transcription pattern in HPV 16-associated cervical carcinomas and in established cell lines derived from these tissues, which may be responsible for HPV associated cancers in cervical tissues. The most abundant viral transcripts in these cells were from the HPV 16 genes E6 and E7. The presence of these transcripts in cervical carcinomas and transformed cell lines (e.g., CaSki cells which have been in culture for years, but have maintained the transcription of these viral genes) suggest that the E6 and E7 gene products of HPV 16 may be transforming proteins that are needed for the maintenance and probably the

initiation of transformation. The E6 and E7 proteins have also been detected in cellular extracts from established cell lines and carcinomas. The E7 protein was determined to be a short half life (40 minutes) phosphoprotein with a molecular weight of 11 kilodaltons (kD). The E6 protein is significantly larger (18 kD) and has two, as yet uncharacterized forms, with half lives of 1/2 and 4 hours. It also appears to have a cellular counterpart of 22 kD. A form of the E6 protein has been found associated with the membrane of HPV-transformed cells and, thus, could be the target for the cellular immune response which may play a role in the regression of HPV-associated lesions. Other investigators (not funded by this Branch) have confirmed that the DNA which encodes the E6 and E7 genes can transform established cells and, when co-transfected with c-ras, can transform primary cells. The identification of these two proteins may lead to the development of specific diagnostic tests to help predict the clinical course of human lesions. Preliminary studies by our grantees indicate that immunochemical assays using these early HPV proteins are feasible (3,101, and DNA I component, reference 16).

The spatial distribution of HPV transcripts has recently been examined in condylomas (genital warts) and precancerous lesions (dysplasias) removed from patients. These studies were made possible by novel asymmetric RNA probes for many of the genes of HPV 6 and 16. These probes were used to examine the relative abundance of HPV mRNAs by in situ hybridization in epidermal tissue sections. The transcripts of the HPV E4-E5 genes were the most abundant particularly in the middle and upper, most differentiated strata of the tissues. E1 signals were occasionally found, but were associated only with nuclei. The capsid messages, encoded by the L1 and L2 genes, were restricted to the cytoplasm of the top most layers, as expected from previous immunochemical studies of the capsid proteins. Surprisingly, in both condylomas (HPV 6 specific) and precancerous tissues (HPV 16 specific) the E6-E7 mRNAs gave only moderate signals, which were found predominantly in the middle to upper strata. Thus, the event(s) that led to the predominance of E6 and E7 mRNAs in cervical cancers may not have yet occurred even in the high grade precancerous tissues (CIN III or carcinoma in situ) that were examined. Analysis of the germinal or basal stratum (the bottom and least differentiated layer) of the epidermis revealed mRNA transcripts from only early genes of HPV 16 in conditions where HPV DNA was undetectable by current technology. Since the germinal layer is a rapidly dividing stratum of cells which is presumably more susceptible to transformation by viruses or other agents, this new information indicates that HPVs do infect and are active in this layer and, thus, could contribute to its malignant conversion. This result also emphasizes the great sensitivity obtainable with the new mRNA probes. The temporal pattern of HPV DNA transcription in developing epidermal tissue has also recently been examined in experimentally induced condylomas in nude mice. Fragments of human genital tissues were infected with HPV 11 and placed under the renal capsule of nude mice (the Kreider procedure). These tissues would ordinarily develop into condylomatous masses; however, for this experiment they were harvested at 2 week intervals. After fixation, tissue sections were probed using single-stranded RNA sequences specific for RNA transcripts from various early and late genes. The experiments demonstrated that all HPV 11 genes were activated simultaneously and very abruptly at about 40 days after infection. The RNA transcription of all the HPV genes appears to be tightly linked, both spatially and temporally, to the morphological development of the condyloma. Much more needs to learned about the interaction of HPV with its host tissues in order to understand this tight developmental control and how it relates to oncogenesis (18,22,47,87, and RNA I component, reference 4).

The progression of papillomavirus-associated precancerous lesions to carcinoma usually has a very long latency period (5 to 20 years). Thus, it is felt that other cofactors are necessary to promote the development of tumors. Recent studies have suggested that activated proto-oncogenes could represent possible cofactors. A junction fragment of HPV 16 DNA integrated into the genome of a lung carcinoma has been isolated. This fragment contains the E6/E7 open reading frame (ORF) and adjacent cellular DNA sequences. Transfection of 3T3 cells with the whole junction fragment produced transformants at high frequencies; however, transfections with either the viral or the cellular sequences alone did not cause transformation. The transformed cells produced tumors in mice and expressed both the HPV and cellular sequences. The cellular sequences are currently being characterized. In another related study, the chromosomal locations of integrated HPV DNA sequences have been determined in several established cervical tumor cell lines and in one primary cervical carcinoma. In two established cell lines, HeLa and C4-I, HPV 18 DNA is integrated on chromosome 8 near the location of the c-myc proto-oncogene. Steady state levels of c-myc mRNA are elevated in these cell lines. In a primary genital carcinoma that was examined, HPV 16 DNA was found integrated onto two chromosomes, 3 and 20, in regions that contain the c-raf-1 and c-src-1 proto-oncogenes, respectively. Thus, in at least some genital tumors, cis-activation of cellular oncogenes may be involved in malignant transformation of HPV-infected cervical cells (4, and RNA II component, reference 44).

Other suggested cofactors for HPV-associated carcinogenesis are herpes simplex virus (HSV) infection and carcinogens from cigarette smoke. There is a known epidemiological association between smoking and cervical carcinoma. HSV, a common infectious agent of the genital tract, is also known to cause mutations in infected cells. A grantee funded by this program area found that HSV-1 or HSV-2 infection of human cervical carcinoma cell lines (HeLa, C4-I, and C4-II) containing HPV 18 sequences results in a two- to eightfold amplification of HPV DNA in these cells. Infection by adenovirus or cytomegalovirus (CMV) does not cause amplification of HPV DNA. Treatment of these cells with the carcinogen 4-nitroquinoline-1-oxide induces a five- to tenfold amplification of HPV 18 sequences. Thus, the synergistic interactions between HPV, HSV and cigarette carcinogens may play a role in the initiation and progression of cervical carciroma (DNA I component, reference 43).

SV40 is another major focus of transformation research in this Branch component. This small DNA tumor virus was originally isolated from monkeys and has long been used as a model system to study viral transformation of cells in culture and tumorigenesis in susceptible rodents. Previous research has demonstrated that the transformation properties of SV40 are associated with the viral gene that encodes the large T-antigen. This protein dominates both the lytic and transforming interactions of SV40 with the host cell. It provides many biochemical functions for viral infection, including modulation of DNA transcription and replication, and the production of cell surface antigens. In contrast to a growing number of viral oncogenes that require multiple proteins acting cooperatively to produce a fully transformed phenotype, the large T-antigen alone can convert primary cells into tumorigenic cell lines. All of the viral functions required to immortalize cells and to confer the transformed phenotype reside in this protein. Thus, the gene for the large T-antigen and its protein product have become primary areas of investigation in SV40-mediated transformation studies.

Intensive genetic and biochemical analysis of the SV40 large T-antigen has demonstrated that the gene and its product can be divided into several domains, each associated with specific functions. In the past, domains were identified which corresponded to the binding site of the SV40 origin of DNA replication, a binding site for the p53 protein, adenovirus helper activity, nuclear transport signal, a putative metal ion binding region, and sequences required for host-specific transformation. Recent studies have confirmed these results, increased the resolution of the domains, and have identified new functional domains. A number of studies have indicated the amino terminal portion of the large T-antigen is involved in transformation. This function has been further localized to amino acids 106-115 of the protein. Mutants in this region retain the ability to establish primary rodent embryo cells, but cannot morphologically transform them. These data argue that this small sequence is at least part of the functional domain required for transformation. This result has also led to the finding that there was substantial similarity in both the sequence and the predicted secondary structure of the protein between the amino terminal portion of the T-antigen and the conserved region 2 of adenoviruses. The region of the T-antigen between amino acids 127 and 250 appears to be necessary to confer the ability to grow in low serum to transformants. Previous results suggested that the p53 protein binding site on the T-antigen may also be the domain for the ATPase function of T-antigen. This result has been strengthened by recent studies that show a close functional link between ATPase activity and the binding of p53. The p53/T-antigen complex has five- to tenfold enhanced ATPase activity. Finding additional functional domains, such as the one responsible for DNA polymerase binding, and understanding the relationship of these functions to the mechanism of transformation is the goal of future research in this area (63,64, 80,90,93).

One of the unsolved problems in SV40 transformation research has been the function of small t-antigen. This small protein is conserved among a number of SV40-like viruses (e.g., BK, JC and polyomavirus), but its importance in transformation or even lytic infection was unknown. Large T-antigen by itself is the only viral protein needed to transform cells. Recent work has shed some light on this issue. It was found that the small t-antigen enhances the activity of large T-antigen when the large T-antigen's concentration was at relatively low levels in infected cells. Small t-antigen alone had no effect on cells. Without the enhancing effect of small t-antigen, the rate of transformation (the number of infected cells that are transformed) by low levels of large T-antigen is dramatically less than the rate at high levels of T-antigen. Models have been proposed to explain this enhancing effect and they are currently being tested (54,55).

The p53 protein which binds to the large T-antigen and apparently participates in the transformation process has also been a subject of intense study. This protein can probably be considered a cellular proto-oncogene, since the gene for p53 can transform cells when transfected with the c-ras proto-oncogene. Both genetic and structural studies on p53 have progressed this year. p53 has recently been shown to have a covalently attached RNA moiety. The attachment is through a phosphodiester bond between a terminal guanosine in the RNA and a serine in the protein. Characterization and function of this RNA/p53 complex will be investigated. A cDNA clone of p53 has also been isolated and used in genetic experiments. Two mutants of p53 have been characterized which have some paradoxical properties. The mutants contain small DNA insertions of 12 nucleotides at either location 158 or 215 in the amino acid sequence. These mutants cannot bind T-antigen, but they can transform cells in cooperation with ras at

much higher efficiency than normal p53 cDNA. The mutant proteins also have significantly higher affinity for the heat shock protein hsc70 than normal p53. In cells transformed by p53 and c-ras, 30% of the p53 is complexed to hsc70 and 70% is free. These data suggest that the heat shock protein hsc70 may be involved in the transformation mechanism. This hypothesis is currently being tested (4,53).

A few years ago, an in vitro SV40 DNA replication system which faithfully reproduced the in vivo process was developed. Recent studies with this system have demonstrated the important role of topoisomerases in DNA replication. Functional information about these enzymes is of clinical interest, since one class of anticancer drugs appears to inhibit these enzymes. Topoisomerases either allow unwinding of parental DNA to accommodate the progression of the replication fork (type I) or allow separation of concatenated or intertwined DNA strands (type II). The new SV40 studies have shown that the type I topoisomerase supplies the main "swiveling" action necessary for most of the replication of the SV40 minichromosome, but that a type II topoisomerase is necessary for the last 5% of the replication to allow separation of the daughter DNA strands. These SV40 studies made extensive use of antitumor topoisomerase inhibitors. It is known that these inhibitors interfere with the breakage/reunion cycle of these enzymes causing crosslinking of protein (presumably topoisomerases) to replicating DNA. The type I topoisomerase inhibitor camptothecin apparently promoted covalent attachments in early and intermediate phases of SV40 DNA replication, while the type II inhibitor, VM26, promoted attachments in late replication where daughter DNA strands are almost fully formed. The SV40 studies have also shown that the type II inhibitors prevent the productive binding of the type II topoisomerases to DNA as well as interfering with topoisomerase action once it has begun. Individual drugs vary widely in their relative ability to act by these two modes. This new information will be useful in the design of future drugs of this class. The in vitro SV40 DNA replication also provides a good system for initial screening of such anticancer drugs (43,44,84).

A cellular transcription factor that interacts with SV40 DNA appears to be the cellular homolog of the product of a known avian viral oncogene. The cellular protein, AP-1, was first isolated by one of our grantees as a factor that enhances the transcription of specific SV40 and cellular genes. It appears to be inducible by 12-O-Tetradecanoyl phorbol-13-acetate (TPA), and it can have both positive and negative effects on the transcription of SV40 genes. This year, AP-1 has been recognized as the protein product of the proto-oncogene c-jun, the cellular homolog of the v-jun oncogene. AP-1 has an 80% amino acid sequence homology with the v-jun protein and antibodies specific for v-jun also recognize AP-1. These findings demonstrate that the proto-oncogene product of c-jun interacts directly with specific DNA sequences to regulate gene expression. It may now be possible to identify genes under the control of c-jun gene product (AP-1) that affect cell growth and neoplasia. In addition, other proteins related to c-jun have been found in cells stimulated to grow with serum or purified growth factors. The mRNAs of two such proteins have been cloned as cDNAs and have been named junA and junB. The role of these related proteins in cell growth is under investigation (43,50).

A third virus whose study is supported by this Branch component is mouse polyomavirus, which is similar to SV40. The SV40 and mouse polyomavirus virions are morphologically identical and contain nearly the same amount of DNA. However, the viral genomes are organized differently, particularly with respect to the

early tumor antigen region which is responsible for transformation. Polyomavirus DNA codes for six proteins, including three tumor antigens: large T-antigen, middle T-antigen, and small t-antigen. The relationship among the tumor antigens with regard to cellular transformation is complex. The large T-antigen (which is localized in the nucleus) appears to be able to immortalize primary cells in culture, whereas the middle T-antigen (which becomes membrane bound) is able to induce the transformed phenotype in previously immortalized cell lines. In the past year, studies have focused on characterizing the structure and mechanism of action of these T-antigens. In particular, the intriguing observations that middle T-antigen can form tight complexes with the cellular protein product of either the c-src or the c-yes proto-oncogenes, have been vigorously pursued.

Functional analysis of the polyoma middle T-antigen has made significant progress in the last year. Both the possible binding sites for pp60c-src, the phosphorylated product of the c-src proto-oncogene, and the mechanism of regulation of its protein kinase activity have been identified. Previously, the carboxyl half of the middle T-antigen protein was found to be largely dispensable for transformation except for the putative membrane binding domain. Four regions of the amino terminal half of the protein have now been thoroughly analyzed by site-directed mutagenesis. Mutations in these regions render the proteins defective for both transformation and pp60c-src binding. Whether these mutations perturb the c-src binding site(s) or the overall conformation of the middle T-antigen is not known. However, it is clear that the putative c-src binding site is in the amino terminal half of the protein (81).

The middle T-antigen is known to increase by 20-fold the tyrosine kinase activity of the associated pp60c-src, thus disrupting the mechanisms which normally act to modulate the kinase activity of pp60c-src. It was recently proposed that the protein kinase activity and the transformation potential of pp60c-src may be regulated by the state of phosphorylation of its carboxyl terminal tyrosine 527. The action of middle T-antigen on pp60c-src may be to prevent the phosphorylation or enhance the dephosphorylation of this tyrosine residue. A series of mutant pp60c-src proteins were prepared with phenylalanine substituted for tyrosine at positions 416, 519 and 527. Kinase activity was enhanced in the 519 and 527 mutants which were also able to readily participate in the transformation of cells. Adding a 416 mutant to the 519 or 527 mutations only partially inhibited the kinase activity, yet abolished all transformation potential. These results suggest that two events must occur to activate the full transforming potential of the middle T-antigen/pp60c-src complex: hypophosphorylation at tyr 527 and hyperphosphorylation at tyr 416 of pp60c-src (81).

Significant progress has also been made on another protein which appears to bind to middle T-antigen: the 85 kD protein. This protein is a candidate for the phosphotidylinositol kinase activity associated with middle T/pp60c-src complexes. It is important because this same protein is a substrate for the platelet-derived growth factor, suggesting a common pathway for the regulation of cell growth. Phosphotidylinositol (PI) metabolism in the cell is a source of intracellular second messenger molecules which can promote changes in cellular phenotype. The 85 kD protein, as expected, can be found in association with the v-src and c-src proteins in cells in the absence of polyomavirus infection. Further, it is not associated with middle T-antigen mutants which are transformation-negative. Multiple phosphorylations have been detected in the 85kD protein. The extent of these phosphorylations correlates with the amount of PI activity in immunoprecipitates. Efforts will be made in the future to purify and characterize this protein (71,73).

An understanding of the normal processes of growth and division in cells is essential for a full understanding of viral-induced neoplastic growth. A number of investigators supported by this Branch component have begun to apply some of the techniques of modern virology to help study normal cell function. For example, Dr. Claudio Basilico, a recipient of an Outstanding Investigator Grant, has used the procedure of marker rescue to isolate two proteins which appear to regulate the progression of cells into G1, the part of the cell cycle in which a cell becomes committed to division. Two temperature-sensitive mutants of established rodent cell lines with a block at the non-permissive temperature for entry into G1 were transfected with human DNA and revertants able to grow at the non-permissive temperature were selected. Two different human cDNA clones were found and characterized. One encodes a 540 amino acid protein. Experiments with serum-synchronized cells indicate that the expression of this protein is necessary for G1 progression and is, itself, cell-cycle regulated, being induced in approximately mid-G1. The second cDNA clone appears to encode a 395 amino acid protein which consists of periodic repetitive clusters of acidic and basic amino acids. Another investigator, Nobel laureate Dr. Daniel Nathans, has been using similar techniques to identify those genes in cells which are induced when cells are stimulated to grow and divide by exposure to serum or purified growth factors. Four mRNAs have been isolated as cDNAs. One appears to be a homolog of a human tissue factor, another is a zinc-associated transcription factor, and the last two are homologs of the c-jun proto-oncogene, junA and junB, and are also presumably transcription factors. The regulation and function of these genes are being intensively studied (5,43).

An RFA, entitled "Studies on Papillomavirus-Host Interactions," was published in January 1987 with a receipt date for applications of August 3, 1987. This RFA was based on the recommendations of a BCB-sponsored workshop held in 1986. The primary objective of this RFA was to stimulate basic research on the interactions between papillomaviruses and their hosts which lead to tumor formation. Thirteen applications were received and subsequently reviewed for scientific merit by an NCI-convened peer review panel. Five were judged to be of high scientific merit and were funded in FY88.

On September 22, 1987, a workshop on "Prospects for Papillomavirus Vaccines and Immunotherapies" was jointly sponsored by this Branch and the Microbiology and Infectious Diseases Program, NIAID. The goals of the workshop were to assess the desirability and feasibility of papillomavirus vaccines and related immunotherapies and to ascertain whether NCI or NIAID should initiate specific programs to facilitate the development of preventative and curative modalities. The workshop participants concluded that development of such vaccines was premature at this time. They recommended that more research was needed on the nature of the host immune response leading to the natural regression of papillomavirus lesions that is often observed in both animals and human patients. Studies using animal models were particularly stressed. An RFA concept entitled "Animal Models for Human Papillomavirus-Associated Neoplastic Diseases" was developed by program staff and approved by the DCE Board of Scientific Counselors. Its goal is the promotion of studies on the host immune mechanisms involved in the regression of HPV-associated lesions and on the molecular mechanisms which may lead to the progression of these lesions to carcinoma. Established animal models or new animal models were felt to be the best experimental systems to generate the

needed data. The RFA was issued in May 1988 with a receipt date of September 15, 1988. Awards under this RFA are anticipated in FY89.

