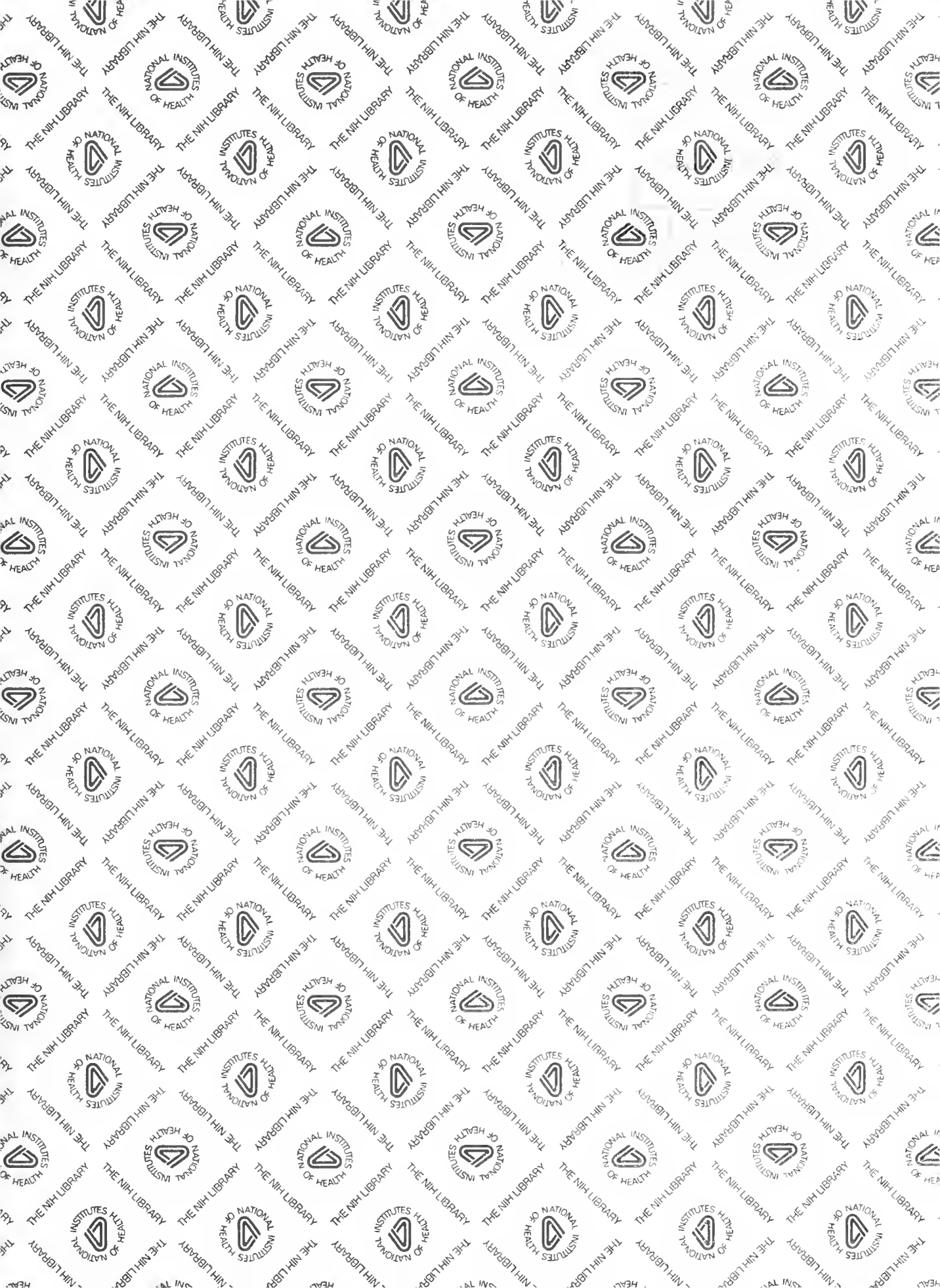
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October 1, 1994 to September 30, 1995

PART 3

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00707 11 LCMN

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Pharmacological Studies of Synaptic Transmission In Vitro

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

P.I.:	M.L. Mayer	Visiting Scientist	LCMN, NICHD 12 months
Others:	K. Partin	NRC fellow	LCMN, NICHD 12 months
	D. Bowie	Visiting Fellow	LCMN, NICHD 12 months
	M. Fleck	IRTA Fellow	LCMN, NICHD 9 months
	R. Bä hring	Visiting Fellow	LCMN, NICHD 8 months
	C. Winters	Chemist	LCMN, NICHD 8 months

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cellular and Molecular Neurophysiology

SECTION
Section of Neurophysiology and Biophysics

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.1	4.4	0.7

CHECK APPROPRIATE BOX(ES)

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

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

This section investigates the functional properties of excitatory amino acid receptors in the vertebrate CNS, utilizing electrophysiological and molecular biological techniques. Concentration jump techniques are used to apply glutamate receptor selective agonists and antagonists to cells and membrane patches under voltage clamp. Preparations in use include recombinant receptors expressed in transfected mammalian cells and Xenopus oocytes, and native receptors generated in primary cultures of hippocampal neurons and glial cells. Analysis of the molecular basis of allosteric regulation of AMPA receptors is being investigated by generation of mutants in which conserved amino acids which differ between the alternative flip/flop exons are tested for sensitivity to potentiation by cyclothiazide and noncompetitive inhibition by 2,3-benzodiazepines. In experiments on hippocampal neurons multiple rate constants for recovery from potentiation by cyclothiazide suggest the existence of heteromeric receptors with different ratios of subunits in their flip and flop splice versions. Inward rectification of glutamate receptors unedited in the second membrane domain was shown to result from ion channel block by micromolar concentrations of cytoplasmic polyamines.

Project Description:

Objectives: To characterize the functional properties of excitatory amino acid receptors, and their role in synaptic transmission in the vertebrate central nervous system. To investigate neurotransmitter and drug action at glutamate receptors. To study the molecular properties of ion channels in neurons and glia, and to characterize mechanisms regulating neuronal and glial cell excitability.

Methods employed: Electrophysiological techniques are used to study neurotransmitter action on a variety of preparations including native receptors expressed in neurons and glial cells, and recombinant glutamate receptors expressed in oocytes and transfected human embryonic kidney cells. Ion channels activated by excitatory amino acids are studied using whole cell patch clamp, the perforated patch technique, outside-out and nucleated patches, and single channel recording as appropriate for individual experiments. Drugs are applied by concentration jump using an array of computer controlled valves and microperfusion tubes which form a rapid drug application system; two methods are currently in use and involve either stepper motor or piezoelectric systems. Simultaneous intracellular recording from pairs of synaptically connected neurons, and analysis of autaptic responses from neurons grown in microculture are used to study excitatory synaptic transmission. Computer analysis of the kinetic properties of agonist evoked membrane currents, single channel activity, synaptic release probabilities and the simulation of the gating properties of glutamate receptor channel plays an important role in the design and interpretation of our experiments.

Major findings: Two major projects were undertaken this year. (1) Analysis of the action of cyclothiazide and some 2,3-benzodiazepines on mutant and native AMPA receptors. (2) Characterization of the mechanisms underlying mRNA editing sensitive inward rectification of AMPA and kainate receptors. Both projects were considerably aided by the use of cotransfection with a reporter plasmid encoding a jellyfish green fluorescent protein, which allowed transfected cells to be identified by fluorescence microscopy prior to recording.

Allosteric modulation by cyclothiazide of mutant AMPA receptors. Recent studies in our laboratory established that cyclothiazide, an allosteric modulator of glutamate receptor desensitization with complete selectivity for activity at AMPA versus kainate preferring receptors, preferentially modulates the flip versus flop splice variants of AMPA receptors. Sequence alignments of the flip/flop domains of each of the four AMPA receptor subunits (which are generated via alternative splicing of two modules each encoding 38 amino acids) reveal 7 amino acids consistently differing between the two splice variants. These 7 amino acids are arranged in groups of 2 + 1 + 4 and are separated by sets of highly conserved amino acids which do not differ appreciably between subunits or splice variants:

Consensus Flip Thr-Pro-(8 AA)-Ser-(21 AA)-Lys-Asp-Ser-Gly-(2 AA)
Consensus Flop Asn-Ala-(8 AA)-Asn-(21 AA)-Gly-Gly-Gly-Asp-(2 AA)

***

To determine the molecular basis for the difference in sensitivity to cyclothiazide of the flip and flop splice variants we performed site-directed mutagenesis, exchanging amino acids between flip and flop versions in each of these three regions, and expressing the mutant receptors either in Xenopus oocytes or transiently transfected 293 cells, using as an assay each of the multiple changes in AMPA receptor activity which occur due to allosteric modulation by cyclothiazide. Comparable sets of mutants were made for the GluRA and GluRB subunits, with GluRA assayed alone and in combination

with GluRB. These experiments revealed that the marked attenuation of desensitization by cyclothiazide for flip splice variants requires the isolated serine residue shown by *** in the above alignment, with no difference in behavior when the C-terminal set of 4 conserved amino acids are in their flip or flop forms. Conversely, in GluRAflop the presence of an asparagine residue at this position is sufficient to produce lower sensitivity to cyclothiazide with no effect observed on exchange of the C-terminal set of 4 amino acids to their flip forms. In heteromeric AMPA receptors assembled from wild type GluRA and GluRB the flip splice forms are dominant for regulating sensitivity to block of desensitization by cyclothiazide. Experiments for heteromers generated from mutant GluRAflop and GluRBflop subunits show that high sensitivity to cyclothiazide typical of wild type AflipBflip is achieved when a serine residue is introduced into either the GluRA or GluRB subunits again with no effect of the C-terminal set of 4 amino acids. Together these experiments show convincingly that all of the splice dependent effects of cyclothiazide are determined by exchange of a single amino acid, Ser in flip and Asn in flop.

Agonist Efficacy for activation of mutant AMPA receptors. Prior work revealed an 8-fold difference in the amplitude of equilibrium responses to maximally effective concentrations of the agonists kainate and glutamate for AflipBflip compared to AflopBflop, with intermediate behavior observed for AflipBflop and AflopBflip. We performed experiments performed on mutant AMPA receptors which indicate that compared to the site controlling sensitivity to cyclothiazide, additional residues in the flip/flop modules of heteromeric AMPA receptors determine the relative amplitude of equilibrium responses to kainate versus glutamate. The amplitude ratio (kainate/glutamate) of responses for these agonists was highest for wild type AflopBflop, decreased approximately 3-fold on exchange of Asn to Ser at positions 750 and 754 in both GluRA and GluRB, with a similar 3-fold decrease on exchange from GGGD to KDSG of the C-terminal set of 4 amino acids in both subunits, but remained below the value for AiBi. The combined exchange of both Asn to Ser and the conserved set of the C-terminal 4 amino acids to generate Ao[ii]Bo[ii] produced a 5.4-fold increase in the relative amplitude of equilibrium responses to glutamate versus kainate. This was still significantly lower than the 11-fold difference observed for wild type AflopBflop versus AflipBflip and the additional exchange of residues at the amino terminal end of the flip/flop modules which differ between splice variants in GluRA and GluRB was required fully restore the behavior of wild type flip receptors. Thus, although amino acids which regulate potentiation by cyclothiazide in heteromers generated from GluRA and GluRB are also critical for attenuation of desensitization, they are not sufficient to determine the relative amplitude of responses to kainate versus glutamate.

A Gln residue blocks sensitivity to cyclothiazide.
Considerable homology between kainate and AMPA receptors in the sequences which align with the alternatively spliced region in AMPA receptors raised the possibility that the lack of sensitivity of kainate receptors to cyclothiazide might reflect small sequence differences between kainate and AMPA receptors over this region. At the position corresponding to the Ser/Asn site in AMPA receptors all 5 kainate receptor subunits express a glutamine residue. Substitution of a Gln at position 750 in both AflipS750Q and AflopN750Q essentially abolished sensitivity to cyclothiazide without altering agonist potency, indicating that although the introduction of Gln at position 750 does not confer the high sensitivity to kainate typical of wild type kainate receptors and which is seen in AMPA receptor chimeras containing kainate receptor sequences thought to encode the agonist binding site. To control for non-specific effects resulting from mutations at position 750 in AMPA receptors, we introduced an alanine at position 750; for this mutant potentiation by cyclothiazide was intermediate between that for GluRAi and GluRAo (data not shown), indicating that substitution with an amino acid other than Ser or Asn is not sufficient to prevent modulation by

cyclothiazide. To determine if the absence of kainate receptor sensitivity to cyclothiazide might arise from the presence of the conserved Gln residue at the position which in kainate receptor subunits aligns with the Ser/Asn site in AMPA receptors we constructed the mutant GluR6Q755S. In contrast to results obtained for the AMPA receptor mutant AiS750Q, the desensitization kinetics of control responses to 1 mM kainate for GluR6Q755S were not identical to those for wild type GluR6, with GluR6Q755S showing a reduced rate of onset of desensitization, and with less desensitization at equilibrium for GluR6Q755S than for wild type GluR6. However, in contrast to the cyclothiazide insensitive responses obtained for wild type GluR6, the rate of onset of desensitization for GluR6Q755S was slowed approximately five-fold in the presence of 100 μM cyclothiazide. Other functional properties characteristic of GluR6, including a high affinity for kainate, lack of sensitivity to AMPA, and pronounced block of desensitization by concanavalin A were maintained in GluR6Q755S.

Noncompetitive inhibition by 2,3-benzodiazepines. AMPA receptors show negative allosteric regulation by 2,3-benzodiazepines such as GYKI 53655 and GYKI 52466 which act as non-competitive antagonists. It has been hypothesized that cyclothiazide and GYKI 53655 act at a common site. Our identification of a serine residue that is critical for directing the interaction of cyclothiazide with AMPA receptors has allowed us to test whether the mutation of this residue to glutamine, which abolishes potentiation by cyclothiazide, can in addition block antagonism by 2,3-benzodiazepines, as would be predicted for action at a common site. We found that the S to Q mutation does not alter antagonism by 2,3-benzodiazepines, suggesting that the molecular determinants directing the interaction between GYKI 53655 and AMPA receptors are not identical to those controlling sensitivity to cyclothiazide. Additional support for this was obtained from analysis of the responses of AMPA receptor flip/flop splice variants, which despite differences in equilibrium desensitization and sensitivity to cyclothiazide, do not show differential sensitivity to 2,3-benzodiazepines. Of interest, experiments with AMPA receptors generated from forebrain poly A mRNA revealed greater sensitivity to 2,3-benzodiazepines than receptors generated by expression of recombinant AMPA receptors, possibly indicating the existence of an accessory protein or novel receptor subunit which modulates the activity of AMPA receptors.