In summary, investigators sponsored by this Branch component have made significant progress in a number of areas. Two probable transforming genes from human papillomaviruses have been identified and their protein products partially characterized. Major advances in mRNA probes have permitted the spatial and temporal characterization of HPV transcription in tissue sections from benign and premalignant lesions from patients. Activation of proto-oncogenes has been suggested as a major step in HPV-associated oncogenesis. In SV40 and polyomavirus studies, there has been continued characterization of the functional domains of the major viral transforming proteins, the T-antigens, and a great deal of genetic and biochemical characterization of cellular proteins, such as p53, pp60c-src and the 85 kD protein, which bind to and mediate the transforming actions of these antigens. Finally, basic studies using an in vitro SV40 DNA replication system have revealed the important role of topoisomerases in this process and the modes of action of one class of anticancer drugs which are inhibitors of these enzymes.

DNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ALONI, Yosef Weizmann Institute of Science 5 RO1 CA 14995-14	Control of Gene Expression in Tumor Viruses and Cells
2. ALWINE, James C. University of Pennsylvania 5 RO1 CA 28379-08	Regulation of DNA Tumor Virus Gene Expression
3. ANDROPHY, Elliot J. New England Medical Center Hospital 5 RO1 CA 44174-02	Characterization of Papillomavirus E6 Proteins
4. BASILICO, Claudio New York University 5 PO1 CA 16239-14	Biosynthesis in Normal and Virus Transformed Cells
5. BASILICO, Claudio New York University 5 R35 CA 42568-03	Viral and Cellular Gene Expression and Growth Regulation
6. BECKMANN, Anna M. Fred Hutchinson Cancer Research Center 1 RO1 CA 47619-01	Pathobiology of Anogenital HPV Infection
7. BENJAMIN, Thomas L. Harvard Medical School 5 RO1 CA 19567-11	Mechanism of Cell Transformation by Polyomavirus
8. BENJAMIN, Thomas L. Harvard Medical School 5 RO1 CA 25390-09	Effects of HR-T Mutations on Polyoma Gene Expression
9. BENJAMIN, Thomas L. Harvard Medical School 5 R35 CA 44343-02	Natural and Unnatural Roles of the Polyoma HR-T Gene
10. BOTCHAN, Michael R. University of California (Berkeley) 2 R37 CA 30490-08	Regulatory Interactions Between Tumor Viruses and Cells
11. BOTCHAN, Michael R. University of California (Berkeley) 5 RO1 CA 42414-03	Bovine Papillomavirus - Model Systems

12.	BRADLEY, Margaret K. Dana-Farber Cancer Institute 5 RO1 CA 38069-05	Nucleotide Binding Properties of SV40 Large T Protein
13.	BRAUN, Lundy A. Brown University 1 R29 CA 46617-01	Oncogenes and Growth Factors in Human Gynecologic Cancers
14.	BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 22555-11	Biological Properties of SV40 Early Proteins
15.	BUTEL, Janet S. Baylor College of Medicine 5 RO1 CA 25215-10	Tumor Viruses, Oncogenes and Mammary Epithelial Cells
16.	CARMICHAEL, Gordon G. University of Connecticut Health Center 5 RO1 CA 45382-02	Processing and Function of Polyoma RNA
17.	CARROLL, Robert B. New York University 2 RO1 CA 20802-12	Biochemical Properties of the SV40 T-antigens
18.	CHOW, Louise T. University of Rochester 5 RO1 CA 36200-05	Human Papillomavirus Gene Expression
19.	COLE, Charles N. Dartmouth College 2 RO1 CA 39259-04	The Molecular Biology of SV40 Large T-antigen
20.	CONRAD, Susan E. Michigan State University 5 RO1 CA 37144-05	SV40-Induced Changes of Growth Regulation in Host Cells
21.	CONSIGLI, Richard A. Kansas State University 5 RO1 CA 07139-25	Studies in Polyoma Transformed Cells: Virion Proteins
22.	CRUM, Christopher P. University of Virginia (Charlottesville) 1 RO1 CA 47676-01	Pathology of Cervical Intraepithelial Neoplasia
23.	DAS, Gokul C. University of Texas Health Center at Tyler 5 R29 CA 47611-02	Regulation of Transcription in Polyoma Virus
24.	DAWE, Clyde J. Harvard University 5 RO1 CA 38722-03	Molecular Pathology of Polyomavirus-Host Interactions

25.	DIMAIO, Daniel C. Yale University 5 RO1 CA 37157-05	Analysis of Cell Transformation by Bovine Papillomavirus
26.	DYNAN, William S. University of Colorado (Boulder) 5 RO1 CA 44958-02	Functional Organization of the BK Virus Promoter/Enhancer
27.	ECKHART, Walter Salk Institute for Biological Studies 5 R37 CA 13884-16	Viral Gene Functions and Regulation of Cell Growth
28.	FARAS, Anthony J. University of Minnesota (St. Paul) 5 RO1 CA 25462-09	Human Papillomaviruses and Malignant Disease
29.	FLUCK, Michele M. Michigan State University 2 RO1 CA 29270-07	Genome Integration and Control of Viral Gene Expression
30.	FOLK, William R. University of Texas (Austin) 5 RO1 CA 38538-05	Mammalian Cell Transformation by Oncogenic Viruses
31.	FOLK, William R. University of Texas (Austin) 5 RO1 CA 45033-02	Mechanism of Transformation by BK Virus
32.	FRISQUE, Richard J. Pennsylvania State University (University Park) 2 RO1 CA 38789-04	A Molecular Approach to the Unique Biology of JC Virus
33.	FRISQUE, Richard J. Pennsylvania State University (University Park) 5 RO1 CA 44970-02	Human Polyomaviruses: Oncogenic Potential and Mechanisms
34.	GARCEA, Robert L. Dana-Farber Cancer Institute 5 RO1 CA 37667-05	Mechanisms in Polyomavirus Assembly
35.	GREEN, Maurice St. Louis University 2 RO1 CA 28689-07	Human Papillomaviruses
36.	GURNEY, Elizabeth T. University of Utah 5 RO1 CA 21797-08	Growth Control and Viral Gene Expression
37.	HANAHAN, Douglas Cold Spring Harbor Laboratory 1 RO1 CA 47632-01	Oncogenesis by Papillomavirus DNAs in Transgenic Mice

38. HANSEN, Ulla M.
Dana-Farber Cancer Institute
5 R01 CA 38038-03
Mechanism of Initiation at RNA Polymerase II Promoters

39. HEARING, Patrick
State University of New York (Stony Brook)
5 R01 CA 44673-02
Analysis of a Polyomavirus Enhancer and Binding Protein

40. HOWETT, Mary K.
Pennsylvania State University (Hershey Medical Center)
2 R01 CA 25305-09A1
Modulation of the Tumorigenicity of Transformed Cells

41. IMPERIALE, Michael J.
University of Michigan (Ann Arbor)
5 R01 CA 19816-13
Role of SV40 Gene A in Cellular Transformation

42. KADISH, Anna S.
Mount Sinai School of Medicine
1 R01 CA 47630-01
Host Immunity to Genital Human Papillomavirus Infection

43. KELLY, Thomas J.
Johns Hopkins University
5 P01 CA 16519-14
Program on Molecular Biology of Viral Tumorigenesis

44. KELLY, Thomas J.
Johns Hopkins University
5 R01 CA 40414-04
Replication of the SV40 Genome

45. KHALILI, Kamel
Jefferson Medical College
Jefferson University
1 R29 CA 47996-01
Tissue Specific Transcription of JCV in Glial Cells

46. KREIDER, John W.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 42011-03
Human Papillomaviruses in Cervical Cancer

47. KREIDER, John W.
Pennsylvania State University (Hershey Medical Center)
1 R01 CA 47622-01
Studies on Papillomavirus Host Interaction

48. LANCASTER, Wayne D.
Georgetown University
5 R01 CA 32603-06
Role of Papillomavirus DNA in Cell Transformation

49. LANCASTER, Wayne D.
Georgetown University
5 R01 CA 32638-07
Role of Papillomavirus in Cervical Neoplasia

50.	LANCASTER, Wayne D. Georgetown University 1 R13 CA 45050-01	International Papillomavirus Workshop
51.	LANFORD, Robert E. Southwest Foundation for Biomedical Research 5 RO1 CA 39390-05	SV40 T-antigen: Model for Nuclear Transport of Proteins
52.	LEHMAN, John M. Albany Medical College of Union University 2 RO1 CA 41608-03	Pathology of Neoplastic Transformation
53.	LEVINE, Arnold J. Princeton University 5 RO1 CA 38757-04	Viral Induced Tumorigenesis
54.	LIVINGSTON, David M. Dana-Farber Cancer Institute 5 RO1 CA 15751-15	Structure and Function of SV40 Non-Virion Proteins
55.	LIVINGSTON, David M. Dana-Farber Cancer Institute 5 RO1 CA 24715-10	Isolation and Function of Small SV40 T-antigen
56.	MANLEY, James L. Columbia University 5 RO1 CA 46121-02	Mechanism of Alternative Splicing of SV40 Pre mRNA
57.	MANN, Kristine E. University of Alaska (Anchorage) 1 R15 CA 41660-01	Enzymatic Activity of SV40 Tumor Antigen
58.	MERTZ, Janet E. University of Wisconsin (Madison) 5 RO1 CA 37208-05	Involvement of T-antigen in SV40 Late Gene Expression
59.	MOUNTS, Phoebe Johns Hopkins University 5 RO1 CA 35535-06	Analysis of Papillomavirus in Laryngeal Papillomatosis
60.	MOUNTS, Phoebe Johns Hopkins University 5 RO1 CA 42089-03	Role of Human Papillomaviruses in Cervical Cancer
61.	OZER, Harvey L. Hunter College 5 RO1 CA 23002-12	Host Functions Related to Tumor Virus Infection
62.	PALLAS, David C. Dana-Farber Cancer Institute 5 R29 CA 45285-02	The Role of Cellular Proteins in Polyoma Transformation

63. PAUCHA, Eva U.
Dana-Farber Cancer Institute
5 R01 CA 42339-03
Mechanism of Transformation by SV40 Large T-antigen

64. PIPAS, James M.
University of Pittsburgh
2 R37 CA 40586-04
Genetic Analysis of the SV40 Large Tumor Antigen

65. POGO, Beatriz G.
Mount Sinai School of Medicine
5 R01 CA 29262-06
The Expression of Oncogenicity of Shope Fibroma Virus

66. POLLACK, Robert E.
Columbia University
5 R01 CA 38883-03
Tumor DNA Transformation of Diploid Cells: New Oncogenes

67. PRIVES, Carol L.
Columbia University
5 R01 CA 26905-09
Function/Expression of SV40 and Polyoma Tumor Antigens

68. PRIVES, Carol L.
Columbia University
2 P01 CA 33620-05
Directed SV40 Mutation: Cell Molecular Consequences

69. REEVES, William C.
Gorgas Memorial Institute of Tropical Medicine, Inc.
5 R01 CA 42042-03
Human Papillomavirus and Cervical Cancer in Panama

70. RICCIARDI, Robert P.
Wistar Institute of Anatomy and Biology
5 R01 CA 44960-02
Role of BKV Enhancers in Virus Regulation and Cancer

71. ROBERTS, Thomas M.
Dana-Farber Cancer Institute
2 R01 CA 30002-07
Molecular Mechanisms of Polyoma-Induced Transformation

72. RUNDELL, Mary K.
Northwestern University
5 R01 CA 21327-11
Functions of the Simian Virus 40 Small T-antigen

73. SCHAFFHAUSEN, Brian S.
Tufts University
5 R01 CA 34722-06
Products of the Transforming Genes of Polyomavirus

74. SCHOOLNIK, Gary K.
Stanford University
1 R01 CA 43871-01
Cervical Neoplasia: Detection of HPV 16 Gene Products

75. SCHWARTZ, Dennis E.
MicroProbe Corporation
1 R43 CA 45914-01
DNA Probes to Detect Human Papillomavirus in Oral Cancer

76. SHAH, Keerti V.
Johns Hopkins University
5 ROl CA 13478-15

Investigation of SV40-Related Infections of Man

77. SHAH, Keerti V.
Johns Hopkins University
5 ROl CA 42074-02

Outcome of Papillomavirus Infections of the Cervix

78. SHAH, Keerti V.
Johns Hopkins University
5 ROl CA 44962-02

Role of Polyomaviruses in Human Malignancies

79. SHENK, Thomas E.
Princeton University
5 POl CA 41086-03

Viral and Cellular Oncogenes: Mechanism of Action

80. SIMMONS, Daniel T.
University of Delaware
2 ROl CA 36118-04

Structure and Function of the SV40 Tumor Antigen

81. SMITH, Alan E.
Integrated Genetics, Inc.
5 ROl CA 43186-03

Mutagenesis of Papovavirus Transforming Proteins

82. SMITH, Janet L.
Gordon Research Conferences
1 R13 GM 39801-01

Diffraction Methods in Molecular Biology

83. SMOTKIN, David
University of Utah
1 R29 CA 47127-01

Human Papillomavirus Gene Expression in Cervical Cancer

84. SNAPKA, Robert
Ohio State University
5 R29 CA 45208-02

Aberrant Papovavirus Replication after Genotoxic Damage

85. SOMPAYRAC, Lauren M.
University of Colorado (Boulder)
5 ROl CA 34072-05

SV40 Deletion Mutants: Oncogenic Proteins

86. STEINBERG, Mark L.
City College of New York
5 ROl CA 27869-08

Oncogene Expression in SV40-Infected Keratinocytes

87. STOLER, Mark H.
University of Rochester
5 ROl CA 43629-02

Human Papillomavirus Expression in Squamous Neoplasia

88. SUBRAMANI, Suresh
University of California (San Diego)
5 ROl CA 44997-02

Mechanisms of Gene Regulation and Transformation in BK

89. SYRJANEN, Kari J.
University of Kuopio
5 R01 CA 42010-03
Natural History of Cervical HPV Infections

90. TACK, Lois C.
Salk Institute for Biological Studies
5 R01 CA 37081-06
SV40 T-antigen, Chromatin Structure and Viral Function

91. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 R01 CA 18808-14
Tumor Virus SV40: Protein Function and DNA Replication

92. TEGTMEYER, Peter J.
State University of New York (Stony Brook)
5 P01 CA 28146-08
Tumor Virus-Host Interactions

93. TEVETHIA, Mary J.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 24694-11
Mutagenesis of Specific Regions of the SV40 Genome

94. TEVETHIA, Satvir S.
Pennsylvania State University (Hershey Medical Center)
2 R37 CA 25000-11
Biology of SV40 Specific Transplantation Antigen

95. TJIAN, Robert T.
University of California (Berkeley)
5 R37 CA 25417-10
The SV40 Tumor Antigen

96. VILLARREAL, Luis P.
University of California (Irvine)
5 R01 CA 42004-03
Gene Expression of a Small DNA Tumor Virus: SV40

97. WALTER, Gernot F.
University of California (San Diego)
5 R01 CA 36111-05
SV40 and Polyomavirus Transforming Proteins

98. WATTS, Susan L.
University of North Carolina (Chapel Hill)
5 R01 CA 42085-03
Cervical Neoplasia: HPV Epidemiology and Molecular Biology

99. WEISSMAN, Sherman M.
Yale University
5 P01 CA 16038-15
Program on the Molecular Basis of Viral Transformation

100. WETTSTEIN, Felix O.
University of California (Los Angeles)
5 R37 CA 18151-13
Analysis of the Shope-Papilloma Carcinoma System

101. WETTSTEIN, Felix O.
University of California (Los Angeles)
5 R01 CA 42126-03

Human Papillomaviruses in Genital Tract Dysplasias

102. WILSON, John H.
Baylor College of Medicine
5 R01 CA 15743-15

Pathways of Information Exchange in Somatic Cells

103. WOODWORTH-GUTAI, Mary
Roswell Park Memorial Institute
5 R01 CA 28250-06

SV40 DNA Replication in Animal Cells

104. YOUNG, Donald A.
University of Rochester
1 R01 CA 47650-01

Papillomavirus Actions on Host Cell Gene Products

CONTRACTS ACTIVE DURING FY88

Investigator/Institution/Contract Number — Title

105. SCHWARTZ, Dennis E.
MicroProbe Corporation
N44-CP-81045

DNA Probes for the Diagnosis of Human Papillomavirus Types in Man

106. SMITH, Alan E.
Integrated Genetics, Inc.
N44-CP-85655

Specific Antibodies to Human and Animal Polyoma Virus Tumor Antigens

107. TAUB, Floyd
Digene, Limited
N44-CP-85652

Identification of HPV by Non-Radioactive DNA Probes

SUMMARY REPORT
RNA VIRUS STUDIES I

The RNA Virus Studies I component of the Branch primarily involves studies of murine and primate tumor viruses and also includes projects on feline and bovine tumor viruses. In this program, extramural research is supported by several funding mechanisms: traditional research grants (R01), program project grants (P01), conference grants (R13), cooperative agreements (U01), outstanding investigator awards (R35), and FIRST awards (R29). The overall effort consists of 110 grants, with a funding level of 17.11 million dollars. These grants involve studies in the murine (74%), primate (16%), feline (6%), and bovine (4%) model systems in the following areas: gene organization and expression, including studies of oncogenes; virus-cell interactions; characterization of the biological activity of retroviruses; studies of the inhibition of viral replication; investigations of virus-induced cell transformation; and development of retroviral vaccines.

Studies in the RNA Virus Studies I component are concerned with the elucidation of the molecular events associated with the viral conversion of normal cells to the malignant phenotype. Since the malignant phenotype is a stable inherited trait, oncogenic transformation may be the consequence of genetic alterations. This is clearly the case for cells transformed by oncogenic viruses, where specific viral genes are responsible for the initiation and maintenance of the neoplastic state. The question arises as to the function and identity of the genes responsible for naturally occurring tumors and the type(s) of genetic rearrangements thought to result in the aberrant activation of these genes. The observation that cellular homologs of viral oncogenes, in many instances, appear to be responsible for the in vitro conversion of normal cells in culture to the transformed phenotype, has spurred a search for the mechanisms by which these endogenous cellular genes may become altered to produce products with a potential for causing malignant transformation. The mechanisms responsible for the activation of cellular oncogenes may involve: local changes or mutations in genes involving base changes or small deletions which alter the functional properties of the gene product; gross changes in the relative position of genes which may either involve translocations of structural gene information from one chromosomal location to another, or the introduction of activators (such as viral long terminal repeats) adjacent to cellular genes such that the level of gene expression is enhanced; gene amplification mechanisms which may increase the amounts of specific gene products; or changes in the activity of oncogene promoters by changing either the base sequence itself or by altering the genome structure in the vicinity of regulatory information (e.g., through changes in the pattern of methylation, the degree of supercoiling, or other aspects of chromatin structure).