Multiple populations of heteromeric AMPA receptors revealed by kinetic analysis of sensitivity to cyclothiazide. The rate of recovery from potentiation by cyclothiazide for two populations of cultured hippocampal neurons, P1 pyramidal-shaped neurons and P8 multipolar interneurons dissected from stratum radiatum area CA1 shows considerable variability suggesting the occurrence of multiple populations of AMPA receptors with different sensitivity to cyclothiazide. Recovery kinetics ranged from predominantly fast, flop-like fast dissociation, to predominantly slow, flip-like dissociation, but included combinations with both fast and slow components as well as intermediate dissociation rates which are not seen with homomeric receptors. To determine the likely molecular basis for this effect we examined the rate of recovery from potentiation by cyclothiazide for recombinant AMPA receptors formed by coexpression of GluRA and GluRB. Recovery kinetics were found to vary with both subunit and splice-variant composition. Recovery time constants were 0.2 sec for AoBo, 3.3 sec for AiBo and > 20 s for AoBi or AiBi. Experiments with GluRA coexpressed at flip/flop ratios from 1:1 to 1:4 revealed that the intermediate time constant is not a result of heteromerization between GluRA and GluRB, but reflects the flip/flop ratio in these heteromers, with recovery becoming faster in heteromers with high levels of flop subunits. The differential expression of recovery rate constants in hippocampal neurons likely reflects a similar mechanism.

Rectification mediated by cytoplasmic polyamines. When AMPA and kainate

receptor subunits were first cloned it was discovered that they contain an RNA editing site in an exon encoding part of the ion channel pore. The resulting codon change, which causes substitution of an Arg for Gln residue in the GluRB, GluR5 and GluR6 subunits, has profound functional consequences for ion flow. Receptors with a GLN residue at this site exhibit pronounced inward rectification, which is absent in subunits with an ARG residue introduced by RNA editing. Although functional studies on native AMPA receptors typically reveal outward rectification strongly inward rectifying AMPA receptor responses with high Ca^{2+} permeability have been observed in subpopulations of hippocampal interneurons and in Bergmann glial cells, as well as in native kainate receptors in embryonic hippocampal neurons and in glial progenitor cells. The mechanisms underlying inward rectification of glutamate receptor responses have only now been established, and suggest that the pore region of glutamate receptors has structural and functional characteristics similar to those for inward rectifying potassium channels, a gene family previously thought to have no homology to glutamate receptors. The strong inward rectification typical of whole-cell responses for the unedited forms of the kainate receptor GluR6 and the AMPA receptor GluRA is lost following the excision of outside-out membrane patches, suggesting that a cytoplasmic diffusible factor accounts for inward rectification of glutamate receptor responses. Consistent with this hypothesis, and similar to recent studies on inward rectifying potassium channels, the inclusion of the polyamine spermine in the patch-pipette solution restored strong inward rectification. In outside-out patches the IV relationship for glutamate receptor responses in the absence of spermine was close to linear at potentials between -100 to +50 mV, with weak outward rectification at membrane potentials between +50 and +100 mV. However, with spermine added to the internal solution, there was rectification with pronounced voltage dependent block over the membrane potential range -50 to +50 mV. However, different from results obtained for inward rectifying potassium channels there was essentially no block by 1 mM internal Mg^{2+} for glutamate receptors, indicating that the pore regions are similar, not not functionally identical. Further evidence for this was obtained from analysis of the effects of extreme depolarization. This revealed pronounced relief of block over the membrane potential range +50 to +100 mV for both AMPA and kainate receptors, strikingly different from the maintained responses observed for potassium channels. This most likely reflects differences in the pore sizes for potassium channels, which are narrow and have high selectivity, while glutamate receptors, which are known to show weaker selectivity, would be expected to have large pores, and perhaps allow polyamines to permeate at high membrane electric field strengths.

Estimation of intracellular polyamine concentrations. By using a Boltzman model for voltage dependent block by internal polyamines in outside patches we were able to determine values for the affinity (Kd values calculated for 0mV membrane potential) of spermine (5.5μM) , spermidine (25.4μM) and putrescine (1.2 mM) for kainate receptors generated by GluR6(Q). For AMPA receptors assembled from GluRAflip spermine affinity (1.5 μM) was slightly higher. To analyze the effect of membrane potential on receptor affinity for spermine, the Kd was determined by dose response analysis at membrane potentials of -80, -40 and +40 mV. At any given membrane potential, concentration-dependent block by spermine was well fit by a single binding site isotherm, with Kd values decreasing progressively with depolarisation, from 467 μM at -80 mV, to 1.15 μM at +40 mV. Comparison with data calculated for potassium channels at +50 mV reveals the affinity of spermine for GluRA flip to be only 3-fold lower than for the IRK1 and BIR10 inward rectifying potassium channels.

Knowledge of the affinity and voltage dependence of binding of spermine and spermidine were then used to estimate the cytoplasmic concentration of polyamines which would account for the rectification of whole-cell responses. Assuming both spermine and spermidine to be present in the cytoplasm at a

ratio of 1:3 (as determined by Watanabe and colleagues in biochemical experiments) we obtained an estimate for the free spermine and spermidine concentrations in intact cells of 51 and 153 μM respectively, in excellent agreement with the results of biochemical analysis which revealed that in mammalian cells the free concentrations of polyamines closely match these values, but are typically < 10% of total because RNA and DNA generate binding sites with high capacity and low affinity for spermine and spermidine. The latter observation most likely explains why the washout of rectification during whole cell recording is exceedingly slow. Our results help to explain a paradoxical observation concerning the high Ca2+ permeability but outward rectifying properties of AMPA receptor responses in patches removed from cortical interneurons. Based on the properties of recombinant AMPA receptors, which link Ca2+ permeability to inward rectification, it would have been predicted that native AMPA receptor responses with high Ca2+ permeability would show strong inward rectification. That this was not seen in outside out patches removed from cortical interneurons can be explained by loss of the cytoplasmic gating mechanism generated by polyamine mediated ion channel block. In contrast, the extent of inward rectification of glutamate receptor responses observed during whole-cell recording for hippocampal neurons expressing native kainate receptors, and for Bergmann glial cells which lack the GluRB subunit, agrees well with that expected for intracellular polyamine concentrations in the range 50 - 200 μM.

Significance to Biomedical Research and the Program of the Institute. The role of glutamate receptors in mediating excitatory synaptic transmission in all parts of the brain is now widely appreciated, but far from completely understood. Experiments with molecular cloning have identified multiple families of receptor subunits, many of which exist in several splice variants. The functional role of this diversity is still largely unknown but provides rich potential for regulation during development, and for therapeutic manipulation. In addition to a basic signalling function regulating information flow across synapses, excitatory amino acids are known to markedly influence the development of neuronal circuitry; to participate in the genesis of abnormal electrical activity associated with disease states; and to contribute to the pathophysiology of stroke and cerebral anoxia. The profound changes in central nervous system development which occur following the experimental manipulation of glutamate receptor function in embryos and in juvenile animals points to an important role in regulating growth of the human nervous system during childhood. Recent experiments with selective NMDA and AMPA receptor antagonists suggest that drugs targeted at both subtypes of the glutamate receptor family have anticonvulsant activity, and show neuroprotection in animal models of stroke. In addition, AMPA receptor antagonists produced a profound amelioration of motor dysfunction in animals models of Parkinson's disease. Preliminary gene mapping experiments to locate glutamate receptors in human chromosomes suggest that abnormalities in their expression could underlie CNS diseases of previously unknown origin; a clearer understanding of this issue is likely to emerge as maps are constructed for more subtypes.

A basic understanding of the mechanism of action of excitatory synaptic transmitters, and factors controlling their release, will be needed to understand the cellular basis of the complex behaviors regulated by the family of glutamate receptor ion channels. Research in the laboratory is directed towards this goal.

Proposed course:

(1) Currently we are making and analysing additional mutations of the residues in around the critical serine/asparagine site in the flip flop module of GluRA to probe this region for structural requirements involved in block of desensitization by cyclothiazide. Our major task during in the immediate future will be to assay these mutants by rapid perfusion using

transfected fibroblasts for desensitization properties in the presence and absence of cyclothiazide. Information gained from the effects of the amino acid side chain at position 750 on the kinetics of recovery from potentiation by cyclothiazide would be expected to give insight into the molecular nature and function of this site.

(2) Our analysis of the rectification properties of AMPA and kainate receptors raises the obvious question as to residues involved in binding polyamines. This will be addressed by constructing mutants in the second membrane domain, initially targeting conserved aspartate and glutamate residues surrounding the RNA editing site.

(3) The most likely explanation for our observation that the inward rectification of glutamate receptors shows relief with strong depolarization is permeation of the blocker at strong membrane electric field strengths. Currently little is known concerning the permeability properties of AMPA and kainate receptors. This will be addressed using organic cations of known sizes in an attempt to construct a realistic model which can describe the action of polyamines.

(4) As time and resources permit we still intend to analyse the molecular pharmacology of kainate receptors. Structure activity analysis of the responses of native kainate receptors on DRG neurons has revealed a hydrophobic pocket in the agonist binding site which is likely to contribute to the substantial difference in affinity for the action of 5-substituted willardiine derivatives on DRG versus hippocampal neurons. Because the subunit composition of kainate receptors in DRG neurons has not been established it is not known whether this represents a general feature of kainate preferring receptors from the family GluR-5 through GluR-7, whether coassembly with subunits from the KA-1 KA-2 family is required or alters the relative selectivity of action of individual willardiines. To understand more about agonist action at kainate receptor subtypes dose response curves will be obtained for the key compounds 5-fluoro, 5-iodo, and 5-methylwillardiine, using oocytes treated with concanavalin A to block desensitization, and expressing GluR-5Q, GluR-5/KA-1, GluR-5/KA-2, GluR-6Q, GluR-6R, GluR-6/KA-1, GluR-6/KA-2. In addition the same agonists will be tested in oocytes expressing the AMPA preferring subunit GluR-A flip, using cyclothiazide to block desensitization.

Publications

Benveniste, M and Mayer, ML. Trapping of glutamate and glycine during open channel block of rat hippocampal neuron NMDA receptors by 9-aminoacridine. Journal of Physiology 1995;483:367-384.

Bowie, D and Mayer, ML. Inward Rectification of Both AMPA and Kainate Subtype Glutamate Receptors by Intracellular Polyamine Mediated Ion Channel Block. Neuron 1995 (In Press).

Dani, JA and Mayer ML. Structure and function of glutamate and nicotinic acetylcholine receptors. Current Opinion in Neurobiology 1995;5:310-317.

Mayer, ML, Partin, KM, Patneau, DK, Wong, LA, Vyklicky, L Jr., Benveniste, MJ and Bowie, D. Desensitization at AMPA, Kainate and NMDA Receptors. In: Excitatory Amino Acids and Synaptic Function. Ed. H. Wheal and A. Thomson. Academic Press (In press).

Mayer, ML, Benveniste, M and Patneau, DK. Molecular pharmacology of NMDA receptors. In: The NMDA Receptor. 2nd Edition. Ed. G.L. Collingridge and J.C. Watkins. Oxford University Press, 1994;132-146.

Partin, KM, Bowie, D and Mayer, ML. Structural determinants of allosteric regulation in alternatively spliced AMPA receptors. Neuron 1995;14:833-843.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01205-03LCMN

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Cellular and Synaptic Physiology of Hippocampal Interneurons

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

C. J. McBain, Ph.D. Senior Staff Fellow;NICHD-LCMN 12 months

Others:

Lei Zhang, Ph.D. Visiting Fellow, NICHD-LCMN 7months
Gianmaria Maccaferri, Ph.D. Visiting Fellow, NICHD-LCMN 9 months
Jing Du, Ph.D. Visiting Fellow, NICHD-LCMN 12 months
Adaora Chikwendu, Pre-IRTA Fellow, NICHD-LCMN 10 months

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cellular and Molecular Neurophysiology

SECTION

Unit on Cellular and Synaptic Physiology

INSTITUTE AND LOCATION

NICHD, NIH Bethesda MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.1	3.3	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human ☐ (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 project is to characterize the properties of voltage gated channels and synaptic transmission of hippocampal inhibitory neurons in the developing brain and how these properties impact hippocampal function under both physiological and pathological conditions. Although much is known about their neurochemistry, their role in the local circuits and the basic electrophysiological properties of inhibitory interneurons, little is known about the specific ionic or ligand gated channels expressed on this highly divergent population of cells. A major part of our effort is to understand the ionic mechanisms which regulate the activity of these cells and how these mechanisms impact hippocampal function using patch clamp, immunohistochemical and molecular techniques. Our work over the past year has focussed on particular populations of inhibitory neurons of the CA1 stratum oriens/alveus and pyramidal cell layers. Particularly we have characterized the complement of potassium channels present on these cells using both a combined electrophysiological and immunohistochemical approach. We have determined the roles of voltage-gated currents in st. oriens-alveus interneurons in determining the action potential and firing patterns of these cells. In addition we have investigated the developmental expression and functional role of the K channel 2subunit Kv3.1 in parvalbumin containing interneurons of st. pyramidale.The role of various types of interneuron during the plastic phenomena of long term depression and potentiation using perforated patch and paired reording techniques has also been studied .

Project Description

Objectives:

The aim of this project is to characterize the properties of voltage gated channels and synaptic transmission of hippocampal inhibitory neurons in the developing brain and how these properties impact hippocampal function under both physiological and pathological conditions. Although much is known about their neurochemistry, their role in the local circuits and the basic electrophysiological properties of inhibitory interneurons, little is known about the specific ionic or ligand gated channels expressed on this highly divergent population of cells. A major part of our effort is to understand the ionic mechanisms which regulate the activity of these cells and how these mechanisms impact hippocampal function.

Methods Employed:

Whole cell patch clamp studies under current and voltage clamp configurations have been performed from visually identified inhibitory neurons of the st. oriens /alveus, st. lacunosum-moleculare and stratum pyramidale, maintained in hippocampal slices from neonatal animals. In addition outside-out macroscopic patches have been excised from the soma of interneurons held in the slice preparation in order to provide an accurate estimation of the activation and inactivation profiles of novel potassium channels expressed on these cells. Simultaneous whole cell patch clamp from interneurons and pyramidal neurons together with field recordings have been made in studies observing the role of interneurons in synaptic plasticity. Perforated patch techniques are now employed in studies investigating long-term potentiation in three different subtypes of interneuron. Combined electrophysiology, morphology and immunohistochemistry techniques are used on single inhibitory cells in the slice preparation to correlate interneuron physiology with the expression of particular potassium channel subunits. Patch clamp recordings from primary cultures of st. lacunosum-moleculare interneurons are being used to characterize the complement of potassium channels expressed on these cells.

Major Findings:

1. Passive propagation of LTD to st. oriens-alveus inhibitory neurons modulates the temporoammonic input to the hippocampal CA1 region.

In order to study the role of st. oriens cells during a long-term depression (LTD)-inducing protocol **Gianmaria Maccaferri** has made whole cell patch clamp recordings from horizontally oriented st. oriens interneurons while simultaneously monitoring the field potential (fPS) of CA1 pyramidal neurons in the hippocampal slice. Morphological characterization was determined by post hoc biocytin staining techniques. Application of a low-frequency stimulation protocol to st. radiatum afferents has been shown to be a reliable method to elicit homosynaptic LTD in CA1 pyramidal cells. Under our experimental conditions, this protocol induced LTD of both the fPS and St. oriens/alveus EPSPs. The simultaneous depression of the fPS and st. oriens/alveus EPSP could be explained either by the induction of an identical phenomenon at the feed-forward synapse between the St. radiatum afferents and the St. oriens interneuron or alternatively, St. oriens/alveus EPSPs could be generated by the feed-back recurrent collaterals of CA1 pyramidal cells. In the former hypothesis st. oriens EPSPs would undergo a real, direct LTD process, while in the latter the effect would be the result of the feed back circuitry synapses onto these interneurons and not a direct induction of LTD.

If the observed LTD of st. oriens/alveus was a direct cellular phenomenon similar to that described in CA1 pyramidal cells, its induction should be Ca^{2+} dependent. Accordingly, strong buffering of $[Ca^{2+}]_i$ levels in the St. oriens/alveus should

disrupt the observed synaptic plasticity. Under conditions to buffer intracellular calcium levels, both LTD of the fPS and the St. oriens/alveus EPSPs were still observed. Moreover, application of a repeated high frequency tetanic stimulus after 30 min. of stable depression could restore or even potentiate the original synaptic response. This experiment ruled out the possibility that the LTD elicited in the St. oriens cells was a direct cellular phenomenon. Alternatively it may simply result from a reduction of the feedback excitation of St. oriens cells by pyramidal neurons due to the direct depression of the CA1 pyramidal neurons as suggested above.

Analysis of the temporal relationship between the peak of the fPS and the onset of the interneuron EPSP revealed that the onset of the St. oriens EPSP always occurred after the peak of the fPS. Since the field population spike reflects the discharge of a large population of pyramidal cells, we conclude that under our experimental conditions horizontal st. oriens interneurons receive their excitatory drive from CA1 pyramidal neurons and are involved in pure feedback mechanisms of inhibition. This was an extremely important observation since until now interneurons of the St. oriens-alveus subfield have been considered to additionally receive feedforward afferents from the CA3 pyramidal subfield.

To determine whether the monosynaptic input to the st. oriens interneurons could undergo cellular LTD we directly stimulated the axon collaterals of the CA1 pyramidal neurons which drive the excitation of the St. oriens/alveus interneurons. Under these conditions pure antidromic fPS were recorded from St. pyramidale however the low-frequency protocol consistently failed to elicit any form of synaptic plasticity suggesting that st. oriens cells do not demonstrate a direct form of LTD. We next made perforated patch recordings to prevent the possible washout of some essential intracellular component necessary for the induction of LTD. Using the antibiotics nystatin, amphotericin and gramicidin to "perforate" the area of membrane under our electrodes we were still unable to identify any direct form of cellular LTD in these neurons. This is the first demonstration of a network effect that results in a long-term modification of the excitatory synaptic responses of CA1 interneurons.

This form of plasticity which we refer to as "passively propagated plasticity" could play an important role in the regulation of information processing in the hippocampal circuit. Morphological analysis of the cells used in the present study show that recordings were obtained from cells with a horizontal dendritic arborization restricted to the St. oriens-alveus layer and whose axons terminate on the distal dendrites of pyramidal cells in st. lacunosum-moleculare. This region of the pyramidal neuron dendritic tree receives direct excitatory input from the temporoammonic pathway arising in the entorhinal cortex (EC). We next demonstrated that the induction of LTD in CA1 pyramidal neurons by Schaffer collaterals, not only depresses synaptic activity onto pyramidal neurons, but depress excitation of the horizontal interneurons of St. oriens which results in a reduction of their inhibitory output onto the distal portion of the pyramidal neuron dendrites. The net result of the LTD formation acts as a "switch" in the source of synaptic input onto CA1 pyramidal neurons from the CA3 region to the EC. This allows afferent activity to bypass the classic trisynaptic circuit and to directly activate the distal dendrites of the CA1 pyramidal cells by a mechanism involving the disinhibition of the temporoammonic pathway.

<u>2. Do direct forms of synaptic plasticity occur on any of the subpopulations of CA1 interneuron?</u>

During the course of performing the above experiments two reports were published stating that in contrast to our observations, cellular plasticity could indeed be observed in two distinct populations of CA1 interneurons. Neither of these studies sufficiently controlled for the possibility of a passive propagation of plasticity induced in the CA1 pyramidal neurons to these types of interneurons. To directly address this possibility and to determine whether any other subpopulation of interneuron with the CA1 subfield did indeed posses cellular

plasticity, we designed experiments utilizing perforated patch clamp techniques. Using this technique **Gianmaria Maccaferri** was able to discriminate between direct cellular forms of plasticity and those induced by the passive propagation of plasticity from the principal pyramidal neurons. Using a pairing protocol (to elicit direct forms of plasticity) followed by a tetanic stimulation paradigm (to elicit a large population plasticity) we identified a cellular form of long-term potentiation in only one of the three morphologically identified cell types so far tested. In an interneuron type located in stratum radiatum, the pairing protocol induced robust long lasting cellular LTP. In contrast, a distinct population of st. radiatum interneurons lacked both cellular and "passively propagated" forms of LTP. In all interneurons of the stratum oriens we were unable to find a cellular form of LTP but similar to the data reported above these cells only posses a "passively propagated" form of LTP. Experiments are in progress to determine the underlying mechanisms involved in the cellular form of LTP observed in the stratum radiatum interneurons.

3. Potassium conductances underlying action potential repolarization and afterhyperpolarization in rat CA1 hippocampal interneurones

Lei Zhang and **Chris McBain** have determined the role of a variety of potassium conductances in action potential repolarization and afterhyperpolarization. Whole-cell current clamp recordings were made from visually identified interneurons in hippocampal slices (300μm) of neonatal rats (11-20 days old). Biocytin was introduced during the whole cell recording to permit visualization of the interneurons of interest. 4-Aminopyridine prolonged the action potential repolarization. The effects of 4-AP persisted in Ca^{2+}-free conditions. Action potentials evoked from hyperpolarized potentials possessed an increased rate of repolarization. These data suggest an involvement of the rapidly activating transient current, I_A, in spike repolarization. Action potential duration was increased in the presence of Ca^{2+}-free, Cd^{2+}-containing solution, iberiotoxin or 1mM TEA. The fast component of the AHP was attenuated by these agents suggesting that the Ca^{2+}-activated K^+ conductance I_C underlies both the spike repolarization and fast AHP.

In Ca^{2+}-free conditions, TEA (>1mM) dose-dependently prolonged the action potential duration by blocking a late conductance in action potential repolarization, suggesting a role for the sustained current, I_K.
The slow AHP was attenuated by Ca^{2+}-free medium, apamin or the Ca^{2+} chelator EGTA, suggesting a role for the Ca^{2+}-activated K conductance, I_{AHP}. We conclude that action potential repolarization and AHPs of St. oriens-alveus interneurones result from the activation of pharmacologically distinct, temporally overlapping potassium conductances. These findings were then correlated with the voltage clamp data obtained in a parallel study from the same neuron class.

4. Voltage-gated potassium currents in stratum oriens-alveus inhibitory neurones of the rat CA1 hippocampus.

Lei Zhang and **Chris McBain** have recorded voltage-activated K^+ currents from visually identified inhibitory interneurones of the CA1 St. oriens-alveus region in neonatal rat hippocampal slices using outside-out patch and whole-cell voltage clamp techniques. Voltage-activated outward K^+ currents comprised both a transient and a sustained component when elicited from a holding potential of -90 mV. Tail current analysis of both transient and sustained current reversal potentials showed that both outward currents were predominantly carried by potassium ions. The transient current, I_A, was activated with a time to peak within 5 ms, inactivated with a time constant ~15 ms at 0 mV and possessed half activation at -14 mV. Half-inactivation of the transient current occurred at -71 mV. At -90 mV, the transient current recovered from inactivation with a time constant of 142 ms. The properties of the transient current are virtually identical to the transient current observed in the principal pyramidal neurons

of the hippocampus. Activation of currents from a holding potential of -50mV permitted isolation of a sustained current, I_K. In Ca^{2+}-free conditions the sustained current showed rapid activation, reaching about 80% of its maximum within 1.5 ms, and showed little inactivation during 1 second depolarizing steps. The majority of sustained outward currents showed no voltage-dependent inactivation. In ~20% of cells, a slow time-dependent inactivation of the sustained current was observed, suggesting the presence of a second type of sustained current in these cells. A Ca^{2+}-dependent K^+ current comprised a significant portion of the total sustained current, this current was activated at voltages positive to -30 mV and showed no time dependent inactivation over a 1 second depolarizing step. This current component was removed in Ca^{2+}-free conditions or by iberiotoxin. Low concentrations of 4-AP (50 μM) attenuated both the transient and sustained current components recorded in a Ca^{2+}-free solution. Higher concentrations of 4-AP (< 10 mM) were without further effect on the sustained current but completely blocked the transient current with an IC_{50} of 1.8mM. TEA blocked the sustained current with an IC_{50} of 7.9mM without significantly reducing the transient current. Both current components were resistant to dendrotoxin (500nM). The properties of the sustained current components are distinct from the sustained currents recorded in pyramidal neurons suggesting that these currents in part determine the fast-spiking properties of this interneuron population

5. Properties of the hyperpolarization-activated current (Ih) in CA1 hippocampal St.-oriens-alveus interneurons.

Despite the fact that interneurons constitute only about the 15 % of the total hippocampal cell population, their role in the regulation of excitability is believed to be crucial. The intrinsic electrophysiological properties of these cells play an important role in the generation of their spontaneous activity. Among the many different kind of voltage-operated conductances which are present in these interneurons, the hyperpolarization-activated current (Ih) is particularly interesting in this respect. In fact, Ih has been shown to be involved in the regulation of the firing pattern of thalamic neurones, is present in many different neuronal preparations, and was firstly described as the current responsible for spontaneous cardiac pacemaking. In order to study the basic properties of this current in St. oriens-alveus inhibitory neurons, **Gianmaria Maccaferri** has made whole-cell voltage-clamp recordings from visually identified interneurons in the hippocampal "in vitro" slice preparation. Ih was activated as an inward current by hyperpolarizing voltage pulses in the range -60 to -130 mV from a holding potential of -40 mV. The activation kinetics was very slow (τ ~ 200 ms at -130 mV) and no inactivation properties were detected. The activation curve showed a threshold at ~ -60 mV, was saturated at ~ -130 mV and had a mid-activation point at ~-90 mV. Consistent with its mixed ionic nature (Na^+ and K^+), its reversal potential was ~-30 mV, as obtained by extrapolation of the fully activated Ih I/V relationship. Finally, complete blockade of the Ih conductance could be achieved after cell exposure to external Cs^{2+} (0.25-5 mM) or the specific Ih antagonist Zeneca ZD7288 (100 um). To determine whether Ih played a role in the regulation of any action potential firing properties in these cells, recordings were made under current clamp conditions in the presence of these selective Ih antagonists. Following blockade of excitatory synaptic transmission with DNQX (20 μM), application of either Cs or ZD7288 caused a dramatic decrease in the rate of spontaneous firing, together with a large membrane hyperpolarization. In conclusion, these experiments reveal the presence of the hyperpolarization-activated current in st. oriens-alveus interneurons and demonstrate its role in determining the spontaneous firing pattern. Finally, experiments are in progress to determine any possible modulation of this conductance by putative neurotransmitters, which could add a new tool in the regulation of the physiology of these interneurons.

6. Developmental Expression and Functional Characterization of the Potassium Channel Subunit Kv3.1b in Parvalbumin-Containing Interneurons of the Rat

Hippocampus.

Previous *in situ* hybridization studies of the distribution of the *Shaw* (Kv3) K channels have shown that two members of this subfamily, Kv3.1 and Kv3.2, are expressed in low levels in the principal cells of the hippocampus i.e. the pyramidal cells. The expression of these subunits is diffuse and only a small number of densely labeled cells, scattered throughout the hippocampus were observed, tentatively identified as interneurons. In the CA1 region, cells labeled with Kv3.2 probes were more abundant in the st. oriens and occasionally along the border of st. pyramidale and were rarely seen in the st. radiatum or lacunosum. In contrast cells hybridizing with Kv3.1 are seen both along the stratum radiatum-pyramidale border and in stratum oriens/alveus. The significance of the selective expression of these potassium channel subunits into the inhibitory neuron population is at present unclear.

In a study led by **Jing Du**, the expression of the voltage-gated potassium channel subunit Kv3.1b in the developing hippocampus was determined by immunoblot and immunohistochemical techniques. Kv3.1b protein was first detected at postnatal (P) day 8 using a specific polyclonal antibody obtained from Dr. Bernardo Rudy (NYU). The Kv3.1b-immunopositive cell number per tissue section reached a maximum at P14 and was maintained through P40. In contrast, the Kv3.1b protein content of isolated membrane vesicles in immunoblots progressively increased through P40 suggesting an increase in Kv3.1b content per cell throughout this time period. Kv3.1b protein was selectively expressed in the somata, proximal dendrites and axons of cells lying within or close to the pyramidal cell layer consistent with their being GABAergic inhibitory interneurons. Kv3.1b was present in ~80% of parvalbumin-positive interneurons. The developmental onset of Kv3.1b and parvalbumin immunoreactivity was identical. In contrast, Kv3.1b was largely absent from the subset of somatostatin-positive inhibitory interneurons, which are known to be a distinct class of inhibitory interneurons from the parvalbumin containing cells. Electrophysiological recordings were made from St. pyramidale interneurons whose morphology and Kv3.1b-positive immunoreactivity were confirmed *post hoc*. Outward currents in these cells had voltage-dependent and biophysical properties resembling those of channels formed by Kv3.1b. The current blocked by low concentrations of 4AP showed marked inactivation, suggesting that Kv3.1b may coassemble with other members of the Kv3 subfamily. In current clamp recordings, concentrations of 4AP which blocked the current through Kv3.1b channels allowed us to tentatively assign a role to Kv3.1b containing channels in action potential repolarization. The developmental onset of parvalbumin-immunoreactivity in the cells has long been associated with the acquisition of their "fast-spiking" characteristics. In the present study however, we have demonstrated that the onset of expression of Kv3.1b is coincident with acquisition of parvalbumin immunoreactivity. This suggests that the fast-spiking activity of these interneurons is not determined by the onset of parvalbumin-immunoreactivity *per se* as previously thought, but may instead be determined by the onset of Kv3.1b expression, a channel which may have a direct involvement in the determination of the action potential characteristics in these cells. The observation that the Kv3.1 subunit is preferentially expressed in many cells known to be GABAergic and possess high firing rates is probably not coincidental. The activation of Kv3.1 channels may act to keep the action potential short. In addition, the calcium-binding protein, parvalbumin, could serve to buffer the intracellular calcium concentration. Both factors could act to prevent the accumulation of Ca^{2+} and the activation of Ca^{2+}-activated outward currents. Furthermore, the rapid deactivation of Kv3.1 channels, will help decrease the membrane conductance soon after the peak of the spike. Taken together these electrical properties of these neurons will ensure a high frequency of action potential firing. More likely, the observation that Kv3.1b-containing channels help to shape the action potential of "fast-spiking" interneurons will also extend to action potentials of other cells strongly expressing Kv3.1b or other Kv3 related subunits. These data demonstrate that Kv3.1b is developmentally regulated in a specific subpopulation of hippocampal interneurons and that channels containing this subunit may be a major determinant in imparting "fast-spiking" characteristics to these and other

cells throughout the CNS containing the Kv3.1b subunit.