Retroviruses first attracted widespread attention as oncogenic agents that replicate via DNA intermediates and involve integration of DNA copies of their genomes in the host chromosomes. No other class of animal viruses exhibits such profound intimacy with the host genome. Thus, information gathered concerning this relationship should increase our understanding of the transformation process.

Retroviruses were previously classified into two groups: those that contain oncogenes and those that do not. Members of the first group (acute transforming

retroviruses or rapidly transforming retroviruses) induce neoplastic disease in infected animals within a few weeks after infection and cause rapid transformation of target cells in tissue culture. These viruses contain oncogenes (v-onc genes) that are derived from normal cellular genes, the proto-oncogenes, by recombination. Viruses of the second group (slowly transforming retroviruses) lack oncogenes, induce neoplastic disease in animals only after a long latent period (4-12 months), and do not cause transformation of tissue culture cells at a detectable frequency. Two additional types of retroviruses have been recognized. The first of these are viruses such as spleen focus-forming virus (SFFV) and mink cell focus-forming virus (MCF) which appear to be envelope gene recombinants. Although these viruses, in some cases, rapidly induce lesions in infected animals, they do not appear to carry an oncogene of the classic type (i.e., a cell-derived oncogene). Sequences located within the env region appear to be responsible for their pathogenic properties. Recently, another group of viruses without oncogenes, the lymphocyte-transforming retroviruses (T-cell lymphotropic viruses, HTLV), have been recognized. They apparently bring about cellular transformation through a novel transactivation mechanism involving a trans-acting protein encoded by the pX region (now termed tat gene) of the viral genome. Extramural research involving all four types of viruses is being administered by this component of the Biological Carcinogenesis Branch.

Highlights of studies on human retroviruses, retrovirus-like elements and a human retrovirus vaccine can be summarized as follows: HTLV-2, an antigenic variant of the human T-lymphotropic virus-1 (HTLV-1) has been isolated only very rarely and usually from cases of hairy cell leukemia. Further support for a causal role of HTLV-2 in human malignancy has awaited additional cases of HTLV-2 associated with human neoplasia. Recently, such evidence was obtained by the isolation of HTLV-2 from a case of promyelocytic leukemia in a 81-year old patient, an immigrant of Russian-Jewish ancestry in the New York area. Antibodies directed against HTLV-I and HTLV-2 were detected in the sera of this patient. Upon cocultivation with cultured blood lymphocytes, the virus could be isolated from leukemic cells. The identification of the isolate as subtype 2 was confirmed in the laboratory of Dr. Robert Gallo, NCI (46).

Human cells contain multiple copies of divergent sequences similar to or identical with retroviral elements and retroviral particles have been consistently observed in human tissues, such as human placenta and cell lines derived from human germ cell tumors. However, infectious, particulate retroviruses have not been isolated. Studies supported under an RFA are currently investigating the medical significance of these retrovirus particles and sequences in human cells. A c-DNA clone, presumed to contain a retrovirus sequence of 300 base pairs (bp), has been isolated from a cell line derived from a human testicular tumor. This clone is being characterized in depth. Specificity for retroviruses while circumstantial, is convincing. Viral RNA preparations having very low levels of cellular mRNA contaminants hybridized extensively with this clone. The clone hybridized with mRNA of a greater than 9 kilobase (kb) from induced human teratocarcinoma cells, but only very weakly with appropriate control materials. The clone did not cross-react with viral RNA from the baboon endogenous retrovirus. The cDNA clone hybridized to specific bands of digested proviral DNA obtained by the Hirst extraction. The sequence of the 300 bp insert is being determined. These studies may provide important leads to unravel the nature and role of these retrovirus-like sequences in human cancers (12).

The production of a HTLV-1 vaccine has been pursued in a manner similar to that described for the development of a feline leukemia virus (FeLV) vaccine, now commercially available as Leukocell TM. The HTLV-1 persistently infected cell line MT-2 was characterized as to maximum HTLV-1 antigen expression during the cell cycle by fluorescent-activated flow cytometric analysis. Characterization of HTLV-1 products released from the MT-2 cells indicated that all HTLV-1 viral components were present. Vaccine material was prepared as cell- and virus-free preparation and was concentrated 50-fold and stored at -70 C until further use. Rabbits were immunized with the HTLV-1 vaccine candidate emulsified in complete Freund's adjuvant (CFA). The humoral immune response was tested by the indirect fluorescent antibody (IFA) test, using the HTLV-1 persistently infected cell line MT-2. From this study, it was found that the anti-HTLV-1 titer of the rabbit serum increased dramatically upon repeated immunization. Western blot analysis, using purified HTLV-1 as an antigen, demonstrated that this vaccine material evoked antibodies directed to several HTLV-1-specific polypeptides, including the envelope and core components. Furthermore, the vaccine-induced antibodies inhibited syncytia formation in the MT-2(HTLV-1):XC syncytia inhibition assay. The HTLV-1 vaccine candidate has been administered to two pigtailed macaques. IFA data and Western blot analysis have demonstrated that the antisera from the immunized animals recognizes the spectrum of HTLV-1-specific polypeptides, including the core and envelope components of the virion. These studies demonstrate the feasibility of vaccination approaches for the eventual prevention of human retrovirus infections (71).

Studies of virus-host interactions and mechanisms of retroviral pathogenicity continue. Highlights of recent research are as follows. HTLV-1 and -2 are unusual among replication-competent retroviruses in that they contain a fourth gene, (x), necessary for replication. The x gene product, px, transcriptionally transactivates the viral long terminal repeat (LTR), and thus is a positive regulator. To investigate px transactivation, sequences from the U_3 regions of the LTRs of HTLV-1 and -2 were inserted into the Moloney murine leukemia virus (M-MuLV) LTR by recombinant DNA techniques. Transient expression assays of the chimeric LTRs indicated that the HTLV sequences conferred responsiveness to the HTLV px protein. M-MuLV enhancers were not required for function of the chimeric LTRs. Infectious recombinant M-MuLVs containing chimeric LTRs were also generated. These viruses showed higher infectivity when assayed in mouse cells expressing HTLV-2 px protein compared to normal mouse cells. Thus, the HTLV sequences were able to confer px responsiveness to infectious M-MuLV. These studies clearly demonstrate and confirm the important function and role of the transactivating proteins of HTLV-1 and HTLV-2 viruses in stimulating their own replication via sequences in the U_3 regions of the LTRs of these viruses (33).

Although many slowly transforming retroviruses do not encode onc gene proteins, they do cause tumors in vivo. However, none have been shown to rapidly affect the growth of cells in vitro. It was recently shown that M-MuLV, a replication competent virus that lacks an onc gene, can immortalize mouse B-cells. In this system, prior infection with Abelson murine leukemia virus (A-MuLV) or lipopolysaccharide (LPS) pre-treatment appears sufficient to allow M-MuLV to immortalize the cells. The immortalized cells are dependent on LPS for their growth and carry multiple copies of M-MuLV. The cells are similar to those isolated using A-MuLV and LPS with respect to immunoglobulin expression. Cells synthesizing both membrane and secreted forms of IgM and IgG have been isolated. This in vitro system to study the cell immortalization/transformation effects of a slowly transforming retrovirus, such as M-MuLV, should facilitate an understanding of

the viral genes responsible for oncogenicity in this group of slowly transforming retroviruses (16,83).

Recently, the importance of enhancer sequences in the viral LTR in conferring tissue tropism and/or enhancement of leukemogenicity has been recognized. In collaborative studies between two grantees in this program, the regions of mink cell focus forming virus (MCF) recombinant MuLV required for thymoma induction were determined. Three pairs of reciprocal recombinant viruses were constructed by Dr. Holland between pathogenic (MCF 247) and non-pathogenic (MCF 30-2) isolates of AKR MCF viruses in which the p15E region of the viral env gene or the viral LTR was exchanged. The experiments were designed to specifically examine viral determinants of leukemogenicity and not viral determinants of tissue tropism, since all of the viral isolates replicated equally well in thymocytes. Studies of thymoma induction following intrathymic injection of the respective viruses showed that the determinant of leukemogenicity mapped to the viral LTR. Sequence analysis showed that the LTR of MCF 247 virus contained two copies of a 109 bp sequence in tandem, each of which contained a 10 bp core enhancer sequence that differed by a single base change. The LTR of a non-pathogenic parental virus, MCF 30-2, and the LTR of the non-pathogenic molecular clone of MCF 247 (MCF 2471b) contained only one of the repeat sequences. Thus, the repeat sequences shared by all three viruses appeared sufficient to confer thymotropism to the MCF viruses, but the MCF 247-specific repeat sequence was necessary for oncogenicity. One speculation is that the dual enhancer sequence plays a critical role in activating cellular proto-oncogenes by the mechanism of insertional mutagenesis (51,107).

The envelope antigens of retroviruses control their host range. Although mouse ecotropic retroviruses multiply in mouse cells and fail to replicate in heterologous primate cells, the xenotropic mouse retroviruses preferentially replicate in heterologous host cells. The HIX strain of mouse retrovirus is an envelope recombinant virus with the dual host range of the mouse ecotropic virus as well as the mouse xenotropic virus. Serial passages of this virus in the human RD cell line further modified its host range characteristics. Thus, the interesting observation was made that RD-HIX somehow lost its dual tropism during this passage; i.e., it became xenotropic in that it would only infect heterologous species cells such as RD or mink CCL64 cells, but could no longer infect mouse cells. To understand how the gp70 interacts envelope glycoprotein with the host cell, the envelope gene of this variant of HIX recombinant virus, termed RD-HIX, was cloned and sequenced. Preliminary analyses indicated that loss of ability to infect mouse cells was due not to any major recombinational event, but to much more subtle point mutations within the envelope gene. Interesting preliminary results have shown that in the 5′ substituted portion of RD-HIX, there were only three amino acid changes relative to HIX gp70. These three changes were all within a 10 amino acid contiguous region of the molecule. More importantly, they were the changes which were also present in xenotropic viruses. Thus, it appears that the region of gp70 which dictates binding of the virus to the host cell has been identified. Further studies are in progress. These studies provide an understanding of the importance and role of particular sequences of the viral envelope in conferring host range, and thus, determining susceptibility, and pathogenicity (29).

Certain strains of leukemogenic retroviruses are able to infect the central nervous system (CNS) and thus induce hind leg paralysis in infected mice. This phenomenon was first described by Gardner et al., in murine leukemia viruses

isolated from wild mice in California. Subsequently, mutants of other murine leukemia viruses have been created which have these same properties. Since the ability to cause CNS infection is a central feature of infection with the AIDS retrovirus, detailed studies of this animal model were undertaken to define the sequences of the virus which confer paralytogenic capacity. Two regions in the viral envelope gene involved in the induction of paralysis were defined. One of these regions is responsible for the inability of the virus to process the env gene product, $Pr80^{env}$, and the other enhanced the virus' ability to invade the CNS. The DNA of these two env gene domains in the prototype paralytogenic mutant, tsl, has been sequenced and the encoded amino acid sequences have been deduced from the DNA sequence. Four alterations in the amino acid sequence which may be responsible for the tsl mutant's ability to induce paralysis have been identified. Fine recombination-mapping for the amino acid changes are being done to further delineate which of the amino acids are necessary for the neurovirulence of tsl. Giant cell (syncytia) formation in some of the nerve cell cultures infected with the tsl paralytogenic mutant have also been observed. These studies should enhance our understanding of the molecular basis of pathogenesis induced by the paralytogenic mutant and other retroviruses with similar propensity, such as the acquired immunodeficiency syndrome (AIDS) retrovirus (109).

A murine model in which neurotropic retroviral infection can be studied over short periods of time was developed. Microinjection of a neurotropic strain of a murine leukemia virus of Lake Casitas wild mice (Cas-Br-E) into midgestation mouse embryos caused paralysis and death within 25 days after birth. This was in contrast to virus-infected neonates which develop disease only after 4 months. To evaluate whether antiviral drugs could cross the placental barrier and influence the course of the disease, the drug 3'-azido-3 deoxythymidine (AZT) was administered to pregnant females. AZT treatment markedly retarded the onset and course of virus-induced central nervous system disease, permitting animals to survive beyond 4 months of age. These results are evidence for effective antiviral treatment during gestation and in the perinatal period and are of potential significance for the management of maternal transmission of the AIDS virus (56).

The major limitation to the use of retroviral vectors for embryonic gene transfer experiments is that preimplantation embryos do not permit retrovirus expression. In many respects, embryonal carcinoma (EC) cells are similar to early mouse embryo cells. The restriction of retrovirus expression in these cells is due, at least in part, to inefficient transcription from the promoter located upstream in the LTR. Despite numerous studies, the identification of the mechanism of repression remains elusive. DNA methylation occurs after provirus integration, but seems to be an effect, not a cause, of inactivation. Transient expression experiments have shown that enhancer sequences are probably involved in the repression mechanism. Competition experiments suggest that EC cells do not lack activating factors, but rather contain repressing factors. Based on these observations, investigators sought DNA-binding proteins that would specifically interact with the retrovirus DNA sequences in both nonpermissive EC cells and permissive cell lines. Any systematic difference in the binding pattern might permit the identification of factors implicated in the repression mechanism. The gel retardation assay and DNase I footprinting were used to identify sequence-specific DNA-binding proteins in crude nuclear extracts obtained from nonpermissive and permissive cells. Four binding sites were located on the murine retrovirus LTRs used in these experiments. Strong binding that occurred in the promoter region was observed specifically with EC cell nuclear extracts. It thus

appears that the inefficient viral transcription in the EC cell may be due to repressors through the observed specific binding of such proteins in EC nuclear extracts to viral sequences (91).

Studies continue on the demonstration and implication of activated oncogenes in the genesis of human tumors. The nuclear oncogene, myc has been implicated in human cancers, such as Burkitt's lymphoma and novel myc-related oncogenes termed N-myc and L-myc have been found to be activated in other human cancers, such as cancer of the lung and neuroepithelioma. Recently, a lambda phage clone containing a human genomic sequence was isolated. This clone hybridized to several probes derived from the human L-myc gene and was named R-myc. Preliminary evidence from co-transfection studies indicated that R-myc can complement activated Ha-ras in the rat embryo fibroblast transformation assay. This gene was mapped to human chromosome 13. The expression of the gene was assayed by Northern blotting analyses of RNA prepared from adult and fetal human tissues. Its expression pattern was strikingly restricted with respect to the tissue and developmental stages. Highest levels of expression were found in developing retina and spinal cord with lower levels in developing brain; no expression was readily detectable in other tissues. In studies of human tumors and cell lines, expression has only been found in a peripheral neuroepithelioma. Studies are continuing to characterize this gene with respect to structure, transforming activity, and expression in various other human tumor cells (4).

A malignant ocular tumor of children appears to be caused by two distinct genetic recessive mutations, each causing loss of function of one of the two homologous copies of a single genetic locus, Rb-1, whose function is required for the prevention of cell overgrowth. This type of oncogenesis is an example of cancer induction by anti-oncogenes. Thus, the development of this cancer is not due to the production of carcinogenic protein factors, but due to the failure of the anti-oncogene to make protective protein factors capable of preventing cell overgrowth. More recently, the Rb gene has been found to encode a 110 kilodalton phosphoprotein that is localized to the cell nucleus. Studies have shown that the Rb protein is able to complex with the proteins specified by the E1A oncogene of adenovirus. This in vitro system may serve as a model for the specific complexing of oncogenes with anti-oncogenes. Such complexes may deregulate cell growth by antagonizing the activity of growth-suppressing anti-oncogene proteins. Inactivation of analogously acting anti-oncogenes may figure largely in the genesis of a number of other tumors, among them commonly occurring tumors, such as colon and lung carcinomas (105).

The Li-Fraumeni cancer family syndrome is manifested in a variety of neoplasms which are transmitted in a dominantly inherited pattern. In experiments to understand the molecular basis of this syndrome, the non-cancerous skin fibroblasts (NCSF) of members of one such cancer prone family were found to exhibit the unique characteristic of being resistant to the killing effects of ionizing radiation (resistant phenotype RR). A three to eightfold elevation of the expression of c-myc was detected in NCSF of several affected family members, but not those of the normal controls. Northern hybridization with other oncogene probes (Ha-ras, Ki-ras, N-ras, sis, erb-B, myb, fos, fes, raf) showed no abnormal levels of expression of these other oncogenes. Interestingly, the NCSF line with the highest expression of c-myc also exhibited the most prominent RR phenotype. High molecular weight (HMW) DNA from these NCSFs could transform NIH 3T3 cells; these transformed cells produced tumors when inoculated into nude mice. The HMW DNA of the human Alu positive mouse primary tumors was used in a second round of

transfection. DNAs from mouse secondary and tertiary tumors shared the same human Alu restriction pattern. Southern blot analysis of these DNAs showed no homology between the human transforming sequences and Ha-ras, Ki-ras, N-ras, sis, src, fos or c-myc oncogenes. However, significant homology was detected between these sequences and the raf oncogene. A single cell clonal line derived from one such tertiary tumor was found to display a high level of RR phenotype relative to the recipient NIH 3T3 cells. This level is comparable to that of the parental NCSF line from which the donor DNA was obtained. These data suggest that the RR phenotype has been transferred in parallel with the human raf sequence to the relatively radiation-sensitive NIH 3T3 cells. These findings also suggest that the inborn defect predisposing to diverse cancer in this cancer-prone family is expressed by at least two abnormalities--elevated c-myc expression and activated raf gene in NCSF of family members. The mechanistic relationship, if any, between these abnormalities remains to be established (14).