7. Two distinct "delayed-rectifiers" determine the voltage-dependent potassium current phenotypes in st. lacunosum-moleculare interneurons in primary culture

Adaora Chikwendu has established primary cultures of interneurons derived from the st. lacunosum-moleculare subfield of the CA1 hippocampus. Whole cell, voltage-clamp recordings were made from cells 4 - 8 days *in vitro*. In cells possessing a bipolar morphology, "delayed rectifier" outward K^+ currents were activated in all cell types at test potentials positive to -40mV (V_H = -60mV). One of two current phenotypes was usually observed in any given cell which were termed "sustained" and "slowly-inactivating". The time to peak of the sustained current was identical at all test potentials. In contrast the time to peak of the slowly inactivating current was markedly voltage dependent, decreasing at more positive test potentials. The voltage-dependence of activation of either current phenotype, could be fit by the Boltzmann equation both of which possessed a half activation close to +4mV (n = 52 and 38 for each current type). These outward currents showed minimal (~20%) voltage-dependent inactivation (Vhalf = -65 and -67mV). In cells possessing predominantly "slowly-inactivating" outward currents, 4-AP dose-dependently (10μM-30mM) blocked a fraction of the total outward current with an IC_{50} of 312 ± 101μM. At a maximal concentration of 30mM, 4-AP selectively blocked 41% of the total current. The block by 4AP was not voltage-dependent at any concentration tested, and blocked an identical fraction of current at all test potentials. The current was however use dependent and the total block was only observed following numerous steps to a given test potential. Isolation of the 4-AP sensitive component yielded a "slowly-inactivating" current with a positive voltage dependence of activation (Vhalf = +10.9 ± 1.7mV). The current remaining in 30mM 4-AP was "sustained" and also possessed a positive Vhalf of 7.2 ± 1.2mV. In cells where a "sustained" outward current dominated in control conditions, 4-AP (30 mM) again removed a "slowly-inactivating" component which represented only 23% (n = 5) of the total outward current. TEA dose-dependently (10μM - 30mM) blocked the total outward currents with an IC_{50} of 142 ±47μM. In 30mM TEA, 85% of the total outward current was blocked regardless of whether the current phenotype was predominantly "sustained" or "slowly inactivating". However, low concentrations of TEA (10 - 100mM) selectively removed a "slowly-inactivating" current component (Vhalf = 10.1 ± 1.3mV, n=5). The block by TEA, like 4AP was not voltage-dependent. Inclusion of TEA (1 and 10mM) in the internal solution caused an apparent increase in the fraction of current blocked by external 4AP, suggesting that these two antagonists act at different sites on the ion channel. In contrast, external TEA blocked a similar fraction of current whether TEA was included in the internal solution or not, suggesting that external TEA may displace internal TEA from its site of action with the ion channel. Inclusion of 4AP in the internal solution caused an apparent reduction in the fraction of current blocked by external TEA suggesting that these agents are blocking a similar current component. Internal 4AP however was without effect on the current fraction blocked by external 4AP suggesting a mechanism similar to that when TEA was present both on the inside and outside of the channel.I n conclusion, bipolar st. L-M interneurons have at least two "delayed rectifier" currents with similar activation profiles which can be differentiated based on their sensitivity to 4-AP and TEA. The differing proportions of either current component usually determines the overall current phenotype in any given cell.

Proposed Course

The future directions of this research program are two-fold. First we propose to continue our studies involving the synaptic plasticity properties of interneurons within the hippocampal slice formation, secondly to continue our studies on the characterization of voltage-dependent potassium currents present on hippocampal interneurons

1. An understanding of the role of each interneuron type in various forms of synaptic plasticity is an important step in elucidating the precise

roles of these cells in the network properties of the hippocampal formation. To this end we are presently studying the roles of several distinct interneuron types of the CA1 subfield during the formation of long-term potentiation. A detailed pharmacological investigation will be made on those cells to determine whether or not this type of LTP is calcium and NMDA receptor dependent. These studies will then be extended to characterize the spiny and aspiny neurons of the dentate hilar region. Since little more than the basic cellular properties of these important cell types are known, an understanding of the types of long-term plastic changes associated with these cells will be important in determining the permissive role these cells play in determining information flow into the tri-synaptic circuit.

2. We propose to use paired whole cell patch clamp recordings from functionally connected interneurons of st. oriens and alveus and pyramidal neurons. Since we are the first lab to demonstrate that horizontal interneurons of the st. oriens are driven purely by the recurrent collaterals of CA1 pyramidal neurons we propose to study this selective innervation more carefully using recordings between connected pairs under both physiological and high-K+ seizure inducing conditions. The use of pairs of whole cell voltage and/or current clamp recording from these cells will permit the unequivocal identification of their role in the synaptic circuit, the "strength" of the synaptic transmission between these two cell types and the identity of the glutamate receptors involved.

3. Since one of our long term goals is to elucidate the specific role of K^+ channels in interneuron physiology, we will continue the electrophysiological characterization of specific K^+ channel conductances present on a variety of interneuron subtypes concomitant with immunological detection of the appropriate subunit on the cells from which electrophysiological recordings have been made. In a continuation of our Kv3.1 subunit study on basket cells of the CA1 pyramidal cell type, we will characterize the role of the subunit Kv1.4 which would appear also to be expressed on these cells. We anticipate having a functional antibody to this channel in the near future and will perform experiments similar to those reported above for Kv3.1. The identification of Kv1.4 in these cells is an important one since its gating mechanism is directly modulated by changes in the extracellular potassium concentration. This property is peculiar to channels formed by this subunit and will likely have important consequences during electrographic activity. To determine any potential role for this channel in regulating interneuron activity during electrographic seizure activity we will directly monitor the properties of this channel in the high-K^+ model of electrographic seizure already used in this laboratory.

4. In a continuation of the study involving potassium currents in primary cultures of st. lacunosum-moleculare interneurons, we propose to interrupt putative potassium channel function in these cells with the delivery of antisense oligonucleotides. In addition, a parallel study will investigate antisense oligonucleotide knock-out of K channels subunits in cultures of cerebellar granule cells (cells rich in the potassium channel subunits Kv3.1 and Kv1.4). Initially antisense oligonucleotides targeted to both Kv3.1 and Kv3.2 will be synthesized and used to block expression of these channels in both these primary cultures since both subunits are thought to be strongly expressed in these cell types. Immunohistochemical and molecular approaches will determine the level of expression of down-regulated K+ channels on these cells. Subsequent electrophysiological experiments will determine the physiological consequences of preventing the protein synthesis of these K^+ channels. As the expression patterns of K^+ channels subunits on hippocampal interneurons is characterized, more relevant K^+ channel subunits as targets for antisense knockout will be identified and tested.

Significance to Biomedical Research and the Program of the Institute

Inhibitory interneurons represent ~10% of the total neuron population of the hippocampus proper and several distinct subpopulations of cell type have been characterized in this lab and others. The high levels of tonic inhibition present within the hippocampal formation under physiological conditions highlights the importance of an understanding of interneuron physiology. The fast spiking characteristics of the st. oriens interneurons present the principal neurons of the hippocampus; the pyramidal neuron, a 10 fold higher level of inhibition synaptic input than excitatory input. The critical role inhibitory neurons play in the regulation of hippocampal excitability is underscored by the large number of existing seizure models which compromise the GABAergic inhibitory mechanism. Thus, an understanding of the basic physiological and pathological properties of these neurons may elucidate mechanisms which could be useful for the management or the prevention of seizure activity in the hippocampus. To this end we have focused on the compliment of voltage gated potassium channels on these cells and their roles in various forms of synaptic plasticity. We anticipate that the characteristic physiological properties of these cells are due largely in part to the compliment of voltage gated channels present on these neurons. It is a long term goal of this lab to exploit the novel potassium channels present on these cells responsible for the regulation of their excitability.

Publications

Journal Articles

Desai, M, McBain, CJ, Kauer, J & Conn, J. Metabotropic glutamate receptor-induced disinhibition is mediated by reduced transmission at excitatory synapses onto interneurons and inhibitory synapses onto pyramidal cells. Neuroscience Letters 1994;181:78-82.

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Zhang, L & McBain, CJ. Potassium conductances underlying action potential repolarization and afterhyperpolarization in stratum oriens-alveus inhibitory neurones of the rat CA1 hippocampus. Journal of Physiology 1995, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 1206 02 LCMN

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
RECEPTOR MEDIATED CALCIUM SIGNALLING IN GLIA AND NEURONS

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

P.I.:	Dr. J. T. Russell	Head,Section on neuronal secretory systems	LCMN, NICHD 12 months
Others:	Ms. L. A. Holtzclaw	Biologist	LCMN, NICHD 12 months
	Dr. C. A. Shepperd	NRC Fellow	LCMN, NICHD 12 months
	Dr. N. Darvish	Visiting Fellow	LCMN, NICHD 10 months
	Dr. P. B. Simpson	Visiting Fellow	LCMN, NICHD 8 months

COOPERATING UNITS (if any)

Dr. V. Gallo	Head, Unit on Neurobiology	LCMN, NICHD
Dr. D. C. Klein	Head Section on Neuroendocrinology	LDN, NICHD

LAB/BRANCH
Laboratory of Cellular and Molecular Neurophysiology

SECTION
Section on Neuronal secretory systems

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.0	3.5	0.5

CHECK APPROPRIATE BOX(ES)

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

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

This Section aims to understand the principles and mechanisms that govern intracellular calcium signalling in glial cells and neurons. Although neurons are the central players in cellular communication and information processing in the brain, recent discoveries of glial cell types, excitability and signalling properties have shown that glial cells may play an important role in long distance signalling in the brain. Glial cells do not show electrically excitable properties similar to neurons. They do, however, possess a form calcium based excitability in response to activation of neurotransmitter receptors. Most receptor mediated regulation of physiological processes utilize cellular calcium signals as triggers. In glia, like in other cells, such calcium signals have recently been recognized to have complex temporal and spatial characteristics. The research focus of the Section includes studies on the signal transduction mechanisms of various receptor systems in glial cells as well as the cell biology of calcium signalling in both glial cells and neurons. One objective is to understand cellular processes that control signal transduction mechanisms for Ca^{2+} signalling and cellular processes that regulate temporal and spatial signalling within cells and between cells at the molecular level. A second objective is to understand the processes that regulate calcium based excitability in glial cells resulting from neuronal activity. Thirdly, we would like to understand the changes in the signalling mechanisms that occur in reactive astrocytes during brain injury. Finally, this Section aims to describe in detail the ionic basis for cellular excitability in pineal cells.

Project Description:

Objectives: The aim of this Section is to describe in detail glial cell signalling in response to neuronal activity in the normal brain and during brain injury. During neuronal activity, it is believed that astrocytes monitor synaptic activity and respond to released transmitters with intracellular calcium signals by the activation of metabotropic receptor systems. These signals are propagated as waves within single astrocytes and are transmitted through a complex network of glial cells connected by gap junctions over long distances, albeit at rates considerably slower than (approximately 1000 times) neuronal signals. This has been termed calcium based excitability. This Section has characterized such signals in tissue cultured glial cells in some detail and has described a mechanism of saltatory propagation of calcium waves within cells. A number of other laboratories also have studied such glial cell signals. All these experiments, however, have been carried out on cells in tissue culture, as purified glial cell cultures or as mixed cultures with neurons. The singular question that remains unanswered is, if such signalling occurs in the intact brain during normal brain function. One set of experiments were carried out in a more intact preparation of hippocampal slices in organotypic culture. Studies on acutely isolated brain slices are not yet available. This Section proposes to examine if glial cells *in situ* participate in signalling as well as to study the cellular mechanisms and processes that support such long distance signalling by glial cells. Secondly, we propose to examine if changes in glial phenotype following neuronal injury (reactive gliosis) result in changes in the signalling modalities in these cells. During the year 1995- '96, we aim to study the following aspects of signal transduction and glial cell calcium signalling: (i) Investigate the receptor repertoire and mechanisms of calcium signalling in the different types of glial cells isolated from rat brain. (ii) Investigate the calcium release process by the endoplasmic reticulum membrane system in glia. (iii) Investigate the structure of endoplasmic reticulum in astrocytes with reference to its Ca^{2+} signalling function. (iv) Investigate calcium based excitability in neuronal-glial networks *in situ*. (v) Investigate the differences in calcium signalling in reactive astrocytes compared with normal cells. (iv) Characterize the ionic conductances that form the basis for cellular excitability in rat pineal cells with particular focus the cyclic-GMP gated Ca^{2+} channel activated by VIP receptors.

Methods Employed:

Primary culture of astrocytes isolated from neonatal rats, high resolution fluorescence microscopy, digital image processing, immunocytochemistry, electrophysiological recordings.

Major Findings:

A. Calcium signalling in astrocytes.

1. Agonist-induced calcium waves

This Section has studied the qualitative and quantitative aspects of cytoplasmic calcium wave generation and propagation within single astroglial cells isolated from new born rat cerebral cortices. Stimulation of astrocytes with glutamate or norepinephrine elicited intracellular calcium signals as waves that always originated at an initiation locus and propagated through the cell. Our analysis has revealed that agonist induced cytoplasmic Ca^{2+} waves initiated at a single locus in a cell and are propagated in a non-linear fashion with regenerative amplification at specific cellular loci. High resolution analysis of the

cellular loci in a number of experiments showed that the diameter of these regenerative loci ranged from 7 μ m to 15 μ m (12 ± 4 μ m, mean ± S.D., n=28). Repeated application of an agonist always initiated Ca^{2+} waves at the same locus with an identical spatio-temporal oscillatory pattern. In any given cell the wave initiation locus and amplification sites remained invariant, We also found that the active loci have independent oscillatory properties and can oscillate at very different frequencies and possess different latencies. Nevertheless, these loci are influenced by diffusional waves reaching them from adjacent cellular regions and thus act as partially coupled oscillators. The local waves generated at the various cellular loci, when out of phase with each other, and are propagated in opposite directions, collide and annihilate.

We extended a previously developed model of calcium oscillations in excitable and non-excitable cells to include spatial diffusion of calcium in a cell with discrete active loci of wave amplification. This model was then used to analyze experimental data and to gain insight into the mechanism of wave collisions and annihilations. Qualitative and quantitative comparisons were made of the computational results with calcium waves measured in astrocytes. The measured and calculated calcium dynamics are strikingly similar in many respects. Furthermore, the model predicted that the intrinsic latencies of each locus are gives the appearance of wave behavior. Nearly simultaneous excitation of the entire cell at high agonist concentrations argues against the idea of a "wave" through an excitable medium, but rather suggests differences in the kinetic behaviour of the different oscillators. No matter which terminology is used, the model predicted behavior consistent with the experimental data.