The Philadelphia chromosome, a result of reciprocal translocation of distal segments of autosomes 9 and 22, is found in greater than 90% of cases of human chronic myelogenous leukemia (CML). The translocation juxtaposes c-abl proto-oncogene sequences on chromosome 9 with a gene of unknown function, denoted bcr, on chromosome 22. A novel hybrid messenger RNA transcribed from this locus directs the translation of a 210 kD phosphoprotein (P210) carrying both bcr and abl antigenic determinants. The P210 bcr/abl protein resembles the v-abl oncogene product of Abelson murine leukemia virus (A-MuLV) in its high tyrosine-specific protein kinase activity. In addition, the P210 and v-abl proteins involve replacements of N-terminal c-abl sequences with bcr and viral gag sequences, respectively. To examine whether the CML-specific bcr/abl protein functions like the gag/v-abl protein of A-MuLV to transform fibroblasts in vitro, various constructs which encode P210 bcr/abl were tested. These constructs did not transform fibroblasts, suggesting that bcr/abl functions differently from the v-abl oncogene product (5,108).

Oncogenesis, in general, appears to be a multi-step process that most likely involves the activation of several oncogenes. The expression of a number of oncogenes, such as int-1, int-2, myc and ras have been shown to be involved in the development of mammary tumors in mice. Furthermore, both myc and ras have been strongly implicated in human mammary tumorigenesis. However, very little is known about the mechanism by which oncogenes act on mammary cells. In addition, it is not known whether or not any other oncogenes play a role in murine mammary tumorigenesis. As an initial step for elucidating how oncogenes participate in the transformation process of normal mammary cells into cancer cells, three carcinoma-associated oncogenes, v-myc, v-Ha-ras and v-mil were introduced into a mouse mammary epithelial cell line, EF43, and the effect of their expression on a number of parameters related to cellular transformation as well as to the ability of the cells to grow in 3-dimensional collagen matrices were determined. Results showed that the expression of v-myc in EF43 cells resulted in no obvious phenotypic changes and did not confer tumorigenic potential upon the cells. By contrast, the expression of v-Ha-ras conferred upon EF43 cells the ability to grow rapidly in tissue culture in an anchorage-independent manner and the ability to cause tumor formation in nude and syngeneic animals. Moreover, it abolished their ability to repopulate the mammary gland and instead resulted in the rapid induction of anaplastic tumors. The v-mil oncogene, like v-Ha-ras, was also found to transform E43 cells, allowing them to grow in an anchorage-independent manner, form tumors in nude mice, and abolished their ability to repopulate the cleared mammary fat pad. However, unlike v-Ha-ras, the tumors arising from the

expression of v-mil showed a differentiated morphology typical of adenocarcinomas. Thus, it appears that different oncogenes show varying degrees of inhibition of the differentiation of mammary epithelial cells in vivo. Here observation should help to develop hypothesis for possible mechanisms of carcinogenesis in the mouse mammary tumor (85).

A role for proto-oncogenes in the regulation and modulation of cell proliferation has been suggested by the findings that the B-chain of platelet-derived growth factor (PDGF) is encoded by the proto-oncogene, sis, and that the erb-B oncogene product is a truncated form of the epidermal growth factor (EGF) receptor. Furthermore, the product of the oncogene fms and proto-oncogene c-fms may be related or identical to the receptor for macrophage colony-stimulating factor (CSF-1). v-fms is the transforming gene of the Sarma-McDonough strain of feline sarcoma virus (SM-FeSV) and belongs to the family of src-related oncogenes which have tyrosine-specific kinase activity. Furthermore, nucleotide sequence analysis of the v-fms gene product revealed topological properties of a cell-surface receptor protein. To elucidate the features involved in the conversion of a normal cell-surface receptor gene into an oncogenic one, the complete nucleotide sequence of a human c-fms complementary DNA was determined. The 972-amino-acid c-fms protein has an extracellular domain, a membrane-spanning region, and a cytoplasmic tyrosine protein kinase domain. Comparison of the feline v-fms and human c-fms sequences revealed that the proteins share extensive homology, but have different carboxyl termini (99).

More recently, another grantee has shown that the v-kit oncogene of the HZ-4 feline sarcoma virus is similar to the fms oncogene and, thus, also codes for a cell receptor oncogene. The sequences of v-kit have been determined and compared to the sequences of v-fms and CSF-1 receptor gene. The nucleotide sequence of the c-kit cDNA predicts a 982 amino acid protein product with a calculated molecular weight of 110,000 daltons. It contains an N-terminal signal peptide, a transmembrane domain and the sequence homolog to v-kit, the C-terminal half of the molecule. c-kit, therefore, contains the features characteristic of a transmembrane receptor kinase. Comparison of c-kit, CSF-1R and PDGFR (platelet derived growth factor receptor) revealed a unique structural relationship of these receptor kinases, suggesting a common evolutionary origin. The sites of expression of c-kit in normal tissues predict a function in the brain, testis and in hematopoietic cells. N-terminal sequences which include the extracellular domain and the transmembrane domain as well as 78 amino acids from the c-terminus of c-kit are deleted in v-kit. These structural alterations are likely determinants of the oncogenic activity of v-kit (9,50).

Transgenic mice provide unusual opportunities to study the effects of isolated genes and combinations of genes in the life cycle of the animal, effects which can be extrapolated to the natural functions of these genes in other intact animals, including man. When virus carrying the human ras gene was microinjected into embryos at day 8 of gestation, epithelial tumors with a long latent period developed in about 15% of the animals. In contrast, infection with a myc oncogene transducing virus did not result in any recognizable phenotype. When embryos were exposed to a virus carrying both the myc and the ras genes, tumors developed in many organs with a short latency period. These results corroborate the in vitro experiments of Land and Weinberg, suggesting that ras and myc oncogenes can cooperate in vivo to elicit a malignant phenotype (both in terms of the rapidity of the tumor formation and the broad spectrum of tissue types involved). The results also suggest that ras alone has a greater ability to form

tumors in the skin than in other tissues as the tropism of the virus changed upon the addition of the myc oncogene. Perhaps the high level of endogenous myc expression known to exist in skin contributes to this phenomenon. Further analysis is necessary to elucidate this observation as well as the observation that the virus carrying the ras and myc oncogenes caused a higher frequency of lesions in the brain than in any other tissue (56).

Important observations on the use of retroviral vectors are described below. Retroviruses are efficient vehicles for the transfer of genes into mammalian cells. Suitably engineered retrovirus vectors are being employed in a number of studies, including attempts to correct gene defects through gene therapy. Recently, retrovirus-mediated gene transfer was used to introduce a recombinant human growth hormone gene into cultured human keratinocytes. The transduced keratinocytes secreted biologically active growth hormone into the culture medium. When grafted as an epithelial sheet into athymic mice, these cultured keratinocytes reconstituted an epidermis that was similar in appearance to that resulting from normal cell engraftment, but from which human growth hormone could be extracted. Transduced epidermal cells applied as grafts may prove to be a general method for the delivery of gene products (5).

Retroviruses containing human factor IX sequences have been generated and these vectors have been used to infect mouse fibroblasts. The infected fibroblasts were embedded in a collagen matrix and implanted under the epidermis. Serum from implanted animals was monitored for the production of human factor IX. Human factor IX could be detected in the mouse serum for only 10-12 days following implantation. The disappearance of factor IX was concomitant with the appearance of antibodies to human factor IX. These results indicated that gene transfer technology works, that factor IX is secreted and that the animal mounts an immune response. Experiments are in progress to generate a dog factor IX cDNA in order to undertake studies on hemophiliac dogs. Thus, general conditions have been established for working with hematopoietic stem cells and with somatic cells as models of gene therapy (101).

One workshop was co-sponsored by the RNA I and AIDS Virus Studies components of the Biological Carcinogenesis Branch. This workshop, entitled "AIDS: Progress and Future Directions for Vaccine Development" was held at the NIH on October 5, 1987.

Thus, grants in the RNA I component have focused on elucidating the biology and diverse characteristics of mammalian retroviruses and on their interactions with their host cells resulting in transformation of normal cells to the malignant phenotype. The authenticity of HTLV-2 as a human oncogenic virus was confirmed by a repeat isolation from a patient with lymphoma. A model vaccine against HTLV-1, prepared using the approach for a commercially available FeLV vaccine, was developed and tested in animals. A murine model with a short latency period for the neurotropic retroviral infection was developed. The anti-oncogene of the human retinoblastoma was characterized and its product found to be a nuclear phosphoprotein of 110 kD size. Techniques were developed for gene therapy through the introduction and expression of foreign genes into mammalian cells via retroviral vectors. Recombinant DNA techniques were exploited for defining the cellular and/or viral sequences involved in conferring viral tissue tropism, in determining the type of cancer induced, and in conferring the ability of viruses to replicate and induce cell transformation. Tissue specificity and oncogenesis

were studied using deletion mutants and recombinants. These studies thus continue to unravel the participation of viral and cellular genes in the genesis of cancer and provide insights on how the eventual control of these diseases might be achieved.

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ALT, Frederick W. Gordon Research Conferences 1 R13 AI 26870-01	Gordon Conference on Animals Cells and Viruses
2. ARLINGHAUS, Ralph B. University of Texas System Cancer Center 5 R01 CA 45125-03	Moloney Murine Sarcoma V-mos Proteins in Cellular Transformation
3. ARLINGHAUS, Ralph B. University of Texas System Cancer Center 5 R01 CA 45217-02	A Temperature-Sensitive Retrovirus Splicing Mutant
4. AXEL, Richard Columbia University (New York) 2 P01 CA 23767-10	Molecular Virology
5. BALTIMORE, David Whitehead Institute for Biomedical Research 5 P01 CA 38497-04	Interactions of Oncogenes With Developing Systems
6. BARKLIS, Eric W. Oregon Health Sciences University 1 R01 CA 47088-01	Targeting of Retroviruses to Specific Cell Types
7. BARON, Samuel University of Texas Medical Branch (Galveston) 5 U01 CA 40764-03	Host Defenses Against HTLV-1 and 2
8. BEDIGIAN, Hendrick G. Jackson Laboratory (Bar Harbor, ME) 5 R01 CA 31102-08	A New Murine Model for the Study of Myeloid Leukemia
9. BESMER, Peter Sloan-Kettering Institute for Cancer Research 2 R01 CA 32926-04	C-KIT & V-KIT: Normal Function and Oncogenic Activation

10.	BHARGAVA, Pushpa M. Centre for Cellular and Molecular Biology (Hyderabad, India) 1 R13 CA 46600-01	Symposium on Frontiers of Tomorrow in Biology
11.	BOLANDER, Franklyn F., Jr. University of South Carolina (Columbia) 5 R01 CA 42009-02	MMTV Regulation in Normal Mouse Mammary Epithelium
12.	BRONSON, David L. Southwest Foundation for Biomedical Research 5 R01 CA 43361-03	Novel Human Retrovirus
13.	BROWN, John M. Stanford University 5 R01 CA 03352-32	Biological Aspects of Carcinogenesis by Radiation
14.	CHANG, Esther H. Henry M. Jackson Foundation (Bethesda, Maryland) 5 R01 CA 45158-03	Oncogenes in Human Cancer Induction
15.	CHEN, Irvin S. Y. University of California (Los Angeles) 2 R37 CA 38597-04	A Molecular Genetic Study of Human T-Cell Leukemia Virus
16.	COFFIN, John M. New England Medical Center Hospitals, Inc. 2 P01 CA 24530-09	Molecular Genetics of Cancer
17.	COMPANS, Richard W. University of Alabama (Birmingham) 5 R01 CA 18611-14	Directional Transport of MuLV Glycoproteins
18.	COOPER, Geoffrey M. Dana-Farber Cancer Institute 2 R01 CA 18689-13	Infectious DNA for Endogenous RNA Tumor Virus Genes
19.	CUNNINGHAM, James M. Brigham and Women's Hospital 1 R29 CA 47075-01	Isolation and Analysis of Murine Leukemia Virus Receptor
20.	DARNELL, James E., Jr. Rockefeller University 5 P01 CA 18213-13	Correlated Program in Viral Oncology

21. DE FRANCO, Donald B.
University of Pittsburgh
5 R01 CA 43037-03

Glucocorticoid Regulation of Retroviral Transcription

22. DESROSIERS, Ronald C.
Harvard University
5 U01 CA 40680-03

T-Cell Lymphotropic Viruses of Macaques

23. DIAMOND, Leila
New York Academy of Sciences
1 R13 CA 48708-01

Viral Oncogenesis and Cell Differentiation: The Contribution of Charlotte Friend

24. DONEHOWER, Lawrence A.
Baylor College of Medicine
5 R01 CA 41476-03

Role of Virus and Cell Genes in Retrovirus Replication

25. DONOGHUE, Daniel J.
University of California (San Diego)
5 R01 CA 34456-05

Expression of Retroviral Envelope Gene Fusion Proteins

26. DUDLEY, Jacquelin P.
University of Texas (Austin)
2 R01 CA 34780-04

Regulation of MMTV in T-Cell Tumors

27. ECKNER, Robert J.
University of Massachusetts Medical School
5 R01 CA 39689-03

Biological and Physical Properties of Friend Virus

28. ELDER, John H.
Scripps Clinic and Research Foundation
5 R01 CA 25533-08

Structural Studies of Recombinant Retrovirus gp70s

29. ELDER, John H.
Scripps Clinic and Research Foundation
5 R01 CA 37830-03

Role of Recombinant Retroviruses in Murine Leukemia

30. ETKIND, Polly R.
Montefiore Medical Center
5 R01 CA 45583-02

Molecular Pathology of Breast Cancer

31. FAMULARI, Nancy G.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 36162-03

Stage Specific Events in Viral Leukemogenesis

32. FAN, Hung Y.
University of California (Irvine)
5 R01 CA 32454-08

Studies of Integrated Murine Leukemia Virus DNA

33. FAN, Hung Y.
University of California (Irvine)
5 R01 CA 32455-08

Expression and Pathogenesis of Murine Leukemia Virus

34. FAN, Hung Y.
University of California (Irvine)
1 R13 CA 47737-01

Workshop on Pathogenesis by Non-acute Retroviruses

35. FARAS, Anthony J.
University of Minnesota at Minneapolis (St. Paul)
1 R01 CA 43472-01

Studies on Novel Human Endogenous Retroviruses

36. FERRER, Jorge F.
University of Pennsylvania
5 U01 CA 40688-03

Regulation of BLV Infection and Leukemogenesis

37. FLYER, David C.
Pennsylvania State University
Hershey Medical Center
2 R01 CA 44633-03

Specificity of the CTL Response to Murine Leukemia Virus

38. FOX, C. Fred
University of California (Los Angeles)
1 R13 AI 26042-01

Conference on Cell Biology of Virus Entry

39. GASPER, Peter W.
Colorado State University
1 R29 CA 46371-01

Marrow Transplant Therapy for Retrovirus Infections

40. GATTONI-CELLI, Sebastiano
Massachusetts General Hospital
5 R01 CA 43499-03

Human Endogenous Retroviruses in Colon Cancer

41. GEIB, Roy W.
Indiana University School of Medicine
1 R29 CA 47944-01

Analysis of a "Friend Virus-like" Disease in Fv-2^{rr} Mice

42. GOFF, Stephen P.
Columbia University (New York)
5 R01 CA 30488-08

Construction and Analysis of Retrovirus Mutants

43. GOLDSCHNEIDER, Irving
University of Connecticut Health Center
5 R01 CA 38762-03

Cellular Targets of Leukemic Transformation

44. GUPTA, Phalguni
University of Pittsburgh (Pittsburgh)
5 U01 CA 42732-03

Mechanism of Action of a Nonantibody BLV Blocking Protein

45. HAAS, Martin
University of California
(San Diego)
2 RO1 CA 34151-07

Viral Malignant Lymphomagenesis in X-Irradiated Mice

46. HAAS, Martin
University of California
(San Diego)
5 RO1 CA 42432-02

Studies of an African Population Endemic for HTLV

47. HASELTINE, William A.
Dana-Farber Cancer Institute
5 RO1 CA 19341-11

The Molecular Biology of Replication RNA Tumor Viruses

48. HASELTINE, William A.
Dana-Farber Cancer Institute
5 RO1 CA 36974-05

Study of pX Region of HTLV 1 and 2

49. HAYS, Esther F.
University of California
(Los Angeles)
2 RO1 CA 12386-14

Development of Lymphoma in the Thymus

50. HAYWARD, William S.
Sloan-Kettering Institute
for Cancer Research
5 PO1 CA 16599-14

Hematopoietic Cell Transformation by Retroviruses

51. HOLLAND, Christie A.
University of Massachusetts
Medical School
5 RO1 CA 41510-03

Determinants of the Oncogenic Potential of MCF Viruses

52. HOOVER, Edward A.
Colorado State University
1 RO1 CA 48594-01

Mechanisms of Retrovirus Induced Aplastic Anemia

53. HOPKINS, Nancy H.
Massachusetts Institute of
Technology
5 RO1 CA 19308-12

Studies on Endogenous and Other C-Type Viruses of Mice

54. HUNTER, Anthony R.
Salk Institute for Biological
Studies
5 R35 CA 39780-04

Role of Protein Phosphorylation in Growth Control

55. HUNTER, Eric
University of Alabama
(Birmingham)
5 RO1 CA 27834-08

Genetics of Primate "D" Type Retroviruses

56. JAENISCH, Rudolf
Whitehead Institute for
Biomedical Research
5 R35 CA 44339-02

Retroviruses, Oncogenes and
Mammalian Development

57. KABAT, David
Oregon Health Sciences University
5 R01 CA 25810-10

Leukemogenic Membrane Glyco-
proteins: gp55s of SFFVs

58. KLINGER, Harold P.
Yeshiva University
5 R01 CA43951-02

Molecular Cloning of
Retroviral Receptor Genes

59. LENZ, John R.
Yeshiva University
5 R01 CA 44822-02

Leukemogenesis by Murine
Retroviruses

60. LERNER, Richard A.
Scripps Clinic and Research
Foundation
5 P01 CA 27489-09

Consequences of Endogenous
Retroviral Expression

61. LICHTMAN, Andrew H.
Brigham and Women's Hospital
5 R29 CA 43651-02

In Vitro Models of Viral
Leukemogenesis

62. LILLY, Frank
Yeshiva University
5 R01 CA 19931-11

Mechanisms of the H-2 Effect on
Viral Leukemogenesis

63. LUFTIG, Ronald B.
Louisiana State University
Medical Center (New Orleans)
5 R01 CA 37380-06

Assembly of Murine Leukemia
Viruses

64. McGRATH, Charles M.
Oakland University
(Rochester, Michigan)
5 R01 CA 44901-03

Endogenous Virus and Hormones
in Mammary Cancer

65. MERUELO, Daniel
New York University
5 R01 CA 22247-11

Genetics of Resistance to
Leukemia

66. MERUELO, Daniel
New York University
5 R01 CA 31346-06

Study of MuLV Sequences in the
MHC: Cloning of Minor H Genes

67. MULLINS, James I.
Harvard University
5 U01 CA 40646-03

Viral Determinants of HTLV-1
Leukemogenesis

68. MURPHY, Edwin C., Jr.
University of Texas System Cancer Center
5 R01 CA 34734-06

MuSV Ts110: Thermosensitive RNA Splicing in Intact Cells

69. O'DONNELL, Paul V.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 31491-07

Kinetic Study of Virus-Accelerated Leukemia

70. OLSEN, Richard G.
Ohio State University
5 R01 CA 30338-06

FeLV Leukemogenesis and Pre-neoplastic Lesions

71. OLSEN, Richard G.
Ohio State University
5 U01 CA 40714-03

Immunoprevention of HTLV Infection

72. PALKER, Thomas J.
Duke University
5 U01 CA 40660-03

Monoclonal Antibodies to HTLV-1 and HTLV-2

73. PAULEY, Robert J.
Michigan Cancer Foundation
7 R01 CA 28999-06

Mammary Neoplasia and the Murine Mammary Tumor Virus

74. PETERSON, David O.
Texas A and M University (College Station)
5 R01 CA 32695-06

Genetic and Molecular Analysis of Steroid Responsiveness

75. PETERSON, David O.
Texas A and M University (College Station)
1 R01 CA 48041-01

Mechanisms of Steroid Hormone-Regulated Transcription

76. PINTER, Abraham
Public Health Research Institute of the City of New York
2 R01 CA 42129-03

Biochemical and Genetic Studies of MuLV Envelope Proteins

77. POGO, Beatriz G. T.
Mount Sinai School of Medicine
5 R01 CA 10000-21

Filterable Agents and Tumor Induction in Mice

78. RACEVSKIS, Janis
Montefiore Medical Center (Bronx, NY)
2 R01 CA 43864-03

MMTV Gene Products and Transformation

79. RADKE, Kathryn
University of California (Davis)
5 U01 CA 40653-03

Cellular Transformation by Bovine Leukemia Virus

80. RADKE, Kathryn
University of California (Davis)
1 R01 CA 46374-01

Target Cell Specificity of Bovine Leukemia Virus

81. RISSER, Rex G.
University of Wisconsin (Madison)
5 R01 CA 41302-03

Biological and Molecular Studies of A-MuLV Tumorigenesis

82. ROSENBERG, Naomi E.
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SUMMARY REPORT

RNA VIRUS STUDIES II

The RNA Virus Studies II component of the Branch primarily involves studies of the avian tumor viruses and hepatitis B virus. This program consists of 101 research grants with an estimated total funding of 20.5 million dollars for FY88. Of these, approximately 83% are involved with studies of avian tumor viruses and 14% concern hepatitis B virus or other hepatitis viruses and their relationship to primary hepatocellular carcinoma. The remaining 3% deal with a variety of agents which are not as closely related to human diseases. The majority of studies funded by RNA Virus Studies II are focused on the molecular nature of the transformation process, the definition and discovery of new oncogenes (genes responsible for the transformation of cells from normal to malignant), and the development and testing of hypotheses about the mechanism(s) of oncogenesis of viruses lacking oncogenes. In addition to 78 traditional R01 grants and 4 P01 program project grants, this component now includes 9 R35 outstanding investigator awards, 2 R37 method to extend research in time (MERIT) awards as well as 2 R13 conference grants, 1 R43 small business innovative research (SBIR) grant, and 5 R29 first independent research support and transition (FIRST) awards.

The elucidation of the mechanism(s) by which protein products of viral oncogenes (v-oncs) initiate and maintain the transformed state, and how their cellular counterparts (c-oncs) or proto-oncogenes acquire transforming potential are key issues in studies of viral oncogenesis and are being actively pursued by investigators in this program component. While less is known about the functions of oncogene products than about the structure of the oncogenes themselves, knowledge of the proto-oncogene functions in the normal cell and their possible modification in the malignant cell is essential to an understanding of virus-cell interactions leading to the transformed cell phenotype. As the techniques for identifying and isolating oncogene products develop further, progress in the search for the essential transforming functions of oncogene products will also be made.

Retroviral onc genes (v-oncs) are derived from the genome of vertebrate cells. The protein products of the normal cellular proto-oncogenes have important regulatory functions, acting on cell growth and cell differentiation. Homologies exist between various cellular regulatory proteins and v-onc proteins. The protein encoded by the oncogene of the simian sarcoma virus, v-sis, is homologous to the platelet derived growth factor (PDGF). Several retroviral onc proteins are related to growth factor receptors, including the proteins encoded by erb-B, fms, kit, ros, and possibly the newly discovered oncogene, sea. The erb-B protein has close homology to the cellular receptor for epidermal growth factor. The fms protein shows a structural homology with colony stimulating growth factor. The protein products of the kit and ros oncogenes show structural and functional features characteristic of a receptor protein: they are integral plasma membrane proteins and have tyrosine-specific kinase activity. Their binding proteins are as yet unknown. The ras proteins appear to correspond to GTP binding g-proteins that act as cellular transducers. The src protein may intervene at the level of an amplifier by effecting the generation of second messengers. Fps, mos, or raf could be homologs of intracytoplasmic effectors, typically protein kinases. Proteins that bind to DNA and may regulate transcription and replication are the products of myc, myb, fos, and ski. The functions of the corresponding cellular proto-oncogene proteins have not yet been identified.

These observations about the origins and functions of onc genes illustrate two points: onc genes are components of growth regulatory networks, and onc proteins interact with each other. As a first approximation, one can define the function of an onc protein by naming its substrates. Many of these substrates will, themselves, be onc proteins or potential onc proteins (e.g., growth factor and its receptor). Part of the problem in defining the mechanism(s) of action of retroviral onc proteins is that those onc genes coding for the relevant interacting substrates have not yet been discovered. Viewed in this context, both the characterization of existing onc genes and the search for new onc genes are clearly important endeavors. It is necessary to define all of the individual components of cellular growth regulatory networks in order to understand their particular function(s) and their interactions as a composite system. In the following discussion, investigations on the properties of several recently discovered oncogenes will be presented.

A number of oncogenes have been isolated from acutely transforming retroviruses. To date, the products of these viral oncogenes have been protein kinases, nuclear proteins, growth factors, or GTP-binding proteins. Hanafusa and his colleagues have recently cloned and sequenced the genome of the previously uncharacterized avian sarcoma virus, CT10. When ^{32}P-labeled complementary DNA made from partially purified CT10 virion RNA was used as a probe, no hybridization was observed with a battery of 19 DNA fragments isolated from molecularly cloned viral oncogenes. This suggested that CT10 contained a novel oncogene. This viral oncogene was found to encode a protein, p47$^{gag\text{-}crk}$, in which the carboxyl half is derived from a cellular proto-oncogene, c-crk (CT10 regulator of kinase). Within the v-crk sequence are two blocks of homology, each consisting of about 50 amino acids, to the N-terminal, non-catalytic domain of p60$^{c\text{-}src}$. In addition, the structure of the p47$^{gag\text{-}crk}$ has a striking similarity to a 180 amino acid region of bovine brain phospholipase C. Since the protein product of this newly discovered oncogene had no homology to the catalytic domain of any known protein kinases, and infection with the parent virus caused increases in the intracellular phosphotyrosine levels, the v-crk gene product must be regulating, directly or indirectly, the activity of endogenous protein kinases. Thus, crk appears to be a new type of oncogene (35).

Last year, Peter Vogt and his colleagues described the isolation of two new onc genes. The first of these oncogenes was called sea, an acronym formed from sarcoma, erythroblastosis, anemia. This oncogene was isolated from the avian erythroleukemia virus S13, which was isolated more than 50 years ago. The virus caused a broad spectrum of tumors in chickens, and induced oncogenic transformation of chick embryo fibroblasts to fusiform cells that were capable of anchorage-independent growth. In further studies of the v-sea oncogene, a temperature-sensitive mutant (ts1 S13) of the S13 virus was isolated and characterized. The temperature-sensitive lesion of the ts1 mutant was found to affect the tyrosine kinase activity, but not the plasma membrane localization of the ts1 S13 v-sea gene product. Erythroblasts transformed by the ts1 mutant could be induced to synchronously differentiate into erythrocytes in an erythropoietin-dependent fashion. An analysis of erythrocyte-specific gene expression in the ts1 S13 erythroblasts revealed that the transformed, self-renewing erythroblasts obtained at the permissive temperature already expressed all of the erythrocyte genes tested for, although at low levels. When differentiation was induced, expression of erythrocyte-specific genes was found not to be coordinately regulated, but to involve complex regulatory mechanisms which appeared to be specific for the

individual genes. These findings provide not only a model system for studying the complex problems of gene expression during erythroid differentiation, but also provide a fruitful system for exploring mechanisms of viral oncogenesis.

In addition, this group isolated a second new oncogene from avian sarcoma virus (ASV) 17. The insert was named jun, an abbreviation from the Japanese ju-nana (=17). The protein product of the jun oncogene is not related to any other known oncogenes, and it is known that jun is cell derived and occurs in diverse vertebrate species. The human c-jun proto-oncogene has recently been localized to the short arm of chromosome 1, at 1p31-32. The most interesting properties of this new oncogene are the structural similarities of its protein product to those of the DNA-binding end of the yeast GCN4 protein, a transcriptional regulator of amino acid synthesis, and the activator protein-1 (AP-1) of HeLa cells. The latter two proteins have well-established functions in regulating the transcription of genes. While transcriptional regulation is relatively common in yeast and mammalian cells, there has been only suggestive evidence to date that it could occur in the viral transformation of cells. The evidence that the cellular progenitor of the v-jun oncogene has a protein product identical to the AP-1 protein provides the strongest basis to date for the hypothesis that transcriptional factors can function directly as transforming proteins. This observation should stimulate efforts to find similar functions in other oncogenes and proto-oncogenes, and to determine if other genes encoding transcriptional factors are proto-oncogenes. The overall significance of these findings is the establishment of transcriptional control elements as direct mediators of oncogenic change (94).

Mike Bishop's group has used molecular cloning to isolate a previously unrecognized human gene that encodes a protein-tyrosine kinase. Expression of the gene is most prominent in lymphoid and myeloid hemopoietic cells and increases as the myeloid cells differentiate to either granulocytes or macrophages. Therefore, the gene was designated as hck ("hemopoietic cell kinase"). The sequence of hck indicates that the gene is related to src and its kin, particularly the gene lck which is expressed primarily in lymphoid cells. These findings strengthen previous indications that there may be a sub-family of protein-tyrosine kinases that are specialized for function in hemopoietic cells (6).

The Varmus group has also made progress in studies of oncogenes. They have extended biological characterization of several mutants of v-src with deletions, duplications, and substitutions in the amino-terminal half of the v-src protein. The highly variable region seems dispensable for transformation, but even a single amino acid deletion in a highly conserved sequence near to, but outside, the kinase domain has a major effect upon function. They have also shown that the seventh amino acid is important for myristylation--lysine or arginine in this position results in a functional protein, but asparagine at position 7 results in a non-functional protein. A large group of non-conditional, kinase-negative mutants are being tested for other interesting lesions in src by cloning, mapping and sequencing.

In addition, they have developed a system in rat cells infected with specially designed vectors that allows isolation of host cell mutants that induce the transforming activity of non-transforming src alleles. Candidate mutants that result in transformation of cells expressing high levels of c-src are now being screened to eliminate intragenic and src-independent mutants.

In studies of the biological activity of the int-1 proto-oncogene, Varmus has found that an established mammary epithelial cell line (C57MG) undergoes morphological transformation and change in growth properties when infected by murine leukemia virus (MLV) vectors carrying int-1. Although several other cell types (e.g., 3T3 or HeLa cells) do not show such effects, when these cells express an exogenous int-1 gene they can induce transformation of adjacent C57MG cells, suggesting a paracrine effect of this oncogene. Introduction of the gene into primary mammary epithelial cells induces no overt effects in culture, but they are currently examining the behavior of such cells when returned to the cleared fat pad of syngeneic animals. Expression of int-1 is extraordinarily restricted, with RNA detectable only in mid-gestational embryos and testis. Moreover, embryonic expression is confined to the central nervous systems (all components save the telencephalon) and only testicular cells with int-1 RNA are post-meiotic, with highest levels in round spermatids. These findings, strongly indicative of specialized developmental roles for this proto-oncogene, are particularly interesting in view of R. Nusse's recent demonstration that the Drosophila homologue of int-1 encodes the wingless phenotype (93).

Other studies from Bishop's group have now isolated five genes from Drosophila that encode protein-tyrosine kinases and have approximate counterparts in mammalian cells (src, erb-b, fps, fgr and abl). The protein product of Drosophila src has been identified--it is a protein-tyrosine kinase, as expected, with properties remarkably similar to those of the enzyme encoded by mammalian src. Immunocytochemistry has substantiated the previous view that expression of src is limited primarily to neural tissues, and has shown that the gene product concentrates in nerve fibers rather than in cell bodies. These findings help focus the search for the physiological function(s) of src. The search for mutants in the src and myb genes of Drosophila continues--results to date suggest that src may not be an essential gene, which agrees with the hypothesis that the gene functions primarily in terminally differentiated cells.

In a continuation of studies of the myc oncogene begun last year in collaboration with David Levens, Bishop's group has now detected a cellular protein that binds with great specificity to a defined nucleotide sequence within the repressor element described last year. It is likely that this element figures in the modulation of myc expression described in neoplastic and differentiating cells. During the differentiation of mouse erythroleukemia cells, the expression of myc is rapidly repressed, returns for a few hours, and then is permanently repressed. The first repression is achieved at the level of transcription; the second by changing the stability of mRNA. Repression of transcription is mediated by attenuation within the first exon of c-myc, at a nucleotide sequence similar to that found in termination regions at the ends of genes. Expression of the beta-globulin gene has been found to be regulated by a similar form of attenuation within an intron.

In investigations of the products and function of myc proteins, it has been found that when carried in MuLV vectors, the biological potency of c-myc differs from that of v-myc in only two regards-transformation of primary rat embryo fibroblasts by c-myc is extremely weak, and established rodent cells transformed by c-myc are less tumorigenic than cells transformed by v-myc. These subtle differences can now be ascribed to the combined effects of several point mutations carried in the allele of v-myc (MV29) employed by the Bishop laboratory. These findings dramatize the versatility of c-myc as an oncogene, and challenge the view that this gene is limited to an "establishment" function during tumorigenesis. The products of c-myc and v-myc co-localize with the antigens of small

nuclear ribonucleoproteins (snRNPs) when studied in either normal or transformed cells by immunocytochemistry. Efforts to find myc proteins in snRNPs by biochemical means have failed, however, and uncertainty remains about the immunocytochemical findings. In collaboration with W. Lee and H. Varmus, at least two domains of c-myc protein have been identified that contribute to localization of the gene product in the nucleus. Two domains of the protein (one aminoterminal, the other carboxyl terminal) are required in concert for neoplastic transformation by c-myc. Remarkably, more than half of the protein can be re-moved without affecting the biological activity. Not all assays for trans-formation give the same result. Thus, some domains of the protein are required for either transformation of established cell lines or cooperative transformation with ras, but not for both of these biological activities (6,93).

In studies of the products and functions of other oncogenes, MuLV vectors carrying intact c-erb-b1 have been found to transform established cells poorly, if at all--sustaining the view that proto-oncogenes encoding protein-tyrosine kinases may be less potent as transforming agents than other types of proto-oncogenes. Bishop's group is now exploring the types of genetic damage required to activate transformation by c-erb-b1. They have also made mutations in v-erb-B designed to test the roles of the extracellular domain and the transmembrane domain of the gene product in transformation and tumorigenesis. By mapping the topography of both the N-myc gene and its mRNAs, it was determined that the gene gives rise to two different forms of mRNA by the use of both alternative transcriptional promoters and alternative splicing. One of the mRNAs potentially encodes a protein not previously recognized as a product of N-myc. A cDNA that encodes the identified product of N-myc has been implanted in a MuLV vector and this virus has been used to demonstrate that the normal allele of N-myc has a virtually full range of transforming activities when expressed in abundance, and to demonstrate that the differentiation of human neuroblastoma cells can be blocked by the sustained expression of N-myc. These findings enhance the argument that amplification of N-myc may contribute to the genesis of several types of human tumors and provide clues as to its physiological function.

In studies particularly relevant to human cancer the Bishop group has used gene transfer to detect an activated oncogene in DNA derived from bone marrow cells of patients suffering from a preleukemic condition known as myelodysplasia. The activated gene appears to be Ki-ras. These findings represent evidence for activation of a human oncogene in advance of overt malignancy. Contrasting findings exist in CML--one of four samples from cells in the chronic phase of the disease yielded a presently unidentified activated oncogene that is not among the ras family, whereas three of five samples from cells in the acute blast-crisis of the disease contained active (mutant) versions of c-Ha-ras. These findings suggest that the initial stage of CML may arise from a combination of genetic lesions (including the translocation of c-abl and the mutational activation of an unidentified oncogene) and that mutations in c-Ha-ras may be one of the events that can trigger the fatal blast crisis. Thus, the discovery and characterization of new oncogenes has the potential for revealing novel mechanisms of transformation. Additionally, knowledge of new oncogenes helps to define all of the various components of the cell growth regulatory network, and thus permits understanding of their individual functions as well as their possible interactions as a whole, composite system (6).

Coffin and his co-workers have studied several important and related areas centered around important areas of the retroviral life cycle. The first of these

involves the mechanism of avian leukemia virus (ALV) integration and is being studied using an ALV genome (called CS8) with a strongly selectable bacterial gene suppressor F (supF) which permits cleaving of integrated viral DNA linked to adjacent cell DNA in an amber mutant bacteriophage lambda. Using this strategy, two independent collections ("libraries") of about 6,000 integration sites from cells infected with CS8 have been made. These libraries have been analyzed for the complexity of their integration target by the frequency with which specific target fragments are repeated in the library. From the relative frequency of "hits" in this experiment, it can be estimated that, on average, the integration target is about 10% of the cell genome. Remarkably, when independent integrations into the same fragment were detected, they were found to be in exactly the same location, implying that there is a class of very highly preferred targets, in which integration occurs at precisely the same location every time. One of these sites was used no less than 6 times in the two experiments, implying that it is used in about 1 in 2,000 infections, an enrichment of about one million-fold. Several such "high frequency" targets have been cloned and are being analyzed to determine whether there is some specific sequence preferred as a target.

A second aspect of these studies is the development of an in vitro system to study integration. For this purpose, the selective power of the CS8 system has been used. At one day after infection of QT6 (quail) cells with CS8 virus, a cell-free extract was made. Preliminary experiments suggested that such an extract contained pre-integration intermediate structures. Integration of proviral DNA from these structures was detected by the addition of amber mutant lambda phage DNA to the extract. After packaging and infection of suppressor (SU) minus E. coli, only lambda phage which received an integrated provirus containing supF could grow. To date, a number of proviruses have been isolated in this way. Sequence analysis showed that they have integrated into a variety of non-essential positions in lambda DNA in "correct" form; that is, with the loss of 2bp from each end of the provirus and the reduplication of 6bp at the integration site. This system will be used to further decipher the biochemical events in the integration process.