2. Endoplasmic reticulum structure in astrocytes

The realization that the endoplasmic reticulum might have specialized regions prompted us to study the distribution of $InsP_3$ receptors (IPCR) on the ER of astrocytes. In addition, we wanted to examine the extent of endoplasmic reticulum within astrocytes using ER specific dyes. IPCR was localized by immunocytochemistry and the ER membrane was fluorescently labelled using Di-O-C_6-3. Carol Sheppard has completed the initial phase of this work and she is currently preparing a manuscript for publication.

We obtained a number of different antibodies against the different types of IPCR from different laboratories in order to study receptor distribution in astrocytes using immunocytochemical techniques. We asked the following questions: (1) What is the IPCR distribution in the ER of tissue cultured astrocytes? (2) Does this distribution support the kinetic pattern of wave propagation measured in the same cell? (3) What is the extent of IPCR containing endoplasmic reticulum calcium stores in astrocytes? (4) Are the different types of IPCR expressed in the same cells, and if so do they show different $InsP_3$ sensitivities in different regions of the cell? In addition, antibodies against specific cell identification markers such as glial fibrillary acidic protein (GFAP) and ganglioside-3 (GD-3) were used to positively identify cells as either type-1 or type-2 astrocytes.

Endoplasmic reticulum in type-1 astrocytes was decorated by antibodies against all three types of $InsP_3$ receptors ie. type 1 IPCR, IPCR1; type-2 IPCR, IPCR2; and type-3 IPCR, IPCR3. The overall distribution pattern of these receptor subtypes, however, was different in any given cell (see later). When the endoplasmic reticulum was stained with Di-O-C_6-3, the ER membrane system was found to extend over the entire cell, including small cellular processes, and the IPCR antibodies in general appeared to decorate this membrane system (see later). This finding would suggest that, like in other cells, in astrocytes the ER is a continuous cistern and participates in calcium signalling. In general

the distribution pattern of the receptors was punctate with occasional clustering and the size of the clusters was not uniform. In some regions of the cell, relatively large clusters of receptors were found compared with other regions.

All three receptor subtypes were distributed throughout the endoplasmic reticulum in type 1 astroglia. There, however, were differences in the intensity of labelling by the antibodies in different regions of the cells. While IPCR1 was distributed intensely around the nucleus and relatively sparsely in the periphery, IPCR2 was distributed relativelyevenly throughout the cell. IPCR3, however was distributed throughout the cell with intense labelling around the nucleus which was different from the distribution pattern observed with IPCR1. IPCR1 labelling appeared to extend into the nuclear matrix or at least the nuclear inner membrane. High resolution analysis of the distribution pattern showed that the different receptor subtypes decorate the same membrane system. It is not clear, however, whether the receptors are made up of heteromeric complexes of different IPCR subtypes.

In an attempt to compare the distribution of IPCR with the wave propagation kinetics, in twelve different type 1 astrocytes, norepinephrine induced calcium waves were analysed in detail to characterize wave kinetics. At the end of the experiment, these cells were fixed and developed for dual labelled immunocytochemistry. One of the labels used was the cell identification marker, GFAP and the other antibodies against IPCR2. This study showed that regions of high calcium release during wave propagation was associated with dense labelling with anti-IPCR2. This was clearly shown in cellular processes where regions corresponding to regeneration loci were found to have high density of receptors. Nevertheless, ample staining was observed in regions where the calcium release kinetics were several fold smaller. This observation may suggest that the ER specializations in regions of regenerative loci may be not only an increased density of IPCR but also differences in the intraluminal contents. b. It is also likely that there is considerable receptor reserve on the ER and only some are functional at any one time. This study on the structural characterization of endoplasmic reticulum is currently in preparation for publication.

3. Functional aspects of calcium release channels on the endoplasmic reticulum.

(i) Ryanodine receptors in glia

In addition to studies on distribution of IPCR on astrocytes, Carol Sheppard and Peter Simpson in the Section have shown that the endoplasmic reticulum of both type 1 and type 2 astrocytes also possess ryanodine receptors. Their function in receptor mediated calcium signalling in these cells, however, has been unclear. We have shown by immunocytochemistry that type 2 astrocytes express ryanodine receptors at a high level in the perinuclear region and at a lower level throughout the cytoplasm. Peter Simpson has shown that perfusion with caffeine activates a peak and plateau $[Ca^{2+}]_i$ elevation in approximately 24% of these cells, the peak being a Ca^{2+} wave which propagates along processes into the cell body in a non-linear, saltatory manner. This is the first report of ryanodine receptor activation in astrocytes. The caffeine-activated $[Ca^{2+}]_i$ peak is unaffected by removal of extracellular Ca^{2+}, while the plateau is abolished, consistent with this phase of the response being due to store depletion-activated Ca^{2+} entry. When caffeine is perfused, followed by perfusion of a phospholipase-C coupled agonist, and the waves activated by each are examined, it is apparent that while initiation sites may differ, the propagation sites for each are at the same loci, and the rates of rise of the responses follow the same pattern through the cell. It has previously been suggested that propagation sites for waves activated by phospholipase-C coupled receptors might correspond to regions of

IPCR specialization, in type 1 astrocytes. The present finding indicates that a more general ER specialization might be the cause of Ca^{2+} response characteristics, at least in type 2 astrocytes, the nature of which remains under investigation. Staining with the ER marker Di-O-C_6-3 indicates that the ER in type 2 astrocytes is not evenly distributed along processes, perhaps consistent with this hypothesized ER specialization. This study is continuing, the aim being to understand the participation of ryanodine receptors in calcium signalling by glial cells.

(ii) Effects of ER calcium pump inhibition

Our studies on protein distribution have shown that type 2 sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps are expressed in type 1 and 2 astrocytes. The pattern of distribution is somewhat correlated with ryanodine receptor expression. Peter Simpson showed that thapsigargin evokes a transient $[Ca^{2+}]_i$ elevation in type 1 and type 2 astrocytes in nominally Ca^{2+}-free medium by inhibition of the SERCA ATPase, whereas a sustained elevation is apparent in normal Ca^{2+}, consistent with an ability of store depletion in both cell types to activate Ca^{2+} entry. The subtracted Ca^{2+} entry component of the response displays a very similar temporal pattern in the two cell types. Unexpectedly, the time required for thapsigargin to mobilize Ca^{2+} differed markedly along the length of a type 1 astrocyte occurring much less rapidly than for agonist-evoked responses,indicative of a, perhaps novel kind of wave phenomenon, different from those described above (t to 50% of maximum response varies from 50 to 75s after onset of agonist perfusion).

4. Calcium responses in other glial cell types

(i) InsP3-mediated Ca^{2+} waves in type 2 astrocytes

By immunocytochemistry with specific antibodies, we have shown that type 2 astrocytes, like type 1 astrocytes, express at least three subtypes of IPCR, with IPCR1 apparently being expressed in the nucleus, IPCR2 in the cytoplasm and IPCR3 in both regions. We have previously demonstrated the existence of Ca^{2+} waves with invariant spatial and temporal characteristics in type 1 astrocytes. It has now been demonstrated that type 2 astrocytes also respond to agonists of phosphoinositide-coupled receptors, such as carbachol or bradykinin, by activation of waves of Ca^{2+} which begin 2-10s after onset of perfusion, and propagate in a saltatory manner in both directions from small initiation sites, typically near the end of processes, usually permeating into the cell body. Waves are seen either in the absence or presence of extracellular Ca^{2+}, and are inhibited by prior store depletion. The average wave velocity is increased at high agonist concentration compared to concentrations around EC50. Cells, particularly those possessing several processes, typically respond with waves initiated in numerous different loci at different times. The rate of wave propagation is non-linear, such that as each wavefront reaches certain specific sites the rise-time is reduced and the response magnitude is increased several-fold. Waves appear to propagate passively between these sites. Both the initiation sites and amplification sites are found at identical loci for several oscillations evoked by a single agonist perfusion, for responses to successive application of a given agonist, or for the responses to different PI-coupled receptors in a given cell.

(ii) Ca^{2+} responses in other glial cell types

Initial studies other cell types indicate that several of the signalling characteristics discussed above may be general features of glial cell behavior.

Ca^{2+} waves have been measured in oligodendrocytes in response to norepinephrine and bradykinin, being initiated at several different sites in the extensive process network of these cells and propagating in a similar manner to that described above for $InsP_3$-mediated waves in type 2 astrocytes. Successive addition of these two PI-coupled agonists each activate very similar wave response patterns in a given oligodendrocyte cell, again suggestive of ER specializations causing wave initiation and amplification. We have also detected caffeine responses in oligodendrocytes, and such responses have also been found in O-2A glial progenitor cells. Very few type 1 astrocytes cultured from neonatal rats respond to caffeine. A significant percentage of type 1 astrocytes cultured from adult rats, however, do respond to this agent with a characteristic peak $[Ca^{2+}]_i$ elevation. Overall these results suggest that ryanodine receptor-mediated Ca^{2+} waves may be widespread among glia, and potentially play an important role in signalling mechanisms in these cells.

In addition, we have investigated intracellular calcium signals triggered by glutamate receptor activation in primary cortical oligodendrocyte lineage cells and in the oligodendrocyte cell line CG-4. Glutamate, kainate and AMPA (30-300 μ M) increased $[Ca^{2+}]_i$ in both types of cells at the stage of oligodendrocyte progenitors (O-2A; GD3+) or pro-oligodendroblasts (O4+). The peak amplitude of Ca^{2+} responses to glutamate receptor agonists was significantly larger in cortical cells. In CG-4 and in cortical cells, the majority (more than 90%) of bipolar GD3+- or multipolar O4+ cells responded to kainate. In all the cells analyzed, kainate was more efficacious than AMPA and glutamate. The percentage of bipolar or multipolar cells responding to glutamate was significantly lower in the CG-4 cell line than in primary cultures. Cellular responses typical of metabotropic glutamate receptor activation were observed in 20% of the cortical O-2A progenitors, but in none of the CG-4 cells. The AMPA-selective antagonist GYKI 52466 blocked kainate-induced Ca2+ responses in cortical O-2A cells. The selective AMPA receptor modulator cyclothiazide (30 μ M) greatly potentiated the effects of AMPA (30-100 μ M) on $[Ca^{2+}]_i$ in cortical and CG-4 cells. Our findings indicate that Ca^{2+} responses in cells of the oligodendrocyte lineage are primarily shaped by functional AMPA receptors. This is a collaborative study carried out together with Dr. V. Gallo and a manuscript describing these results is in Press in the Journal of Neuroscience Research.

B. Pineal cell calcium signals

Nissim Darvish, a visiting fellow from Israel, has established electrophysiological methodologies in the Section. This has been a much anticipated addition to the laboratory, since it allows for exploring ionic channel mechanisms involved in calcium signalling. Although this approach was initially undertaken to characterize the cyclic GMP gated cation channel in pineal cells, the methodology provides a new dimension in our analysis of glial neuronal interactions as well. This system is currently fully functional and only some software upgrades remain. In addition, Dr. Darvish has refined techniques to obtain pineal cells from rats and maintain them in culture for long periods of time, The following major findings have emerged from studies on these tissue cultured pineal cells:

1. PACAP receptor mediated calcium signals in pineal cells

PACAP (Pituitary Adenylyl Cyclase Activating Peptide) is a 38 amino acid peptide which shares considerable sequence homology with VIP and is found in nerve fibres innervating rodent pineal. Since our previous studies showed that the signal transduction of VIP receptor may utilize the cyclic GMP-gated cation channels, we wanted to examine if PACAP also stimulated this receptor complex. Preliminary

experiments showed that PACAP at a concentration of 0.1 μ M caused an increase in intracellular calcium signals in isolated rat pineal cells. The pattern of these signals, however, showed a peak followed by plateau type of response. This pattern is distinct from that seen when VIP receptors are stimulated, which produced a transient peak response. In addition, unlike the VIP stimulated calcium signals, the PACAP response persisted in the absence of extracellular Ca^{2+}. Furthermore, depletion of cellular calcium stores using thapsigargin (0.5 μ M), an inhibitor of ER calcium pumps, abolished the PACAP response. These observations suggested that the PACAP response is dependent upon release of calcium from intracellular stores. The signal transduction mechanisms that underlie this PACAP-induced calcium release from pineal cell ER are under investigation.

The patch clamp studies on pineal cells utilized various different configurations, namely, on-cell, whole-cell, perforated patch and isolated patches. By the use of these approaches the following ionic conductances have been identified in pineal cells. These are in various stages of completion and is outlined hereunder.

2. cyclic GMP-gated currents

At least three different genes encoding VIP receptors have been identified so far and all of them bear the G-protein associated receptor motif. The classical signal transduction mechanism for VIP receptors is activation of adenylyl cyclase and cAMP production. Experiments conducted in our laboratory have shown that one of the VIP receptors may be coupled to cyclic GMP and a cation channel. We concluded that gating of a cyclic-GMP dependent cation channel appears to mediate the VIP induced transient $[Ca^{2+}]_i$ signal. The following evidence from pineal studies support this conclusion: (1) VIP elevates cGMP more than 10-fold, (2) the cGMP analogues, 8-Br-c-GMP and DB-cGMP elevate $[Ca^{2+}]_i$; (3) SNAP, which liberates NO and increases guanylyl cyclase activity in pinealocytes also increases $[Ca^{2+}]_i$ in thee cells; (4) VIP, cGMP and SNAP all depend upon the presence of mM concentrations of $[Ca^{2+}]_o$ for their induction of $[Ca^{2+}]_i$ signals; (5) The effects of VIP and cGMP are blocked by *l*-cis-diltiazem, which is known to specifically block the cGMP gated cation channel in the retinal rods; (6) RT-PCR analysis suggests that the rod-type cyclic nucleotide gated channel is expressed in pineal cells. Accordingly, it seems reasonable to conclude that a VIP-cGMP-$[Ca^{2+}]_i$ mechanism exists in the pineal gland, and that the rod type cyclic nucleotide gated cation channels mediate this effect. In an attempt to characterize this cation channel perforated patch experiments have been carried out in isolated pineal cells.

In the whole cell recording mode, under voltage clamp conditions, 8-bromo-cyclic GMP ellicited an inward current in approximately 50% of pineal cells examined (6 out of 14). These measurements were carried out under conditions where the voltage dependent sodium currents were blocked with tetrodotoxin (20 μ M) and the the recording medium also contained tetraethyl ammonium (10 mM) CsCl (10 mM) and 4-aminopyridine (5 mM) and ibrotoxin (100 nM). This combination of ion channel blockers were added to completely abolish all potassium conductances. Under these conditions, in pineal cells a type of potassium current still persisted. Addition of 8-bromo-cyclicGMP (1 mM) shifted the reversal potential of the whole cell current by 5 mV towards zero. This result will suggest activation of a non-selective cation conductance by cyclic GMP. subtracting the currents measured in the absence of cyclic GMP from that measured in its presence revealed that the residual current had a linear I/V relationship. The reversal potential of this current was close to zero (6 $\pm$ 3 mV), and this result also is consistent with a non-selective cationic conductance.