Investigation of provirus expression involves the use of several assays for expression of viral genomes and analysis of viral RNAs developed by Coffin's group. Transient expression of provirus-like DNA after transfection has been used to analyze the effect of various viral sequences on expression. Of particular recent interest has been the differential usage of the two long terminal repeats (LTR's). The 3′ LTR, despite an identical sequence to the 5′ LTR, is usually completely silent since no transcripts can be detected which are initiated within it. The difference in expression between the 3′ and 5′ LTRs is attributable to a small region in the leader sequence near the 5′ end, which either stabilizes the transcripts or serves to "direct" enhancer activity to the nearby promoter. Further analysis using plasmids with only an LTR with or without the candidate region imply that this region seems to select, on the DNA level, the specific promoter to be used for integration. These studies are expected to yield significant information on variation and integration of retrovirus RNA (19,20,21).

Harold Varmus and his group are carrying out a molecular analysis of retroviruses and oncogenes under an outstanding investigator award. Dr. Varmus' studies on integration have revealed that newly synthesized murine leukemia virus (MLV) DNA, linear and circular molecules, in both cytoplasmic and nuclear forms, is present in nucleoprotein complexes that sediment with coefficients of about 160S in

sucrose. These complexes include viral gag proteins, can be banded in metrizamide gradients and partly purified by gel filtration chromatography, and can meditate precise retroviral integration in vitro (93).

In collaboration with Mike Bishop's laboratory, Varmus' group has developed an in vitro assay for viral integration that uses as a target naked lambda bacteriophage DNA with amber mutations. In this assay, successful integration is scored by using as a source of nucleoprotein complexes extracts of cells infected with an MLV strain carrying an amber suppressor gene and by plating packaged bacteriophage DNA on a suppressor-minus host. The assay has shown that linear DNA serves as a precursor to the integrated form, and the absence of a requirement for a nucleotide energy source argues that circularization need not occur in the reaction. The procedure permits integration of retroviral DNA to occur in vitro and to be detected with great sensitivity. Integration of viral DNA into an artificial target of phage DNA is efficient and accurate--the integrated DNA displays all of the structural features found when provirus is produced in intact cells. Both linear and circular DNA can serve as precursors for the reaction. The availability of such an in vitro system should facilitate the isolation, characterization and purification of components required for retroviral integration, and the assay could be used to screen for agents which block the integration of retroviral DNA (6,93).

Recently, David Boettiger's laboratory has demonstrated that the fibronectin receptor is a control point for myogenic differentiation. Specific cell surface receptors have been described which mediate the interaction between cells and specific components of the extracellular matrix. Functional studies on the receptor have demonstrated that it plays an important role in cell migration and probably in cell positioning. Boettiger has demonstrated that interactions with this receptor can also regulate gene expression and hence provide a direct means for signals from the extracellular matrix to influence cell differentiation.

The integrin gene described in the chicken codes for an integral membrane protein receptor complex which consists of alpha and beta polypeptide subunits. Based on the blocking of the binding of this receptor to extracellular matrix components by monoclonal antibodies directed against the beta subunit and on equilibrium gel filtration studies, integrin functions as the receptor for fibronectin and laminin. In fibroblasts and myoblasts, integrin co-localizes along portions of stress fibers and at their termini in the adhesion plaques of the cell membrane. On the internal cell surface, the integrin molecule has a binding domain which interacts with talin. This provides the initial internal link with the microfilament component of the cytoskeleton. Thus, integrin provides a link between the extracellular matrix and the cytoskeleton.

Functional studies of this integrin complex have been aided by a monoclonal antibody, CSAT, which binds to the external portion of the integrin molecule and displaces both fibronectin and laminin from their attachment to integrin. The CSAT antibody has been shown to have an adhesion perturbing effect on fibroblastic and myogenic cells in vitro. It is known that as myogenic cells differentiate in culture their surface expression of CSAT changes. The earliest identified muscle precursor cell in myogenic cultures prepared from the breast muscle of 11 day chick embryos is the replicating myoblast. These cells are morphologically identical to fibroblasts, but may be distinguished by their synthesis of low levels of desmin and the presence of a cell surface antigen defined on the basis of a monoclonal antibody, L4. Boettiger's study demonstrates a new

function of the CSAT receptor, in that its interaction with the substrate is an essential portion of the mechanism which triggers the metabolic events of terminal differentiation (8).

Another study by Boettiger examined the effect of src on chick embryo lens cells in culture. As shown with other cell types, chick embryo lens cell differentiation is inhibited by the src protein. However, this system is unique for two reasons. First, many functionally linked phenomena can be studied in a concerted manner since these cells undergo at least a partial morphogenesis in culture, organizing lens-like structures known as lentoids. This is accompanied by the formation of extracellular matrix structures and the synthesis of delta-crystallin. Secondly, and most significant for this study, as these lens cultures differentiate, up to 50% of their plasma membrane becomes involved in gap junction structures. This high percent of junctional membrane, far in excess of what has been reported for other cell types, allows a straightforward analysis of the effect of transformation on gap junction protein synthesis and junction formation. The effect of oncogenic transformation on permeable junctions has long been a matter of debate. Studies of these junctions in cultured cell lines have produced a variety of contradictory evidence and the role of the junctions in maintaining or establishing a normal state remains to be demonstrated. Boettiger's results demonstrate definitively that the expression of pp60v-src does not inhibit the synthesis on MP28, the major protein of the lens gap junction. However, the formation of the junctions is inhibited. Thus, the block must occur at a point after the synthesis of MP28 but before the assembly of the lens junctions. Since immunofluorescence studies do not show any specific membrane localization, it is possible that the block is at the point of getting the protein to the plasma membrane. It is interesting that this block has no feedback control on limiting synthesis of MP28 in the transformed cells (7).

A result of interest was the development by Chisari and his group of hepatocellular adenomas and carcinomas in a transgenic lineage of mice that contains the entire envelope open reading frame of hepatitis B virus (HBV). This lineage spontaneously develops ground glass cells and hepatocellular injury due to the storage of cytotoxic amounts of nonsecretable long hepatitis B surface antigen (HBsAG) filaments within the endoplasmic reticulum. This lineage also exhibits a chronic hyperplastic response to the stimulus of ongoing liver cell necrosis. Virtually all mice of this lineage develop hepatocellular neoplasms after 12-14 months of age. These neoplasms are heralded by serum alpha-fetoprotein elevations that can surpass levels 100 times above normal limits. Analysis of restriction digests of genomic DNA from these tumors revealed no evidence of gross rearrangement of the integrated HBV transgene or of cellular flanking sequences, suggesting that transformation may be due to chance mutations occurring elsewhere in the mouse genome in the setting of chronic hepatocellular regeneration. If this is correct, in this model, HBV sequences serve to initiate a series of events that eventually lead to neoplasia. Although they are necessary, the HBV sequences are clearly not sufficient for malignant transformation as they might be if HBV contained an acutely transforming oncogene. Clearly, much needs to be done to elucidate the mechanism responsible for this lesion and its biological characteristics, including the establishment of continuous cell lines, the propagation of the tumors in nude mice, phenotypic characterization of transformed cells with a panel of tumor markers, the cloning and sequencing of the integrated DNA and cellular flanking sequences in tumor and non-tumorous tissues to identify point mutations and minor deletions or rearrangements that might have escaped detection by restriction mapping and to search for neighboring cellular oncogenes, and the search for activated oncogenes in the tumors by DNA-mediated transformation of NIH 3T3 cells.

In additional studies using these transgenic animals, the first attempts at immunological manipulation of this system were successful. Objectives were to use the system to (a) probe the pathogenetic potential of the immune response to HBV-encoded, cell-associated antigens, and to (b) study mechanisms of immunological tolerance. These experiments require the presence of the HBV transgenes on an inbred genetic background to permit cell transfer between animals without induction of an allogenic response (i.e., graft rejection or graft versus host disease). This important intermediate goal has been achieved in two ways. By backcrossing an early hybrid lineage containing the HBV envelope region under control of the inducible mouse metallothionein promoter to each parental line, Chisari's group is now 11 generations into the C57B1/6 background and 8 generations into the SJL background. Starting with a hybrid animal, sufficient genetic homogeneity is achieved by the 12th generation, so experiments should begin with this lineage during the next year. Another approach is by producing transgenic mice on an inbred genetic background. Although the efficiency of producing inbred transgenic mice is much lower (10%) than hybrids, they have succeeded in introducing the HBV envelop region under control of the mouse albumin promoter into inbred, congenic recombinant mice of the B10 series. Two lineages that do not develop the spontaneous lesions have been selected for study. Thus, four related sets of inbred transgenic mice have been produced carrying the same transgene (Alb-HBV<u>env</u>) at two different integration sites in the context of two different major histocompatibility complex (MHC) haplotypes, one of which is immunologically responsive ($H\text{-}2^d$) and one of which is non-responsive ($H\text{-}2^s$) to HBsAG at the T-cell level. Sufficient numbers of the 107-5 ($H\text{-}2^d$) lineage have been generated to perform several pilot experiments with the following results. First, this lineage is immunologically tolerant to its transgene products, i.e., it does not develop antibody or proliferative T-cell response to hepatitis B surface antigen (HBsAG) or the Pre-S antigens either spontaneously or after immunization with either soluble HBsAG or vaccinia recombinants containing the HBV envelope region. Second, tolerance is transgene (ay)-specific, since the mice do mount a normal response to the alternate HBV subtype (ad). Third, adoptive transfer of immunologically naive nontransgenic syngeneic ($H\text{-}2^d$) spleen cells into these transgenics does not lead to a measurable immune response in the recipients, suggesting that suppressor networks may be active in the transgenic mice and partially responsible for the tolerant state. Fourth, adoptive transfer of immunologically primed (i.e., HBsAG immunized) syngeneic nontransgenic spleencells <u>does</u> lead to an immune response in transgenic recipients measured as specific antibody production with clearance of circulating HBsAG for a period of at least 16 weeks after transfer. Fifth, these mice develop hepatocellular injury characterized by elevated serum alanine aminotransferase levels and necroinflammatory changes histologically reminiscent of human viral hepatitis. These observations suggest that the HBV envelope polypeptides can serve as targets of immune attack on the hepatocyte surface. They also afford the opportunity to explore the phenotype of the cells responsible for immunological injury, the specific antigenic determinants recognized and possible specific immunotherapeutic strategies for intervention in hepatic cell injury and subsequent hepatocellular carcinoma (18).

In summary, the search for new viral <u>onc</u> genes and their characterization may uncover interesting new genes that are components of the growth regulatory system of the cell. Since only a minor fraction of these regulatory genes are known, most new ones that are discovered and studied cannot immediately be connected to and integrated into the system as a whole. They resemble isolated, though important, pieces of a puzzle and the <u>sea</u> oncogene is one such piece. Occasionally, however, by serendipity or design, a connection to existing information on

growth control can be established, as with *jun*, GCN and AP-1, and with it emerges the promise of a broader outlook and a deeper appreciation of information necessary to understanding and overcoming human cancer. The use of the new transgene technology should add substantially to our ability to determine the targets of various oncogenes, their mechanisms of action, and perhaps suggest means of interfering with or interrupting the malignant process.

RNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 2 R01 CA 34818-04	Studies on the Replication and Oncogenicity of HBV
2. BALDUZZI, Piero C. University of Rochester 5 R01 CA 32310-06	The Transforming Genes of Avian RNA Tumor Viruses
3. BALUDA, Marcel A. University of California (Los Angeles) 5 R01 CA 10197-21	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. Johns Hopkins University 5 R01 CA 33199-06	Location and Function of M6A in Retrovirus RNAs
5. BISHOP, J. Michael University of California (San Francisco) 5 R01 CA 12705-16	Rous Sarcoma Virus: Replication and Cell Transformation
6. BISHOP, J. Michael University of California (San Francisco) 5 R35 CA 44338-02	Retroviruses and Cancer Genes
7. BOETTIGER, David E. University of Pennsylvania 2 R01 CA 16502-14	Genetic Analysis of RNA Tumor Viruses
8. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 30383-08	Virus-Induced Myeloid Leukemia
9. BOSE, Henry R., Jr. University of Texas (Austin) 5 R01 CA 33192-05	Transformation by Avian Reticuloendotheliosis Virus
10. BRUGGE, Joan S. State University of New York (Stony Brook) 2 R37 CA 27951-09	Regulation of pp60-src and the Polyoma mT Protein Interaction

11.	BURK, Robert D. Albert Einstein College of Medicine of Yeshiva University 5 R01 CA 45476-02	Role of Hepatitis B Virus in Hepatocellular Carcinoma
12.	BUSS, Janice E. La Jolla Cancer Research Foundation 5 R29 CA 42348-03	Attachment of Myristic Acid to P60-*src*
13.	BUTEL, Janet S. Baylor College of Medicine 5 R01 CA 37257-03	Hepatitis B Virus and Human Liver Cancer
14.	CARBON, John A. University of California (Santa Barbara) 5 R01 CA 11034-20	Studies on Centromere Structure and Function
15.	CASPAR, Donald L. Brandeis University 5 R01 CA 15468-15	Assembly of Viruses, Membranes, and Tissue
16.	CASPAR, Donald L. Brandeis University 1 R35 CA 47439-01	Switching in Virus and Membrane Assemblies
17.	CHEN, Ji Hshiung St. Jude Children's Research Hospital 5 R01 CA 42859-02	Transforming Gene of Avian Acute Leukemia Virus E 26
18.	CHISARI, Francis V. Scripps Clinic and Research Foundation 5 R01 CA 40489-04	Pathogenesis of Hepatitis B
19.	COFFIN, John M. Tufts University 5 R01 CA 17659-12	Relationship of Avian Tumor Virus RNA and Host Genome
20.	COFFIN, John M. Tufts University 5 R01 CA 27108-08	Mechanisms of Variability of Tumor Virus RNA
21.	COFFIN, John M. Tufts University 5 R35 CA 44385-02	The Molecular Biology of Retroviruses
22.	COOPER, Jonathan A. Fred Hutchinson Cancer Research Center 5 R01 CA 41072-03	Protein Phosphorylation and Cell Growth Regulation

23.	DUESBERG, Peter H. University of California (Berkeley) 5 R35 CA 39915-04	Retroviral Onc Genes and Cellular Proto-Onc Genes
24.	EISENMAN, Robert N. Fred Hutchinson Cancer Research Center 5 RO1 CA 20525-12	Control Mechanisms in Avian Oncornavirus Replication
25.	FARAS, Anthony J. University of Minnesota (Minneapolis-St. Paul) 5 RO1 CA 18303-13	RNA-Directed DNA Polymerase and 70S RNA of Oncornaviruses
26.	FEITELSON, Mark A. Fox Chase Cancer Center 1 R29 CA 48656-01	Products of the X and Polymerase Genes of Hepadnaviruses
27.	GANEM, Donald E. University of California (San Francisco) 1 R13 AI 26936-01	Hepatitis B Virus Meeting
28.	GILMORE, Thomas D. Boston University 1 R29 CA47763-01	Transformation of Cells by the V-Rel Oncogene
29.	GOLDBERG, Allan R. Rockefeller University 5 RO1 CA 13362-17	RSV Functions Involved in Transformation
30.	GOULIAN, Mehran University of California (San Diego) 5 RO1 CA 11705-19	DNA Synthesis Studies
31.	GRANDGENETT, Duane P. St. Louis University 5 RO1 CA 16312-15	Avian Retrovirus DNA Synthesis and Integration
32.	GRANOFF, Allan St. Jude Children's Research Hospital 5 RO1 CA 07055-26	Studies on Lucke Tumor Associated Viruses
33.	GRODZICKER, Terri I. Cold Spring Harbor Laboratory 1 R13 AI25198-01	Conference on Molecular Biology of Hepatitis B Viruses
34.	HALPERN, Michael S. Wistar Institute of Anatomy and Biology 5 RO1 CA 31514-07	Endogenous Retrovirus as a Determinant of Tumor Immunity

35. HANAFUSA, Hidesaburo
Rockefeller University
5 R35 CA 44356-02

Analysis of Cell Transformation by Retrovirus

36. HARRISON, Stephen C.
Harvard University
5 R01 CA 13202-17

Structure and Assembly of Viruses and of Coated Vesicles

37. HAYWARD, William S.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 43250-03

Mechanisms of Viral and Non-viral Oncogenesis

38. HUMPHRIES, Eric H.
University of Texas Health Science Center (Dallas)
5 R01 CA 32295-06

Characterization of the ALV-Induced Transformed Follicle

39. HUMPHRIES, Eric H.
University of Texas Health Science Center (Dallas)
5 R01 CA 41450-03

Expression and Function of v-rel in Lymphoid Tissue

40. HUNTER, Eric
University of Alabama (Birmingham)
5 R01 CA 29884-08

Site Specific Mutagenesis of the Envelope Gene of RSV

41. JOKLIK, Wolfgang K.
Duke University
5 P01 CA 30246-08

Regulatory Functions of Protein Nucleic Acid Interaction

42. JOVE, Richard
University of Michigan
1 R29 CA 47809-01

Mechanisms of Cell Transformation by the Viral src Gene

43. KNOWLES, Barbara B.
Wistar Institute of Anatomy and Biology
2 R01 CA 37225-04

Hepatitis Virus and Primary Hepatocellular Carcinoma Cells

44. KOPROWSKI, Hilary
Wistar Institute of Anatomy and Biology
2 P01 CA 21124-11

Virology and Genetics of Cancer

45. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 38659-03

Oncogene and Activator: Tumorigenesis by Cloned DNA

46. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 39207-04

Avian Erythroleukemia and c-erb-B Activation

47.	KUNG, Hsing-Jien Case Western Reserve University 1 RO1 CA 46613-01	Oncogene Activation in Avian B & T Lymphoma
48.	LAU, Alan F. University of Hawaii (Manoa) 5 RO1 CA 35578-03	Cellular Substrates of pp60-src in ASV-Infected Cells
49.	LEE, Wen-Hwa University of California (San Diego) 5 RO1 CA 39537-03	Transforming Gene and Protein of Fujinama Sarcoma Virus
50.	LEIS, Jonathan P. Case Western Reserve University 5 RO1 CA 38046-05	Studies of Retroviral Proteins
51.	LINIAL, Maxine L. Fred Hutchinson Cancer Research Center 5 RO1 CA 18282-13	Retroviral Coded Functions
52.	LIPSICK, Joseph S. University of California (San Diego) 5 RO1 CA 43592-02	Mechanism of Transformation by the V-myb Oncogene
53.	MACARA, Ian G. University of Rochester 2 RO1 CA 38888-04	Oncogene and Control of Phosphoinositide Cycle/Kinase C
54.	MAJORS, John E. Washington University 2 RO1 CA 38994-04	Analysis of Retroviral Transcriptional Regulation
55.	MARTIN, G. Steven University of California (Berkeley) 5 RO1 CA 17542-13	Genetics of RNA Tumor Viruses
56.	MILLER, Arthur D. Fred Hutchinson Cancer Research Center 5 RO1 CA 41455-03	Gene Transfer Using Retroviral Vectors
57.	MONTELARO, Ronald C. Louisiana State University A&M College (Baton Rouge) 5 RO1 CA 38851-07	EIAV: Antigenic Variation and Retrovirus Persistence
58.	MOSCOVICI, Carlo University of Florida 5 RO1 CA 10697-22	Avian Leukemia Viruses and Cell Differentiation

59.	NEIMAN, Paul E. Fred Hutchinson Cancer Research Center 5 R01 CA 20068-13	Molecular Mechanisms in Neoplasia
60.	NEIMAN, Paul E. Fred Hutchinson Cancer Research Center 5 P01 CA 28151-09	Program in Retroviruses and Cancer
61.	OGSTON, Charles W. Rush-Presbyterian-St. Luke's Medical Center 5 R01 CA 37276-03	Molecular Biology of Hepatitis Virus In Vivo
62.	PARSONS, J. Thomas University of Virginia (Charlottesville) 5 R01 CA 27578-08	Expression of Avian Retrovirus Transforming Genes
63.	PARSONS, J. Thomas University of Virginia (Charlottesville) 5 R01 CA 29243-08	Avian Sarcoma Virus-Specific Tumor Antigens
64.	PARSONS, Sarah J. University of Virginia (Charlottesville) 2 R01 CA 39438-04	Role of c-src in Retroviral Transformation
65.	PERDUE, Michael L. U.S. Agricultural Research Service Athens, GA 5 R01 CA 45134-03	Regulation of Protein Synthesis by the Retrovirus Leader
66.	PIEHL, Richard F. Molecular Biology Resources, Inc. (Milwaukee) 1 R43 CA 45876-01	Large-Scale Production of Avian Myeloblastosis Virus
67.	PRIVALSKY, Martin L. University of California (Davis) 2 R01 CA 38823-04	Characterization of the v-erb-B Oncogene Protein of AEV
68.	REPIK, Patricia M. Medical College of Pennsylvania 5 R01 CA 38007-03	Gene Expression--Oncogenic/ Chronic Hepatitis B Infection
69.	RHODE, Solon L., III University of Nebraska Medical Center 5 R01 CA 37481-03	Replicon Control in Normal and Transforming Cells

70. ROBINSON, Harriet L.
University of Massachusetts
Medical School
2 R01 CA 23086-11

Retrovirus-Host Interactions

71. ROBINSON, Harriet L.
University of Massachusetts
Medical School
7 R01 CA 27223-09

Avian Leukosis Viruses and Cancer

72. ROBINSON, William S.
Stanford University
5 R01 CA 34514-05

Duck Hepatitis B Virus: Infection and Disease

73. ROGLER, Charles E.
Albert Einstein College of Medicine of Yeshiva University
5 R01 CA 37232-05

WHV and HBV Associated Hepatocellular Carcinoma

74. ROHRSCHNEIDER, Larry R.
Fred Hutchinson Cancer Research Center
5 R01 CA 20551-12

Mechanisms of Oncornavirus-Induced Transformation

75. SEFTON, Bartholomew M.
Salk Institute for Biological Studies
5 R01 CA 17289-13

Membranes and Viral Transformation

76. SHAFRITZ, David A.
Yeshiva University
5 R01 CA 32605-07

Hepatitis B Virus - Chronic Hepatitis - Liver Cancer

77. SHALLOWAY, David I.
Pennsylvania State University (University Park)
2 R01 CA 32317-07

Role of pp60c-src Homolog of the RSV Oncogenic protein

78. SHANK, Peter R.
Brown University
5 R01 CA 32980-06

Stability and Disease Tropism of Proviral DNAs

79. SHENK, Thomas E.
Princeton University
2 R01 CA 39606-04

Functional Analysis of the Adeno-Associated Virus Genome

80. SHIH, Chiaho
University of Pennsylvania
5 R01 CA 43835-02

Integration of Hepatitis B Virus and Liver Neoplasia

81. SIDDIQUI, Aleem
University of Colorado Health Sciences Center
5 R01 CA 33135-05

Expression of Hepatitis B Virus Genes and Hepatoma

82. SMITH, Ralph E.
Colorado State University
5 R01 CA 35984-06

Biochemistry of RNA Tumor Virus Replication

83. STAVNEZER, Edward
University of Cincinnati
5 R01 CA 43600-03

Origin Structure and Biological Activity of SKVS

84. STOLTZFUS, Conrad M.
University of Iowa
5 R01 CA 28051-09

Avian Retrovirus RNA Metabolism

85. SUMMERS, Jesse W.
Institute of Cancer Research (Philadelphia)
5 R35 CA 42542-03

Persistent Infections by Hepadnaviruses

86. SWANSTROM, Ronald I.
University of North Carolina (Chapel Hill)
5 R01 CA 33147-05

Retrovirus Replication: Interaction with Host Genome

87. TATTERSALL, Peter J.
Yale University
5 R01 CA 29303-08

Molecular Basis of Parvovirus Target Cell Specificity

88. TAYLOR, John M.
Institute for Cancer Research (Philadelphia)
5 R01 CA 22651-10

Reverse Transcription

89. TEMIN, Howard M.
University of Wisconsin (Madison)
2 P01 CA 22443-11

Molecular Biology and Genetics of Tumor Viruses

90 TENNANT, Bud C.
Cornell University (Ithaca)
5 R01 CA 37264-03

Hepatitis, Aflatoxin, and Hepatocarcinogenesis

91. TIOLLAIS, Pierre
Pasteur Institute
5 R01 CA 37300-04

Hepatitis B Virus DNA, Oncogenes, and Liver Cancer

92. VARMUS, Harold E.
University of California (San Francisco)
2 R01 CA 37281-05

Oncogenic Potential of the Hepatitis B-Type Viruses

93. VARMUS, Harold E.
University of California (San Francisco)
5 R35 CA 39832-04

Molecular Analysis of Retroviruses and Oncogenes

94.	VOGT, Peter K. University of Southern California 5 R35 CA 42564-03	Onc Genes in Virus and Cell
95.	VOGT, Volker M. Cornell University (Ithaca) 2 R37 CA 20081-12	Avian Retrovirus Structure and Assembly
96.	WANDS, Jack R. Massachusetts General Hospital 5 R01 CA 35711-05	Pathogenesis, Immunodiagnosis, and Therapy of Carcinoma
97.	WANG, Lu-Hai Rockefeller University 5 R01 CA 29339-08	Transforming Genes of Avian Sarcoma Viruses
98.	WANG, Lu-Hai Rockefeller University 1 R01 CA 47386-01	Expression and Function of Proto-Oncogene C-src
99.	WEBER, Michael J. University of Virginia (Charlottesville) 5 R01 CA 39076-05	Early Cellular Changes in Viral Oncogenesis
100.	WEINTRAUB, Harold M. Fred Hutchinson Cancer Research Center 5 R35 CA 42506-03	Generation of Development Mutants with Cloned DNA Vector
101.	WILLS, John W. Louisiana State University Medical School (Shreveport) 5 R29 CA 47482-02	Analysis of Retrovirus Assembly by In Vitro Mutagenesis

SUMMARY REPORT

AIDS VIRUS STUDIES

The AIDS Virus Studies component of the Branch supports research on the human immunodeficiency virus (HIV) and other retroviruses as models of HIV infection. There are 17 research grants in the program with an estimated funding of 3.11 million dollars including traditional research grants, cooperative agreements and outstanding investigator awards. Acquired immune deficiency syndrome (AIDS), first described in 1981, is caused by HIV, and the disease has been characterized clinically, immunologically, and epidemiologically. AIDS is characterized by lymphopenia with a marked reduction in the numbers of T4 helper lymphocytes and a consequent suppression of immune system functions, which greatly increase patients' susceptibility to opportunistic bacterial, viral and fungal infections and to malignant sequelae such as B-cell lymphomas and Kaposi's sarcoma (KS). The control of AIDS is particularly difficult due to: 1) the ability of the virus to evade the immune system upon initial infection; 2) the extended period of time between infection and the first appearance of clinically recognizable symptoms during which the virus can replicate and be transmitted; and 3) the difficulties encountered in the design of experimental prophylactic vaccines because of extensive antigenic heterogeneity of the virus and a lack of appropriate animal models in which to evaluate potential candidate vaccines. Research supported by the AIDS Virus Studies component focuses on the etiologic role of HIV in AIDS and other diseases (18%); viral mechanisms involved in immunopathogenesis, immune system dysfunction and lymphocyte depletion (12%); molecular mechanisms of viral replication and gene expression (12%); development of appropriate animal models for basic research in HIV and lentivirus pathogenesis (23%); basic laboratory research leading to the development of experimental vaccines and preclinical evaluation of vaccine efficacy (23%); and association of HIV with an increased incidence of neoplastic sequelae, such as Kaposi's sarcoma, malignant B-cell non-Hodgkin's lymphomas, and T-cell chronic lymphocytic leukemia (12%).

AIDS is associated with an increased incidence of neoplasia, primarily Kaposi's sarcoma (KS) and malignant lymphomas, including rapidly growing B-cell non-Hodgkin's lymphomas (NHL, malignancy of lymphocytes), Hodgkin's disease (HD, malignancy of macrophages) and T-cell chronic lymphocytic leukemias. AIDS-associated Burkitt's lymphomas (AIDS-BL) are characterized by specific chromosomal translocations involving the c-myc oncogene locus on chromosome 8 and one of the immunoglobulin loci located on chromosomes 2, 14 or 22. C-myc gene rearrangements analogous to those observed in Burkitt's lymphomas (BL) were detected in 12 of 16 AIDS-associated NHL cases; six of the 16 cases had detectable EBV sequences and proteins, but none of the 16 cases displayed other oncogene rearrangements or contained viral sequences from the human T-lymphotropic virus 1 (HTLV-1), suggesting a role for c-myc activation in the pathogenesis of AIDS-associated NHL. Cytological characterization showed that the breakpoints in 5 of 6 cases occurred within or near the immunoglobulin heavy chain switch region on chromosome 14 and within the c-myc locus on chromosome 8, and thus were similar to chromosome breakpoints observed in BL. The nature of these breaks suggests that they occur at the time of heavy chain isotype switching, i.e., relatively late during B-cell development. An in vitro system was utilized to determine whether introduction of activated c-myc genes into human EBV-infected lymphoblastoid cells from infected peripheral blood of AIDS patients was sufficient for transformation. The constitutive expression of the

transfected exogenous c-myc caused negative regulation of endogenous c-myc expression, changes in growth properties typical of transformed cells, and acquisition of tumorigenicity when injected into immunodeficient mice. The degree of tumorigenicity in all myc-transfected populations directly correlated with the level of c-myc mRNA expression. Epstein-Barr virus (EBV) infection and c-myc activation thus appear sufficient for the malignant conversion of human B-cells in vitro, supporting the hypothesis that these same two pathogenetic steps may be involved in the in vivo development of the EBV fraction of AIDS-NHL. These results suggest that lymphomagenesis in AIDS is a multistep process in which the oligoclonal B-cell expansions observed in ARC-associated lymph-adenopathy (AIDS related complex) may represent a "pre-malignant" condition for the development of AIDS-NHL. The state of HIV-induced immunosuppression and continuous EBV reactivations during ARC appears to favor the expansion of multiple EBV-transformed B-cell clones which increases the probability of the occurrence of additional genetic alterations, such as the translocation of the c-myc gene, thus resulting in the malignant transformation of B-cells (1).

KS appears with high frequency in AIDS patients, particularly among the homo-sexual group. Experiments were conducted to search for and characterize trans-forming DNA sequences within the KS tumor that were able to morphologically transform tissue culture cells and induce tumors in rodents. The cloned human DNA sequences present in transformants encompass about 32 kilobases (kB) and contain four rearrangements with respect to normal human DNA. To identify the protein(s) encoded in two novel mRNAs of 1.2 and 3.5 kB expressed in NIH 3T3 transformants, a cDNA library was constructed. One of the cDNA clones isolated (KS3) corresponded to the 1.2 kB mRNA and morphologically transformed NIH 3T3 cells when inserted into a mammalian expression vector. The 1152 nucleotide KS3 cDNA encodes a protein of 206 amino acids with significant homology to the basic and acidic human fibroblast growth factors (FGF). The new K-FGF protein appears to represent a new member of the FGF family. Similar to basic FGF, the K-FGF protein is mitogenic and induces growth of NIH 3T3 mouse cells in serum-free medium. These observations are consistent with an autocrine mechanism of growth in which activation of the oncogene is due to overexpression of the K-FGF protein rather than mutations in the K-fgf gene. Current research focuses on invest-igations of various human tumors, including Kaposi's sarcoma, to determine the involvement of the K-fgf oncogene in these malignancies (DNA II component, reference 4).

A variety of ocular and neurologic disorders have been observed in AIDS patients. HIV has been isolated from brain tissue and cerebrospinal fluid; retinal diseases due to opportunistic infection with cytomegalovirus, Toxoplasma and cryptococcus have also been observed. HIV was isolated from the retinas of two AIDS patients who presented symptoms of fever, headache, nausea, vomiting, difficulty in swallowing, ocular cytomegalovirus infections, and bilateral loss of vision. Postmortem examination of retinal tissue demonstrated positive staining for the HIV-1 gp120 (envelope) and p24 (core protein) antigens in the cytoplasm of capillary endothelial cells and neuroretinal cells. HIV-1 was detected in these retinal tissues seven to ten days after culturing the retinal tissue in vitro. These results indicate that HIV-1 infection of the retina can occur and may contribute to some of the ocular disorders described in patients infected with HIV-1 (11,15).

Heterosexual spread of HIV appears to be the major route of transmission in Africa, whereas in the United States HIV has been spread primarily by male

homosexual activities and contact with HIV-infected blood through intravenous drug abuse or through transfusions. However, heterosexual spread in the United States and Europe now appears to be increasing. Women now account for 7% of the cases of AIDS reported in the United States; of these women, 50% had used intravenous drugs, 29% were sexual partners of HIV-infected men, 11% were transfusion-related AIDS cases and 10% were of undetermined origin. HIV has now been detected in the cervical secretions of HIV-infected women. Inflammatory cell infiltration of cervical epithelium (cervicitis) was shown by immunohistochemical staining in cervical biopsy specimens from four HIV-seropositive women; cervicitis was not found in similar biopsies from four HIV-seronegative women. HIV antigens were detected in monocyte-macrophages and endothelial cells within the submucosa of three biopsy specimens by specific immunohistochemical staining, while HIV was not detected by similar immunohistochemical techniques in cervical biopsy specimens from the four HIV-seronegative women. These clinical observations suggest that HIV enters cervical secretions from selected infected cell populations within the cervical tissue; these HIV-infected cells may be involved in transmission of HIV by heterosexual contact and to neonates of HIV-infected women (11, DNA I component, reference 26).

HIV-2, a fourth T-cell lymphotropic virus, was first described in 1985; however, its presence in parts of West Africa, where AIDS is still a rare disease, dates to 1975. In contrast, antibodies to HIV-2 have not been detected in AIDS patients in Central Africa, in areas where the rate of HIV-1 seropositivity approaches 25%. These results demonstrate that HIV-2 is not seroepidemiologically linked to AIDS or HIV-1 infection of patients in Central Africa. Therefore, HIV-2 is distinct from HIV-1 and apparently is not associated with AIDS (5).

HIV is a retrovirus with a dense cylindrical nucleoid containing RNA, core proteins, and reverse transcriptase. The lipid envelope contains two major glycoproteins, gp41 (the transmembrane protein) and gp120 (envelope glycoprotein). The viral genome consists of gag (group-specific antigen), pol (polymerase), and env (envelope) genes and at least five other genes involved in the regulation of viral gene expression. Viral expression begins when HIV gp120 interacts with the CD4 molecule, the viral receptor on helper-inducer lymphocytes, some monocytes, and possibly on other cells such as glial cells and central nervous system endothelial cells. Once HIV enters the cell, RNA-dependent DNA reverse transcription occurs and some of the proviral DNA is inserted into the host chromosome. Restricted HIV gene expression results in a latent infection, often enabling the virus to evade host immune clearance. Monocytes are relatively resistant to the cytolytic effects of HIV infection, perhaps as a result of their diminished CD4 expression, and they may serve as a reservoir for viral persistence by disseminating virus to target organs such as lung and brain (10,11,15, RNA I component, reference 4).

In addition to the three major structural proteins common to all known retroviruses, HIV encodes several proteins unique to HIV. Since these proteins are involved in transcriptional regulation of HIV gene expression, research continues towards an understanding of the molecular mechanisms of action of these regulatory proteins, with the goal of preparing HIV-specific antiviral agents. One protein, art (anti-repressor transactivator), is encoded by two exons that partially overlap the tat gene (transactivator protein) and env genes. Deletions or insertions of genetic material in either of the art coding exons inactivates the virus and diminishes the production of the gag and env proteins. Such mutants simultaneously inactivate either the tat gene function or alter the

structure of the env gene, which renders the virus replication defective. This replication defect can be complemented in trans by addition of functional art gene product. The art protein therefore may represent a potential target for the development of new antiviral agents that could be useful in the control of HIV infection (10).

HIV infection begins with the viral envelope protein binding to the CD4 lymphocyte receptor on the plasma membrane of uninfected cells, followed by the fusion of the viral lipid bilayer with that of the target cell. The HIV envelope glycoprotein is synthesized as a large polyprotein precursor (gp 160), proteolytically cleaved to gp120-gp41, and transported to the plasma membrane of the infected cells. During viral budding, the envelope protein is incorporated on the outer surface of the mature virion. By interaction between the gp160 on infected cell surfaces and the CD4 receptor on uninfected cells large multinucleated cells (syncytia) are formed which can result in cell death. To assess the importance of the gp160 cleavage event, the tryptic-like endoproteolytic cleavage site was removed from the gp160 precursor by site-directed mutagenesis and replaced with a chymotryptic-like site. The resultant viral mutant was indistinguishable from wild-type HIV when analyzed at the level of proviral DNA replication, RNA transcription and processing, protein expression, and viral assembly. However, the gp160 polyprotein was not cleaved and the mutated virions were biologically inactive until they were exposed to limiting concentrations of chymotrypsin. As is the case for other enveloped mammalian viruses, these results indicate that cleavage of the HIV envelope protein and release of a unique hydrophobic domain is necessary for the full expression of viral infectivity (RNA I component, reference 13).