Under current clamp conditions, addition of 8-bromo-cyclic GMP (1 mM) resulted in depolarization of the membrane potential by 10 mV in three separate trials. In one experiment, this depolarization was transient, however, and the membrane potential returned to resting values even in the presence of cyclic GMP. In two other experiments, the depolarization was sustained in the presence of 8-bromo-cyclic GMP. Currently these limited experimental observations are being extended.

3. Outwardly directed K^+ conductances

We have identified, at least, two different outwardly directed K^+ conductances in rat pineal cells. One is the delayed rectifier K^+ channel and the other a distinct voltage dependent large K^+ conductance. The latter was found in all the 30 pineal cells so far examined in the perforated whole cell configuration. We have investigated this channel at the whole cell level using amphoterecine-B perforated patches and single channel recordings in isolated patches. The whole cell recordings revealed an I/V relationship where the reversal potential was near -60 mV which is close to the calculated reversal potential for K^+ currents under our experimental conditions. Furthermore, the result showed significant outward rectification. The magnitude of the observed current varied a great deal from cell to cell suggesting differences in channel density. This channel was not blocked by pharmacological agents that are used to block other types of K^+ channels ie. TEA (10 mM), CsCl (10 mM), 4-AP (5 mM), Ibrotoxin (1μ M). This channel is completely blocked by cobalt (1 mM $CoCl_2$) and not by barium.

At the single channel level, the I/V relationship of this conductance could be fitted to a polynomial and showed an 'N' shaped curve with remarkable outward rectification. The single channel conductance was measured to be 180 pS in the linear range. The channel gating was voltage dependent and the open probability (P_o) change e-fold for a 50 mv membrane potential change. Plotting the P_o against the command voltage revealed that the channel does not gate at or near the resting potential of the cell. These specific properties are reminiscent of calcium activated K^+ conductances observed in other cell types. This conductance is being further characterized.

4. Inwardly directed K^+ conductance

In pineal cells, while the large outward K^+ conductance was found in all the cells examined, in a subpopulation (20%, 6 out of 30) an inward K^+ current was observed. The I/V relationship showed significant inward rectification and the reversal potential as measured was close to calculated for a K^+ selective conductance. The reversal potential shifted upon changing $[K^+]_o$ constant with K^+ selectivity. Changing $[K^+]_o$ also changed the slope of the I/V relationship in the inward direction whereby increasing $[K^+]_o$ increased the slope. Channel activity can be blocked by Barium (1 mM) added to the recording medium. These observations are reminiscent of the inward rectifier K^+ channels described in various excitable cells.

Significance to Biomedical Research and the Program of the Institute:

The experimental system being established is directed towards the understanding of the cellular basis for glial cell signalling mechanisms, and neuronal and glial cell interactions. Glial cell derived trophic factors are essential in neural development and maintenance of the cytoarchitecture. Understanding of the nature of the glial-neuronal interactions is essential to understanding the development of cellular organization in the central nervous system. The methods developed is expected to provide fundamental insights into the functional and

structural organization of calcium signalling processes in cells. The cyclic nucleotide gated Ca^{2+} channels activated by VIP receptors is a novel mechanism of signal transduction.

Proposed Course:

A. Calcium signalling in astrocytes

This year we aim to set up paradigms to study calcium based excitability in glial cells in response to neuronal activity and to compare cellular calcium signals in normal glial cells with those in reactive glia. For the former, we will use both acutely isolated glial cells and confocal microscopy of glia in isolated brain slices. In studies on reactive glia, we will utilize paradigms that are currently in development in the laboratory to investigate kinetic parameters and specific ion channel types involved in calcium based excitability. Thirdly, we plan to expand our immunocytochemical studies on endoplasmic reticulum structure with respect to distribution of ion channels and ion pumps involved in calcium signalling. These approaches are described in detail hereafter.

B. Agonist-induced calcium waves in astrocytes

1. Ion channels involved in glial cell calcium signalling

Some of the major findings to emerge from our previous work on glial cell calcium waves remain experimentally untested. A major question remaining to be addressed is the nature of the initiation and amplification sites involved in Ca^{2+} waves in astrocytes and oligodendrocytes. By combining immunocytochemistry and Ca^{2+} wave analysis in individual cells, we hope to identify a correlation between protein or ER expression patterns and Ca^{2+} wave characteristics. A variety of ER proteins which could underlie wave phenomena will be analysed in this manner. While agonist-evoked activation of IPCR waves is well characterized and caffeine activated waves have been identified, it is unclear what if any role ryanodine receptors play in agonist responses in glial cells.

(i) Calcium waves in cells of the O-2A lineage induced by caffeine. (Peter B. Simpson)

We have found that although type 1 astrocytes possess ample ryanodine receptors on the endoplasmic reticulum membranes, these channels are not sensitive to caffeine, nor do they appear to participate in agonist-induced calcium signals. In glial cells of the O-2A lineage, although the CICR channels are sensitive to caffeine, it is not clear if they participate in agonist evoked calcium signalling, eg. during activation of ionotropic receptors. The objective of these experiments is to describe in detail the participation of the CICR channels in calcium signalling in the different types of glial cells. The response to caffeine in type 1 astrocytes, type 2 astrocytes, oligodendrocytes and O-2A progenitor cells will be catalogued in detail. In each cell type, concentration response curves will be constructed for caffeine. These experiments will be followed by examination of the source of the calcium signals induced by caffeine. Thapsigargin will be used to deplete the iER store to test if all of the caffeine response is dependent upon release from intracellular stores. Similarly, the effect of depriving extracellular Ca^{2+} on the caffeine response will be tested. Thirdly, the effect of the specific inhibitor of the CICR channel, ryanodine, will be tested to delineate if these channels are sensitive to this pharmacological agent.

The interaction between ionotropic receptor activation and caffeine induced

calcium release will be investigated to assess the contribution of CICR channel mediated calcium release to ionotropic responses. Kainate will be used as the model agonist since all the cells of the O-2A lineage have been shown to have functional kainate receptors. The effect of kainate pretreatment of cells on caffeine responses will be investigated. Similarly, the effect of ryanodine on the kainate-induced calcium signals will be investigated. Finally, characteristics of caffeine induced calcium waves will be studied in detail in type 2 astrocytes and oligodendrocytes. Kinetics of wave propagation will be analyzed, using paradigms previously developed in the laboratory. From these experiments we hope to obtain wave amplitude and local slopes of calcium release in individual cells. Waves produced by consecutive challenges with caffeine will be analyzed to test for fidelity in the responses. This study will provide, for the first time, a comprehensive analysis of CICR mediated calcium signals in glial cells and would set the stage for studies on acutely isolated glial cells from normal rat brain and from animals in which reactive gliosis has been induced.

(ii) $InsP_3$-dependent and caffeine-activated calcium waves in type 2 astrocytes (Peter B. Simpson)

Since type 2 astrocyte CICR channels respond to caffeine, it allows for comparison of calcium waves initiated by two different mechanisms ie. CICR vs IPCR. This comparison will differentiate between specializations in ion channel complement versus ER membrane or ER luminal specialization in the calcium wave regenerative loci and wave initiation sites. Experiments will be carried out to analyze in detail calcium waves induced by methacholine and bradykinin. These results will be compared with caffeine-stimulated waves in the same cells. Specific cellular loci of wave initiation and regenerative release sites will be compared. Initially, dose response relationship will be constructed for the two agonists. This set of experiments will be followed by characterization of the dependence on the ER store for the response by the use of thapsigargin and removal of extracellular Ca^{2+}. Wave propagation kinetics will then be characterized using previously established experimental paradigms. In this way, wave initiation and regenerative propagation will be analyzed. These kinetics will be compared in the same cell for waves initiated with the two agonists and consecutive stimulations with the same agonist. Finally wave kinetics will be compared between bradykinin and methacholine induced waves and caffeine induced waves. If the two waves show identical wave initiation and regenerative release loci, the result would support the hypothesis that the kinetic differences may be due to ER membrane specialization or luminal contents in terms of protein buffers and perhaps not due to differences in the distribution of the IPCR channels and CICR channels.

(iii) Signal transduction in purinergic receptors on glial cells (David B. Langley, Nissim Darvish)

Among the different types of receptor systems found on glial cells, purinergic receptors are the most abundant and are expressed by all the different types of glial cells so far examined. It is believed by some that activation of purinergic receptors by ATP released from damaged and dying neurons acts as the trigger to change glial cell phenotype to a reactive one. Activation of these receptors show a robust $[Ca^{2+}]_i$ response. Two principal mechanisms for ATP-induced increases in $[Ca^{2+}]_i$ have been described: influx of Ca^{2+} through ligand-gated ion channels activated by ATP and release of Ca^{2+} from intracellular stores, a metabotropic response. The metabotropic receptors are coupled to phospholipase-C via G_q. Since ATP has been recognized to act as an excitatory neurotransmitter in both peripheral and central nervous systems, we would like

to characterize the glial cell receptors to ATP.

As mentioned earlier, we have shown that primary cultured type-1 and type-2 astrocytes respond to ATP, and 2-methylthio-ATP with a large increase in $[Ca^{2+}]_i$. Furthermore, an immortalized type-1 astrocyte cell line (DTNC1) also expresses this receptor in abundance and we have established this cell line in culture in our laboratory. In preliminary experiments we have shown that activation of these receptors in DTNC1 cells induces a robust $[Ca^{2+}]_i$ response. We propose to use both the cellular systems to characterize the ATP receptor mediated response and take advantage of the homogeneity afforded by the cell line.

To determine the type of ATP receptor coupled to $[Ca^{2+}]_i$ increase in glial cells dose response curves for ATP and the non hydrolyzable ATP analogue, 2-methylthio-ATP will be constructed in the same cells using $[Ca^{2+}]_i$ measurements. These will be compared with dose response curves constructed in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o = < 5\mu$ M). The results of this analysis will differentiate between the metabotropic response from ionotropic response. If the $[Ca^{2+}]_i$ signal is due to a metabotropic response, a pharmacological characterization of the receptor will be carried out by comparison of dose response curves for a,b-methylene-ATP with 2-methylthio-ATP followed by construction of dose response curves in the presence of selective antagonists for the P_{2g} and P_{2x} subtypes of purinergic receptors.

On the other hand, if the response is due to a ionotropic mechanism, we propose to characterize this signal transduction mechanism using a combined patch clamp and $[Ca^{2+}]_i$ measurement approach. Whole cell voltage clamp experiments will be carried out to determine if the $[Ca^{2+}]_i$ signal is due to gating of voltage-dependent Ca^{2+} channels secondary to depolarization. Simultaneous electrical and $[Ca^{2+}]_i$ measurements will be used to study agonist mediated currents and accompanying Ca^{2+} changes. Cells will be stimulated with 2-methylthio-ATP in the presence and absence of $[Ca^{2+}]_o$ which wil determine if other ions are flowing through the ligand-gated channel. Ion replacement experiments will follow these studies to establish the relative permeabilities of the different monovalent and divalent cations. A screen for possible modulators of this receptor channel will be made to discover regulatory mechanisms. Attempts will also be made to study channels in isolation using detached patches from the glial cell line.

(iv) Calcium signalling in reactive glia (Peter B. Simpson, David B. Langley)

Astrocytes are known to change their protein expression patterns and behavior in response to brain insult, becoming what are known as reactive astrocytes. In order to investigate whether changes in Ca^{2+} signalling may underlie some of the changes which occur in such conditions, preliminary experiments have been initiated, in collaboration with Dr. Joan Schwartz and Dr. Vivian Wu (NINDS), to compare Ca^{2+} responses in astrocytes from normal rat brain with those from animals subjected to a defined chemical lesion. In these experiments, the receptor repertoire and calcium signals will be compared using the paradigms described in detail in sections (i) and (ii) above.

Similarly, we plan to examine acutely isolated glial cells from areas where reactive gliosis has been induced as soon as possible after isolation. Particularly, we propose to study purinergic receptor mediated responses in these cells. In collaboration with Dr. Paul Britton at Walter Reed Army Medical Research Center, we propose to obtain lesioned rats and isolate glial cells from lesioned areas. Cells will be isolated at various times after lesioning and their responses to both ionotropic and metabotropic agonists will be examined. Calcium waves and local kinetics will be measured and compared with cells

isolated from non-lesioned regions.

2. Endoplasmic reticulum structure in astrocytes (James T. Russell, Peter B. Simpson)

We plan to extend our current studies on the ER membranes in astrocytes in culture. Dr. Carol Sheppard has described the distribution of all three types of IPCR and one CICR subtype on endoplasmic reticulum membranes of type 1 and type 2 astrocytes. These studies have raised a number of interesting questions. Chief among them are : 1. What is the extend of the receptor clustering in cellular regions? 2. Does the clustering of IPCR and CICR occur in the same cellular regions? 3. Does the IPCR1 distribution seen in the nucleus extend into the inner nuclear membrane? 4. Can we obtain a three dimensional map of receptor distribution on the ER? 5. Are the SERCA pumps colocalized with the ryanodine receptor channel?

We propose to analyze all of the above questions in the coming year. Experiments will be designed to immunocytochemically decorate the different subtypes of IPCR and CICR. Both single labelling and dual labelling techniques will be utilized as appropriate. Fluorescently labelled microbeads (0.1 μ m diameter) will be used as internal standards to allow for calibration axial resolution. The samples will be visualized using a high resolution cooled CCD camera and digitized. Images will be acquired at multiple focal planes every 0.25 μ m using a piezo-electric device mounted beneath the objective lens. An extensive photon reassignment algorithm will be used to deblurr these wide angle images to obtain confocal images. The confocal images will be utilized to render in 3-D the distribution of the channel types. Subregions in the cell will be analyzed for specific localization, three dimensional reconstruction and receptor clustering. Furthermore, using Z dimensional calibration, the extend of channel distribution will be analyzed in cellular organelles including the nuclear membrane. This analysis is expected to yield a high resolution (0.15 μ m in X and Y; 0.25 μ m in Z) view of the calcium release channels in the ER of astrocytes. This analysis will form the basis for comparisons between different glial cell subtypes and reactive glia. These studies will be extended to acutely isolated glial cells.

3. Glial cell signalling *in situ*

Until now all experiments showing calcium based signals in glial cell have been carried out in tissue cultured cells in purified cultures or in mixed cultures with neurons. Only the set of experiments were carried out in a more intact preparation of hippocampal slices in organotypic culture currently supports the notion that glial cells respond to neuronal activity. Glial cells in culture continue dividing unlike cells *in situ* and express antigens such as vimentin and nestin which are the hallmarks of "reactive astrocytes". In addition, the effects of unidentified growth factors and trophic substances found in fetal bovine serum on glial cells, their receptor expression and signal transduction are uncontrollable under experimental conditions. Because of these problems we would like to study this question of glial cell signalling using two different approaches. In the first stage, experiments will be carried out to ask if acutely isolated glial cells from brian have the expected complement of receptor systems that respond to neurotransmitters. In the second approach, we intend to use the brain slice preparation and confocal microscopy to study glial cell signalling in response to neuronal activity.