Based on precedents from two retroviruses, mouse mammary tumor virus (MMTV) and Rous sarcoma virus (RSV), the precursor protein of HIV-1 reverse transcriptase (RT) is predicted to be a 160 kD polyprotein encoded by both the viral pol gene and the upstream gag gene. These two genes lie in different translational reading frames, with the 3′ end of gag overlapping the 5′ end of pol by 241 nucleotides. Translation of HIV-1 RNA, which was synthesized in vitro, yields significant amounts of a gag-pol fusion protein, indicating that efficient ribosomal frameshifting occurs within the HIV-1 gag-pol overlap region, similar to that observed for MMTV and RSV. Two other retroviruses, HIV-2 and the simian immunodeficiency virus, also contain gag-pol overlaps and could also be expected to use ribosomal frameshifting at this site to produce these fusion proteins. Since a complex set of transcriptional and post-transcriptional mechanisms has been proposed to regulate HIV-1 gene expression, these in vitro results suggest that specific inhibitors of the frameshifting process might be an effective means of interfering with HIV-1 replication (RNA II component, reference 93).

A persistent, non-cytopathic, HIV infection has been detected in a small population of T-lymphocytes, monocytes, macrophages and B-lymphocytes. However, the mechanism which converts a persistent infection to a productive infection is not known. Herpesviruses affect the expression of HIV-1 in dually infected cells. In an in vitro assay, herpes simplex virus 1 (HSV-1) or human cytomegalovirus infection (HCMV) induces transcription of the chloramphenicol-acetyltransferase (CAT) gene directed by the long terminal repeat (LTR) of HIV. This effect is observed in both transient and permanently transfected cells containing the HIV-LTR/CAT hybrid gene on a plasmid. The molecular mechanism of the viral transactivation was examined by co-transfection with isolated regulatory genes from HSV-1, using an in vitro system that mimics the latent integrated HIV provirus.

Immediate early (IE) genes of HSV-1 are involved in early events of HSV gene activation and transcriptional regulation. IE175 is involved in HSV-delayed early gene expression and viral replication. An HSV-1 protein of 65,000 daltons (Vmw65) is a virion transcription factor, and IE110 is involved in transactivation of HSV. Transient-expression assays were conducted in which the HIV-LTR/CAT construct was co-transfected with IE HSV genes. The IE110 protein, either alone or in combination with the IE175 protein, can transactivate the HIV-LTR, while the IE175 gene alone or with the gene coding for the Vmw65 factor did not lead to HIV-LTR activation. In a similar manner, the immediate-early region 2 of HCMV also transactivates HIV-LTR. A 5′ upstream sequence within the HIV-LTR appears to contain a specific recognition sequence necessary for the activation by HSV-1 and HCMV, and which is distinct from HIV sequences required for response to the HIV transactivator protein, tat. Some epidemiological studies suggest that herpesvirus infection can increase susceptibility either to HIV infection or disease progression (DNA I component, references 23 and 29).

Novel approaches and reagents for viral therapy and treatment have recently been developed. The CD4 lymphocyte receptor gene was isolated and expressed in an in vitro mammalian system in which a recombinant, soluble form of CD4 (sCD4) is secreted into tissue culture supernatants. sCD4 retains the structural and biological properties of native CD4 on the cell surface, binds to the HIV envelope gp120 and therefore inhibits the binding of virus to CD4+ lymphocytes in vitro, resulting in a striking inhibition of virus infectivity in vitro. Although significant variance exists among different HIV isolates, all appear to use CD4 as a virus receptor. Since sCD4 has not been shown to possess toxic properties in vitro, it might prove to be a useful inhibitor of HIV virus infections. This type of reagent should also allow a more precise understanding of the structural basis for the association of CD4 with the HIV envelope glycoprotein and with the surface of antigen-presenting cells (RNA I component, reference 4).

Relatively little is known about HIV effects on host cells during persistent noncytopathic infection. Expression of the HIV envelope gene induces syncytia (fusion of infected and uninfected cells to form large multi-nucleated cells) and death of CD4+ cells, but not B-lymphocytes, because of the absence or low level expression of the CD4 antigen on B-lymphocytes. Infection of a continuous human T-lymphoid cell line with HIV results in a noncytopathic persistent infection and the gradual loss, over a period of ten days, of CD4 receptors and four other T-lymphocyte specific receptors. A distinct sub-population of lymphoid cells was not selected upon infection with HIV, and the reduction in receptor protein expression was not directly attributed to similar reductions in messenger RNA levels coding for the receptor proteins. These observations suggest that HIV may induce a simultaneous post-transcriptional down-regulation of several T-cell surface receptors. Other investigators have shown that blocking the expression of cell receptors after retroviral infection is the basis for viral interference. Thus, this newly discovered, efficient down-modulation of viral receptors after the first round of infection, preventing super-infection with the newly produced virus, may be a mechanism of long-term persistence of HIV infections (16, 17).

HIV proteins, expressed in infected lymphocytes, are likely targets for the cytotoxic T-lymphocyte (CTL) response, in which virus-infected cells are killed by the host cellular immune defenses. Continuous cultures of B-lymphoid cells, from individuals seropositive and seronegative for HIV, were infected in vitro with a recombinant vaccinia virus producing the HIV pol gene so that the reverse

transcriptase (RT) protein would be expressed in the cultured B-cells. Circulating lymphocytes capable of killing HIV RT-expressing autologous B-cells were detected in eight of ten HIV seropositive subjects, while HIV-specific cytotoxicity was not observed in any of six seronegative subjects. This is the first observation of CTL directed against the polymerase of retroviruses. HIV RT-specific cytotoxic T-cells may result from chronic antigen stimulation in vivo, as they are detected in fresh blood and do not require in vitro stimulation. CTL have been shown to be important in recovery from cytomegalovirus and influenza virus infections. However, it is not yet clear whether env, gag or RT-specific CTL play a role in protection from disease progression in persons infected with HIV-1. Greater CTL activity against RT-expressing target cells compared to env-expressing target cells was seen only in asymptomatic seropositive subjects. Since the HIV pol gene has a higher degree of genetic conservation than do other HIV-1 genes, attempts to elicit humoral and cytotoxic responses to the RT protein with a vaccine might be considered if it appears that RT-specific CTL plays a role in the prevention of AIDS disease progression (11,15).

A number of retroviruses cause immunologic defects in their hosts, including monkeys, cats, cows, horses and mice. These animal models provide the means of investigating mechanisms of viral pathogenesis in a natural host and may provide information which correlates with AIDS disease in humans. Feline leukemia virus (FeLV) is associated with a fatal feline AIDS (FAIDS), with cats developing either an acute disease or a more chronic disease, depending on the age of the animals. A replication-defective variant of FeLV was molecularly cloned directly from infected tissue and shown to induce rapid and fatal immunodeficiency in the absence of replication competent helper viruses. Characterization of this variant virus demonstrated that these viruses differ from the parental virus by deletions in the polymerase gene (6 of 8 isolates), one isolate contained deletions in both the envelope and polymerase genes and one isolate contained a small deletion in the transmembrane protein coding sequence. These changes convert a minimally pathogenic virus into a disease-causing variant that induces the acute form of immunodeficiency. The replication-defective variant is present in low titers in the overall FeLV virus population. However, in infected tissues, the FAIDS-inducing variants usually occurred at higher copy number than did those of the replication competent viruses, suggesting that the variant forms have the ability to outgrow helper viruses in tissues. These observations that FeLV occurs in nature as a mixture of viruses (defective and non-defective) with varying pathogenicities may explain the decrease of pathogenicity recently observed in tissue culture passage of some retroviruses related to HIV, in which the ratio of pathogenic variants may change from that seen in nature. It is possible that similar replication-defective variants with increased pathogenicity may exist in naturally occurring lentivirus populations, such as HIV, the simian immunodeficiency virus (SIV), and other related retroviruses. In the preparation of vaccine challenge pools, it will be important to verify the presence of the disease-causing variants to assure a proper test of vaccine efficacy (12).

The SIV induces an AIDS-like disease in rhesus monkeys, with clinical symptoms similar or identical to those observed in humans. After inoculation with SIV, activated CD8+ lymphocytes are present in increased numbers in the lymph nodes of SIV-infected monkeys with the lymphadenopathy syndrome. CD8+ lymphocytes from SIV-infected monkeys, but not uninfected rhesus monkeys, can block SIV replication in an in vitro tissue culture system, indicating that these T-lymphocytes may contribute to containing or suppressing the progression of simian-AIDS. This is an important observation, since it is the first indication of a specific T-lymphocyte subset that is effective in suppressing replication of a lymphotropic lentivirus (6).

On October 5, 1987, the Branch sponsored a workshop entitled "AIDS: Progress and Future Directions for Vaccine Development." The workshop focused on defining the state-of-the-art in the field of AIDS vaccine development and concluded with a comprehensive discussion of HIV pathogenesis and strategies for intervention. The participants noted that the technology is available to prepare a variety of antigens for use as prototype vaccines, but because of the lack of basic understanding of the mechanism of viral pathogenesis and the lack of information on the proper preparation, amount and route of virus challenge, there is little basis for an informed decision on the most promising antigen(s) for future vaccine studies. The workshop participants indicated that there is an urgent need for valid animal models for investigating the pathogenesis of HIV infections and their subsequent oncogenic sequelae. An RFA based on these recommendations was developed. At its February 1988 meeting, the DCE Board of Scientific Counselors approved the issuance of this RFA, entitled "Retrovirus Animal Models and HIV Pathogenesis." The goals of this RFA were to 1) encourage applications and studies emphasizing the development of animal models using HIV, the simian immunodeficiency virus or other appropriate retroviruses, which mimic long-term HIV infections including viremia, latency, and disease progression to immune dysfunction and possible neoplastic sequelae; and 2) use these animal models for investigations emphasizing virus-host interactions to better define and understand viral-induced pathogenic and immune function alterations. It is anticipated that applications for this RFA will be received and reviewed in the Fall of 1988, with funding of meritorious applications in FY89.

Investigations administered by the AIDS Virus Studies component have resulted in the discover of chromosome rearrangements associated with c-myc activation in AIDS-associated non-Hodgkin's lymphomas. A new Kaposi's sarcoma-associated fibroblast growth factor has been identified; research continues on the role of this autocrine factor in this AIDS-related malignancy. A soluble form of the T-lymphocyte CD4 surface receptor has been produced in vitro via recombinant DNA techniques and serves as an in vitro inhibitor of virus infectivity. A specific subset of T-lymphocytes is effective in suppressing replication of the simian immunodeficiency virus. A replication-defective variant of feline leukemia virus that induces a feline AIDS disease was molecularly cloned. Human cytotoxic T-lymphocytes directed against the HIV reverse transcriptase protein have been detected. HIV has been detected in the cervical tissues and secretions of HIV seropositive women; these HIV-infected cells may be involved in transmission of HIV by heterosexual contact and to neonates born to HIV-infected women. In summary, these studies continue to advance our understanding of the function of HIV viral genes, and the association of HIV and AIDS with neoplastic sequelae and other diseases.

AIDS VIRUS STUDIES

GRANTS ACTIVE DURING FY88

Investigator/Institution/Grant Number	Title
1. DALLA-FAVERA, Riccardo New York University 5 U01 CA 37295-05	Molecular Biology of AIDS-Related Tumors
2. DE NORONHA, Fernando M. Cornell University, Ithaca 5 R01 CA 37742-03	An AIDS Model
3. DESROSIERS, Ronald C. Harvard University 5 R01 CA 38205-03	Type D Retroviruses and Macaque Immunodeficiency
4. ELDER, John H. Scripps Clinic and Research Foundation 5 R01 CA 43362-03	Development of a Synthetic Vaccine to Retroviruses
5. ESSEX, Myron E. Harvard University 5 R35 CA 39805-04	NCI Outstanding Investigator Grant
6. FINBERG, Robert W. Dana-Farber Cancer Institute 5 R01 CA 34979-05	Animal Models of AIDS
7. GRANT, Christopher K. Pacific Northwest Research Foundation 5 R01 CA 43371-03	Anti-Idiotype Vaccines for Feline Leukemia Virus
8. GREEN, William R. Dartmouth College 5 R01 CA 43475-03	Study on Development and Assessment of Retroviral Vaccines
9. HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 42098-03	Molecular Biology of the AIDS Virus HTLV-III
10. HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 44460-02	Molecular Biology of the ART Gene of HTLV-III
11. HIRSCH, Martin S. Massachusetts General Hospital 5 R01 CA 35020-06	Viruses, Acquired Immunodeficiency, and Kaposi's Sarcoma

12.	HOOVER, Edward A. Colorado State University 5 R01 CA 43216-03	Pathogenesis of Feline Leukemia Virus Induced AIDS
13.	NEURATH, A. Robert New York Blood Center 5 R01 CA 43315-03	Synthetic HTLV-III Env Protein Analogs for Future Vaccines
14.	ROSENTHAL, Leonard J. Georgetown University 5 U01 CA 37259-03	Role of HCMV in KS Associated with AIDS
15.	SCHOOLEY, Robert T. Massachusetts General Hospital 5 R01 CA 37461-05	Cellular Immune Response to HIV
16.	VOLSKY, David J. St. Luke's-Roosevelt Hospital Center 5 U01 CA 37465-03	Studies of the Viral Etiology of AIDS
17.	VOLSKY, David J. St. Luke's-Roosevelt Hospital Center 5 R01 CA 43464-04	Novel Retroviruses from South America: HTLV-Type Viruses

SUMMARY REPORT

RESEARCH RESOURCES

The Research Resources component of the Biological Carcinogenesis Branch (BCB), in conjunction with the various research units of the Branch, is responsible for developing, allocating and maintaining a coordinated program of research material support to meet the needs of extramural investigators funded by the Branch. The planning, initiating, and oversight necessary to generate and maintain specific research resources is the responsibility of the individual Program Directors who administer each of the research components of the Branch. However, the storage and distribution of research materials, the management of some resource contracts, the development and maintenance of a computerized inventory, and the day-to-day general management and direction of all resources distribution are the responsibility of the Research Resources component of the Branch. One research resource contract, the competitive continuation of the cell culture identification service, was awarded in FY 1988. There are currently five research resources contracts with an estimated FY 1988 funding level of 1.01 million dollars.

Laboratory investigations carried out under the sponsorship of the BCB depend on the availability of adequate quantities of viruses, viral reagents, antisera, animals, and clinical and laboratory materials of adequate purity, viability and potency, some of which are not available from the commercial sector. The BCB resources component provides some research materials and other supporting activities through contract operations in four general areas. These include activities directed toward production, characterization and distribution of viral and anti-viral reagents; activities concerned with animal resources, including breeding and maintenance of animal colonies; activities directed toward the provision of specialized testing services for the examination of experimental materials; and activities concerned with the storage, inventory and distribution of human specimens.

A consistently active supply of avian myeloblastosis virus (AMV) reverse transcriptase is vital to biological carcinogenesis studies involving the production of cDNA copies of retrovirus genomes for use as probes to identify viral sequences in normal or malignant tissues, to compare viral and cellular sequences for homology, to permit expression of viral sequences in bacterial systems and for other molecular biological studies. To meet these needs, more than 500,000 units of AMV reverse transcriptase were produced and over 160 shipments were made to domestic and foreign laboratories (2).

In the search for oncogenic viruses, many cell cultures from the same or different species are used concurrently, thus offering frequent opportunities for cross-contamination. In cross-species tumor transplantations, it is important to be able to determine the derivation of induced tumors. Additionally, the significance of the presence of virus in tissue cells, the ability to grow virus, or the validity of virus isolation systems are all dependent upon the assurance of the identity of the cell cultures used. To meet this need, a maximum of three assays were carried out on approximately 400 cultures from over 50 laboratories. In making these interspecies and intraspecies cell identifications, more than 1,000 procedures were performed using the following assays: immunofluorescent staining for species-specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (4,5).

During this period, more than 250 shipments of viral reagents and human specimens were made to domestic laboratories from the inventory of frozen biological reagents. Appropriate demographic, clinical and characterization data were included with each shipment. In addition, over 80 shipments of reagents and data were sent to foreign laboratories (3).

While animals have an important role in the biological carcinogenesis program, the actual use of marmosets has been minimal under the payback system. This contract, providing animal resources, is currently being phased out. However, since marmosets are an endangered species, extra phase-out time is required to identify suitable recipients of the animals (1).

The Branch has been involved in implementation of the resources "payback" system since 1981. The payback system is one in which the recipients of resource materials or services reimburse the resource or production contractor directly for the services or materials received, based on a price schedule agreed upon in advance between the NCI and its resource contractor. The contractor in turn credits those funds received from recipients against its production costs and these are shown on monthly vouchers which it submits to the Government for payments under the contract. Initiation of this system was the result of a variety of influences: the noticeable shrinking of the budget; an interest in seeing that the resource dollars utilized by grantees, intramural scientists, and contractors were included in a peer-review system; and the perception that free distribution of resources did not always result in the most effective utilization of available funds.

All resource contracts operate under the payback system. Total costs of production and distribution of research materials are collected on the contracts in which there are a large number of individual users who are receiving small amounts of material at costs reasonable enough for them to continue to acquire them without financial hardship. In other cases, where past utilization patterns have shown that significant problems would be encountered if total costs were charged, only partial operating costs are collected in order that investigators will not have to unduly curtail their research activities. In either case, as a general rule, all grantees, contractors, and intramural scientists pay for the resources which they receive. The payback system seems to be performing as expected. The demand level for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use of costly resource reagents. This has resulted in a reduced level of effort in several resource contracts or the termination of activities deemed to be no longer necessary.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY88

Investigator/Institution/Contract Number	Title
1. CLAPP, Neal K. Oak Ridge Associated Universities NO1 CP 51006	Operation of a Marmoset Colony for Cancer Research
2. HOUTS, G. E. Molecular Genetics Resources, Inc. NO1 CP 51007	Production, Characterization and Distribution of AMV Reverse Transcriptase
3. MASSAGEE, Pamela D. Microbiological Associates NO1 CP 61020	Repository for Storage and Distribution of Viruses, Viral Reagents and Human Sera
4. PETERSON, Ward D. Children's Hospital of Michigan (Detroit) NO1 CP 21017	Inter- and Intraspecies Identification of Cell Cultures
5. PETERSON, Ward D. Children's Hospital of Michigan (Detroit) NO1 CP 85645	Cell Culture Identification and Cytologic/Karyotypic Analysis

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