(i). Acutely isolated glial cells (Lynne Holtzclaw, James T. Russell)

In order to study acutely isolated glial cells, we will use the tissue print technique. Brains from new born rats (1 to 10 days old) will be isolated and tissue prints prepared in 25 mm glass cover slips. In this technique chunks of tissue are made and treated with pronase and DNAase for a brief period of time. The tissue chunks are placed in test tubes at 37 C and gently agitated. The dissociated cells will be plated on substrate coated cover slips and examined under the microscope following loading with calcium indicator dyes. It is expected that the cells will be examined within 45 to 60 minutes of isolation from the animal. This method has now been set up in the laboratory and our yield of cells which are glial cells on the cover slips is approximately 20 - 25%. The rest of the cells are neurons as shown by dual immunocytochemical staining using antibodies against GFAP and neurofilament. We propose to analyse each cover slip by GFAP immunocytochemistry following calcium measurement to identify cells with certainty. Simultaneously improvements in the isolation technique will be attempted to improve the yield of glial cells over neurons.

Cells on cover slips will be perfused while measuring $[Ca^{2+}]_i$ using fluorescence microscopy. Dose response curves will be constructed using neurotransmitters appropriate for the brain region and other candidate agonists. These experiments will establish the receptor repertoire of glial cells from the different regions of the central nervous system. Depending upon the responses that we record, we propose to test if the signal transduction mechanisms for these receptor-mediated responses resemble mechanisms identified in cultured cells. These experiments will unequivocally establish that appropriate receptor systems to neurotransmitters exist in glial cells from the different regions of the brain and that these receptors correspond to the neurotransmitters used by neurons in that region. Such a conclusion will show that glial cells *in situ* can respond to neuronal activity but leave the question of whether such signalling occurs unanswered.

(ii) Studies on brain slices (James T. Russell)

The brain slice preparation will be used to study glial cells *in situ* using the Noran confocal microscope. The experiments planned will take the course of previously published work on organotypic cultures of rat hippocampal slices. The goal of the studies on brain slices is to find answers to a number of questions: (1) does neuronal stimulation result in glial cell responses in nerve terminal fields in the intact brain slice? (2) How far are these signals propagated? (3) Are there special glial cell phenotypes involved in this form of glial signalling in the brain? (4) are the gap junctional coupling between glia modulated depending upon neuronal activity? (5) What is the status of coupling between neurones and glial cells?

This project will be initiated only after the establishment of the confocal microscope in the laboratory after its initial calibration experiments have been completed. We expect this to be completed by December 1995 at the latest. This involves minor modifications to Room 5C-28 in building 49 and installing black out curtains. I expect to install the microscope and carry out initial calibrations during the fall of 1995. In the meantime, we have begun processes to recruit a postdoctoral fellow with extensive experience in brain slice methodology. The project would be begun when the new fellow arrives in collaboration with Dr. Chris McBain (LCMN) and a number of experiments are planned. We are hiring the postdoctoral scientist together and would seek funds from external funding agencies for the research project for fellowship support.

To investigate the initial question of whether neuronal activity results in glial

cell signals we need to establish methods to monitor $[Ca^{2+}]_i$ in glial cells in a brain slice. We intend to use hippocampal slices because of the anatomical uniqueness and because the preparation is well established. We intend to take advantage of Dr. Chris McBain's extensive experience in the slice technique. A number of difficulties are expected, including satisfactory loading of glial cells with reporter dyes. Since reporter dye loading using acetoxymethyl ester forms is expected to degrade signals due to light scattering during optical measurements, we intend developing techniques to inject cells with dyes. Stimulating electrodes will be placed in the region of the dentate gyrus to stimulate mossy fiber tracts and glial cells in the CA3 region where the mossy fiber terminal fields exist will be imaged to monitor $[Ca^{2+}]_i$ changes. In design the experiments are identical to the ones carried out by the Smith group in organotypic cultures of the hippocampus. If signals such as the ones measured by these authors are recorded, control experiments will be carried out to establish that the $[Ca^{2+}]_i$ signals result from neuronal activity. One of the most interesting possible outcomes of these experiments would be the understanding of the extent of glial cell signalling in response to neuronal activity. This knowledge will be crucial in order to reveal the functional significance of such signalling. Two other projects are planned and would follow these experiments. One involves studies of $[Ca^{2+}]_i$ changes in dendritic spines in hippocampal interneurons and the second a fluorescence recovery after photobleaching (FRAP) study to investigate the extent of coupling and its regulation in glial cell networks in the brain slice preparation.

C. VIP receptors on rat pineal cells

We have described a novel signal transduction mechanism for the VIP receptors in rat pineal cells. Our studies showed that VIP receptors evoke a $[Ca^{2+}]_i$ signal in pineal cells by activation of guanylyl cyclase which gates a retinal rod-type cyclic nucleotide gated channel on the plasma membrane. A number of questions remain to be answered, namely (1) What is the coupling mechanism between VIP receptors and guanylyl cyclase? (2) What is the molecular identity of the cyclic nucleotide gated channel? (3) What are the ionic channels that underlie cellular excitability in pineal cells? (3) how does this mechanism interact with the adrenergic activation of pineal cells and melatonin production? We propose to characterize the putative cyclic nucleotide gated cation channel and establish its molecular identity. In addition, we plan to describe in detail the ionic channels that regulate cellular excitability in pineal cells. Thirdly, the signal transduction mechanism for the PACAP induced calcium signals will be investigated. Dr. Nissim Darvish who joined us in December of 1994 is involved in this study. He has established the necessary instrumentation for simultaneous recording of electrical and fluorescence signals from cells. A number of experiments are underway.

1. Identification and characterization of the channels mediating the VIP-induced $[Ca^{2+}]_i$ signal
   (Nissim Darvish)

Patch clamp techniques in conjunction with $[Ca^{2+}]_i$ measurements will be used to study the channel and its receptor coupling which will allow for biophysical characterization of the channel. VIP receptors are found in high density in pinealocytes and we plan to use cells acutely isolated by mechanical dispersion as well as by papain treatment. $[Ca^{2+}]_i$ measurements will be carried out both together with electrical measurements and in parallel to monitor functional coupling of the channel.

Perforated patch experiments will be carried out to establish if VIP induce

membrane currents. Under voltage clamp conditions, VIP evoked membrane currents will be measured to rule out the possibility that VIP responses are mediated by membrane depolarization. Dose response relationship curves will be constructed for these VIP induced currents under voltage clamp conditions. Secondly, the depolarization induced by VIP-will be measured in current clamp. In order to investigate if the VIP currents are due to cyclic GMP generation, currents induced by 8-bromo-cyclic GMP will be measured in perforated patch experiments. The pharmacological characteristics of the currents induced by both VIP and cyclic GMP will be studied and compared. l-*cis*-diltiazem will be used as the antagonist to verify our earlier identification of the channel.

Macroscopic current recordings will establish kinetics of channel gating and inactivation if any. Using the perforated patch technique with the input resistance of the cell being high (>1.0 GΩ), and since the pineal cells are small, single channel currents will be recorded under steady state conditions. Channels will be activated using cyclic GMP or VIP. From this data, we will analyze the dose dependent activation of the single channel and the Hill coefficient of activation. Furthermore, kinetic parameters such as mean open time, mean close time and open probability will be measured. Channel mean open time will be measured at various times after addition of cyclic GMP to establish the kinetics of inactivation of the channel. Using this paradigm, we will investigate the mechanism of inactivation. Ion selectivity for channel permeability will be established using I/V plots constructed in ion replacement experiments and calculating permeability ratios. Sensitivity to l-*cis*-diltiazem of the channel will be determined.

2. Ion channels that regulate pineal cell excitability (Nissim Darvish)

In preliminary experiments, we have identified two distinct K^+ currents in rat pineal cells. Previous studies by different laboratories have shown that K^+ currents in pineal cells may be important in the regulation of pineal function which is controlled by adrenergic input from the superior cervical ganglion. One of these currents is an outwardly directed large conductance channel and the other is an inward current which has not been previously observed. We propose to characterize both of these channels in detail.

(i) Outward K^+ channel

Whole cell patch experiments and single channel conductance measurements will be utilized to investigate the biophysical characteristics of this channel. Initially the single channel conductance and ion selectivity will be established from I/V plots derived from experiments under different ionic conditions. Voltage dependence of the channel will then be investigated using conventional electrophysiooogical methods. From this data channel gating kinetics will be derived. Since this channel is outwardly rectifying as shown in our preliminary experiments at the single channel level, we intend to explore the mechanism of rectification (ie. $Ca^{2=}$, Na^+, H^+ etc.). Since this channel is not blocked by conventional K^+ channel blockers other than cobalt we would explore other candidate blockers. Finally, based on our preliminary results potential modulation of the channel by Ca^{2+}, cyclic AMP and cyclic GMP will be investigated. Cyclic nucleotide dependent effect will be investigated to differentiate direct effects from effects mediated via phosphorylation of the channel protein through protein kinase activation.

(ii) Inward K^+ channel

Biophysical characterization of the inward K^+ channel will be carried out in

detail in whole cell and isolated patch experiments. These include single channel conductance and ion selectivity from I/V relationships measured under different ionic conditions. Channel open probability and its relationship to membrane potential will be investigated as well as the dependence on K^+ concentration out side. Pharmacological studies will characterize the blocking action by Ba^{2+} ions. The mechanism of inward rectification of the channel will be studied and candidate agents such as Mg^{2+} and polyamines will be tested. Finally channel modulation by putative modulators such as ATP and Ca^{2+} will be examined.

Secretion of melatonin by pineal cells is tightly regulated by adrenergic input from the superior cervical ganglion. Activation of adrenergic receptors is coupled to increase in cellular Ca^{2+}, cyclic AMP, and cyclic GMP. The cellular physiology of coupling these second messengers to melatonin production and secretion is currently unknown. It is likely that the two K^+ channels described here play a crucial role in not only controlling cellular excitability but also in regulating the circadian control of melatonin production and secretion. Experiments to study modulation by adrenergic agents and their second messengers are expected to reveal underlying mechanisms that control pineal function.

3. PACAP-induced $[Ca^{2+}]_i$ signals in pineal cells (Nissim Darvish)

We have established that PACAP causes increase in $[Ca^{2+}]_i$ in pineal cells by release from intracellular thapsigargin-sensitive stores. The mechanism of coupling receptor activation to $[Ca^{2+}]_i$ elevation, however, is not known. It appears that although PACAP shares significant sequence homology with VIP, it acts through a different mechanism. Initially dose response relationships for VIP and PACAP will be established using $[Ca^{2+}]_i$ measurements to ensure that they are acting through different receptor systems. Experiments will be carried out to establish the second messenger system that mediates PACAP-induced $[Ca^{2+}]_i$ signals. In other cellular systems, in addition to increasing cyclic AMP, PACAP has been shown to activate phospholipase-C and $InsP_3$ production. Heparin injection experiments will be attempted to verify if in the pineal cell PACAP receptors act via the phospholipase pathway.

Publications:

Fleishman, LF, Holtzclaw, L, Russell, JT, Mavrothalassitis, G and Fisher, RJ. ETS-1 in astrocytes: Expression and transmitter-evoked phosphorylation. Mol Cell Biol 1995;15:925-931.

Gallo, V and Russell, JT. Excitatory amino acid receptors in glia: Different subtypes for distinct function? J Neurosci Res 1995;42 (In Press).

Holtzclaw, L, Gallo, V and Russell, JT. AMPA receptors shape Ca^{2+} responses in cortical oligodendrocyte progenitors and CG-4 cells. J Neurosci Res 1995;42 (In Press).

Roth, BJ, Yagodin, S, Holtzclaw, L and Russell, JT. A mathematical model of agonist-induced propagation of calcium waves in astrocytes. Cell Calcium 1995; 17:53-64.

Schaad, NC, Vanacek, J, Rodriguez, IR, Klein, DC, Holtzclaw, L and Russell, JT. Vasoactive intestinal peptide elevates pinealocyte $[Ca^{2+}]_i$ by enhancing influx: evidence for involvement of a cyclic GMP-dependent mechanism. Mol Pharmacol 1995;47:923-933.

Yagodin, S, Holtzclaw, L and Russell, JT. Subcellular calcium oscillators and calcium influx support agonist-induced calcium waves in cultured astrocytes. Mol Cell Biochem 1995 (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 02000 04

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Neurotransmitter Receptors in Glia

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

PI:	Vittorio Gallo	Visiting Scientist, LCMN, NICHD 12 months
Others:	Mario Pende	Visiting Fellow, LCMN, NICHD 12 months
	Steve Scherer	IRTA Fellow, LCMN, NICHD 12 months
	Montse Molne	Visiting Fellow, LCMN, NICHD 7 months
	Fei Huang	IRTA Fellow, LCMN, NICHD 7 months
	Li Jin Chew	Visiting Fellow, LCMN, NICHD 2 months
	Jia Min Zhou	Biologist, LCMN, NICHD 11 months
	Robert Hagan	Summer Student, LCMN, NICHD 2 months

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Cellular and Molecular Neurophysiology

SECTION
Unit on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.2	5.2	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

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

The molecular and functional properties of neurotransmitter ligand-gated ion channels in glial cells were studied, in order to understand their regulation and function in the brain. Studies were carried out to: A. characterize the physiological role of glutamate-gated ion channels in glial development and B. to clone a glutamate receptor gene and analyze its 5' regulatory region in order to understand its transcriptional regulation in glia. A. Physiological properties and functional role. Primary cultures of rat oligodendrocyte progenitors (O-2A) were used as a model system. O-2A cells develop into oligodendrocytes *in vitro* similarly to O-2A progenitors *in vivo*. Kainate- and AMPA-preferring glutamate receptors are co-expressed in O-2A cells. AMPA receptor activation inhibits O-2A cell proliferation and prevents lineage progression at early developmental stages. Intracellular Ca^{2+} transients, due to transmembrane influx of the divalent cations, and immediate early gene (*NGFI-A* and *c-fos*) transcription are regulated by AMPA receptors. Immediate early gene induction occurs at the transcriptional level through protein kinase C-mediated phosphorylation of the transcription factor CREB. Genes encoding separate kainate-preferring glutamate receptor subunits are co-expressed in undifferentiated, nestin-positive multi-potential precursor cells of the rat neural tube. B. Glutamate receptor genes and analysis of their 5' regulatory region. Genes encoding kainate receptor subunits are highly expressed in cells of the oligodendrocyte lineage. The cloning of the entire rat GRIK5 gene, which encodes the kainate-preferring subunit KA2, was accomplished, and its intron-exon organization was partially determined. Two kb of the 5' flanking region of the rat GRIK5 were sequenced and found to comprise 3 independent transcription start sites. The transcriptional potential of the 2kb region was analyzed in an oligodendrocyte cell line as well as in PC12 cells.

Project Description:

Objective:

To study the molecular and functional properties of glial neurotransmitter-gated ion channels. To understand the physiological role of glutamate-gated ion channels in glia. To study the transcriptional regulation of glutamate receptor genes in neural cell development.

Major Findings.

1. Functional role of glutamate receptors in oligodendrocyte development

Oligodendrocyte development is controlled by several growth factors, including platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), which regulate proliferation and differentiation of oligodendrocyte progenitors (O-2A). O-2A cells co-express functional AMPA- and kainate-preferring glutamate receptors (GluRs). We hypothesized that the neurotransmitter glutamate may regulate oligodendrocyte development through the activation of membrane receptors expressed early on during O-2A proliferation and migration. Vittorio Gallo and Jia Min Zhou used *in vitro* bioassays to analyze potential functions of GluRs in modulating proliferation, migration and differentiation of O-2A progenitor cells. O-2A progenitor cells purified from the embryonic rat cerebral cortex were cultured under different conditions. The different culture conditions tested were as follows: PDGF, bFGF, PDGF+bFGF, 30% B104 (neuroblastoma cell line) conditioned medium (B104-CM). All these culture conditions promoted O-2A cell proliferation, as measured either by ^{3}H-Thymidine (^{3}H-Thy) or by bromodeoxyuridine (BrdU) incorporation. Co-incubation with the GluR agonists glutamate, kainate or AMPA, significantly inhibited ^{3}H-Thy or BrdU incorporation in O-2A cells. The effects of the GluR agonists were dose-dependent and, at all concentrations tested, kainate was more efficacious than AMPA and glutamate. The IC_{50} values for kainate were 32.8μM (cells cultured with PDGF) and 23.3μM (cells cultured with PDGF+bFGF). IC_{50} values for AMPA were 28.1μM (PDGF) and 10.3μM (PDGF+bFGF). Under all culture conditions, the selective non-NMDA receptor antagonist DNQX not only prevented the inhibitory effects of kainate, but also significantly stimulated O-2A cell proliferation. This was due to block by DNQX of the inhibitory effects of endogenous glutamate present in the culture medium, which ranged between 9 (cells in bFGF) and 10.2μM (cells in PDGF), as determined by HPLC analysis of the media after 24 hr in culture (in collaboration with Judy Harvey White, NINDS). Activation of other transmitter receptors (GABA and muscarinic) did not modify O-2A cell proliferation. The inhibitory effects of the selective agonist AMPA on cell proliferation indicate that AMPA

receptors are primarily involved. Low micromolar concentrations of glutamate are sufficient to activate such receptors to elicit biological effects, as demonstrated by the IC50 values for GluR agonists and the stimulatory effects of the antagonist DNQX on O-2A cell proliferation.

The inhibitory effect of GluR agonists on O-2A cell proliferation was likely to be a direct effect on the cell cycle, since the GluR agonists did not induce neurotoxicity or apoptosis. O-2A cells that were cultured with PDGF+bFGF and GluR agonists for 24hr, and then ^{3}H-Thy-pulsed for different lengths of time in a GluR agonist-free medium containing PDGF+bFGF, re-entered the cell cycle with a temporal pattern similar to cells that were never exposed to GluR agonists. These experiments demonstrated that the inhibitory effects of kainate and AMPA were reversible and that the O-2A cells were still viable after a 24 hr exposure to GluR agonists.

Since O-2A cells proliferate much more vigorously than cells at later stages of the oligodendrocyte lineage, inhibition of cell proliferation could also result from promoting O-2A cell differentiation. Therefore, the effects of long-term GluR activation on O-2A lineage progression were investigated by using cell type-specific antibodies to identify pre-O-2A cells ($GD3^{-}$/$nestin^{+}$), O-2A cells ($GD3^{+}$/$nestin^{+}$), oligodendroblasts ($O4^{+}$), and finally oligodendrocytes (O1+). In cultures maintained with PDGF+bFGF for 1 day, the cells maintained the pre-O-2A or O-2A phenotype, which was not significantly altered after a 22hr exposure to kainate or AMPA. Similar results were also obtained in cells cultured in PDGF or bFGF alone. Under culture conditions that permit O-2A lineage progression (i.e. N1, PDGF or bFGF), AMPA significantly inhibited acquisition of O4, while DNQX significantly increased the percentage of $O4^{+}$ oligodendroblasts. Importantly, in cultures maintained in bFGF, the small but significant percentage of dividing $O4^{+}$ oligodendroblasts was not modified by treatment with AMPA or DNQX. In contrast, under conditions that prevented O-2A cell differentiation (i.e. PDGF+bFGF or B104-CM), neither AMPA nor DNQX affected O-2A cell lineage progression. These data indicate that long-term activation of AMPA receptors not only inhibited cell proliferation at the O-2A stage, but also prevented lineage progression to the $O4^{+}$ oligodendroblast stage.

In collaboration with Regina Armstrong (Dept. of Anatomy and Cell Biology, USUHS), we examined whether GluR agonists can act as chemoattractants, or alter migration to PDGF, which is a known chemoattractant for O-2A cells. In a microchemotaxis chamber assay, O-2A cells migrated toward PDGF in a dose-dependent manner. GluR agonists or DNQX, at concentrations which altered proliferation and differentiation, did not affect migration of O-2A progenitors to PDGF. Control experiments showed that GluR agonists and the antagonist DNQX did not stimulate chemokinesis in O-2A cells. These data emphasize the viability of the O-2A cells in the presence of

GluR agonists and are consistent with the previous observation that proliferation is not required for O-2A cell migration.

2. Glutamate receptors, Ca^{2+} transients and stimulus-coupled gene transcription in oligodendrocyte progenitor cells.

Intracellular calcium signals triggered by glutamate receptor activation were studied in primary cortical oligodendrocyte lineage cells and in the oligodendrocyte cell line CG-4 in collaboration with Lynne Holtzclaw and James Russell. Glutamate, kainate and AMPA increased $[Ca^{2+}]_i$ in both types of cells at the stage of O-2A or oligodendroblasts ($O4^+$). The amplitude of Ca^{2+} responses to GluR agonists was significantly larger in cortical cells and, in all cells analyzed, kainate was more efficacious than AMPA and glutamate. The percentage of O-2A or oligodendroblast cells responding to glutamate was significantly lower in the CG-4 cell line than in primary cultures. Cellular responses typical of metabotropic glutamate receptor activation were observed only in 20% of the cortical O-2A progenitors, but in none of the CG-4 cells. The AMPA-selective antagonist GYKI 52466 blocked kainate-induced Ca^{2+} responses in cortical O-2A cells. The selective AMPA receptor modulator cyclothiazide greatly potentiated the effects of AMPA on $[Ca^{2+}]_i$ in cortical and CG-4 cells. Our findings indicate that Ca^{2+} responses in cells of the oligodendrocyte lineage are primarily shaped by functional AMPA receptors.

Previous studies by Mario Pende demonstrated that activation of AMPA receptors and transmembrane Ca^{2+} influx trigger immediate early gene (IEG) transcription in O-2A progenitor cells. Mario Pende performed nuclear run-off assays in nuclei isolated from O-2A progenitor cells after exposure to kainate and demonstrated that AMPA receptor stimulation directly activates transcription of *NGFI-A* and *c-fos*. The intracellular events leading to gene expression after non-NMDA receptor stimulation were further analyzed in primary O-2A cells. It was demonstrated that activation of glutamate receptors causes phosphorylation of the transcription factor CREB and subsequent IEG transcription through a previously unidentified pathway, i.e. through the activation of protein kinase C (PKC). Immediate early gene induction was caused also by stimulation of O-2A cells with the phorbol ester TPA, which directly activates protein kinase C. Pre-treatment with the selective PKC inhibitor bisindolylmalemide blocked not only the increase in IEG RNA transcripts caused by TPA, but also the effects of kainate. Down regulation of PKC induced by long-term treatment with phorbol esters drastically decreased the effects of kainate and TPA on IEG induction. The muscarinic receptor agonist carbachol also stimulated IEG transcription in a bisindolylmalemide-sensitive manner. Incubation with kainate, carbachol or TPA caused an increase in the specific binding of ^3H-phorbol ester to intact O-2A cells, indicating that these agents stimulated PKC translocation to the membrane. Within 5-10 minutes after stimulation of non-NMDA

receptors by kainate, the transcription factor CREB was phosphorylated on serine-133, as assessed by immunoblot analysis with an antiserum specific for the phosphorylated form of CREB. TPA and the muscarinic receptor agonist carbachol had a similar effect on CREB phosphorylation. Surprisingly, forskolin and the growth factors PDGF and bFGF failed to induce CREB phosphorylation in O-2A progenitor cells. Bisindolylmaleimide completely counteracted the effect of kainate, TPA and carbachol on CREB phosphorylation. Immunoblot analysis with a pan anti-PKC antibody of cellular fractions from O-2A progenitors demonstrated PKC translocation to the nucleus after phorbol esters treatment. These results define a novel pathway for CREB activation in neural cells that is dependent on PKC.

3. Kainate receptor expression in precursor cells of the rat neural tube.

Primary progenitors derived from E10 rat neural tube are capable of differentiating along both the neuronal and glial pathways. Steve Scherer showed that plating the neural tube precursor cells on polyamines as opposed to fibronectin not only had a marked effect on the morphology of the cells (more neuron-like), but also increased the percentage of neural vs. smooth muscle precursors. Previous RT-PCR analysis of neural tube/neural crest cell RNA demonstrated that these nestin positive neural progenitor cells expressed transcripts for the kainate-preferring subunits GluR6, GluR7, KA1 and KA2, and that these transcripts were also expressed in E10 embryos *in vivo*. The subunit GluR5 was not expressed *in vivo* nor in culture. The subunits GluR6 and KA2 are capable of forming functional hetero-oligomeric channels. RNase protection assays showed that, in E10 embryos and in cultured neural progenitors, both GluR6 and KA2 are expressed at levels 10 times lower than in the brain at postnatal day 1. RT-PCR analysis of total RNA from neural tube cultures and from E10 embryos demonstrated that, both in the cultures and *in vivo*, GluR6 is almost exclusively (92%) unedited in the transmembrane region TM2. Indirect immunohistochemistry of the cultured cells with anti-GluR5/6/7 and anti-KA2 antibodies showed expression of these subunits in differentiated neurons as well as in immature neuroepithelial precursor cells. These results indicate that heterooligomeric kainate-preferring receptors may be assembled in neural progenitor cells early on during embryonic development.

4. Transcriptional analysis of the GRIK5 5' flanking region.

The gene GRIK5 encodes the kainate-preferring glutamate receptor subunit KA2. We have previously obtained a rat GRIK5 clone comprising 2kb of 5' flanking region. To identify the transcription initiation site(s) of the rat GRIK5 gene, Montse Molne performed primer extension analysis of total RNAs derived from rat cerebral cortex and cerebellum, as well as liver and of total RNAs derived

from primary cultures of rat O-2A progenitors, from the glial cell line CG4 and from PC12 cells. The primer extension analysis revealed the presence of 3 transcription start sites in the tissue-derived and cell-line derived total RNAs. Thus multiple transcription start sites seem to be used for rat GRIK5 gene transcription, a feature commonly found in genes lacking a TATA box in their promoter region. Multiple transcription initiation sites have also been described in the promoter regions of other glutamate receptor subunit genes that also lack a TATA box. In order to detect more distal initiation sites, the total RNA samples used for the primer extension were additionally tested by Northern analysis with genomic and cDNA derived probes. No signal was detected with a genomic probe spanning a region upstream of the putative transcription start sites. So far, we have not found evidence of preferential transcription initiation site use in different cell types or brain regions.

We have placed the bacterial reporter gene CAT under the transcriptional control of the 2 Kb fragment and under the transcriptional control of a smaller 600bp fragment derived from it, containing all the detected GRIK5 transcription initiation sites. We have measured the transcriptional potential of these constructs after transient transfection in different neural and non neural cell types. The 2Kb fragment is able to direct high expression levels of the reporter CAT gene when transfected in the oligodendrocyte cell line CG-4 and in the cell line PC12 differentiated to a neuronal phenotype with NGF. This construct remains silent when transfected in undifferentiated PC12 cells or primary cultures of astrocytes. The 600bp chimeric construct, containing all the transcription initiation sites, is moderately active in CG-4 cells and is not sufficient to drive detectable levels of transcription in differentiated PC12 cells. This construct remains also silent or not detectable in astrocytes and in undifferentiated PC12 cells, and thus only partially reproduces the neural cell type specific transcriptional behavior of the 2Kb fragment driven constructs. These observations indicate that GRIK5 genomic sequences comprised within the 600 bp fragment are sufficient to ensure detectable levels of transcription in neural cell types. In oligodendrocyte cells, the transcriptional levels can be highly increased when additional upstream sequences are present. In PC12 cells, high transcription levels are only observed when upstream sequences are present and the cells are fully differentiated to a neuronal phenotype by NGF. Thus this minimal 600bp fragment can be activated by upstream regulatory elements and/or in response to specific differentiation factors.

The 2Kb and the 600bp fragments are both sufficient to maintain tissue specific expression, as determined after transfection in non neural cell types (Hela, 3T3 and HEK 293 cells). The 600bp fragment retains tissue specific expression and is sufficient to direct basal levels of expression in oligodendrocytes, behaving

typically as a promoter region, it can also be activated in a cell specific manner by sequences located distally, suggesting that sequences able to functionally assemble the transcriptional machinery are present in this DNA region. The primer extension analysis indicates that all transcripts are initiated within this 600 bp region in CG4 and PC12 cells, as well as *in vivo*.

The sequence analysis indicates the presence of several INR sequences and Sp1 sites, and a high G content, but no canonical TATA or CAAT boxes in this fragment. All of this features are commonly observed in the promoter regions of housekeeping genes. In order to determine if these INR and Sp1 consensus sites are equally functional and to further localize the promoter region of the GRIK5 gene, we have obtained new chimeric GRIK5-CAT constructs in which these putative promoter sites have been serially deleted in the presence or absence of distal sequences of the GRIK5 gene and tested these constructs after transient transfection in CG-4 cells and PC12 cells, and in non neural Hela and 3T3 cells. Our analysis indicates that the INR sequences (220 bp) located at the most 5' end transcription start site are sufficient to assemble efficiently the transcriptional machinery, and that INR and Sp1 sites located downstream somehow negatively regulate this INR site. Constructs that do not contain the most 5' INR, but contain all or some of the downstream INR and Sp1 sites give very low, but detectable levels of transcription in CG-4 cells. All the deletion constructs tested retain tissue-specificity of expression and remain silent in non neural cell types. Taken together, these results indicate that the 220 bp region that comprises the most 5' INR contains transcriptional regulatory cis-elements sufficient to direct high transcription levels in CG4 cells and to maintain tissue specificity of expression in other cell types.

5. Molecular cloning of the entire rat glutamate receptor gene GRIK5 and analysis of its exon-intron organization.

The cloning of the entire rat GRIK5 gene was accomplished. Fei Huang screened a rat genomic library with KA2 cDNA-derived and genomic GRIK5-derived probes, and isolated 7 independent, overlapping genomic clones encoding the complete GRIK5 gene. Preliminary restriction analysis indicates that the gene spans 50-60 kb of genomic DNA. In order to study the role of regulatory DNA elements other than those comprised in 5' flanking region (e.g. intronic sequences), Fei Huang has begun to determine the exon-intron organization of the rat GRIK5 gene. The complete sequence and exon-intron organization of the most 5'-end genomic clone, including 4kb of the 5' flanking region, of the rat GRIK5 was obtained. Eleven small exons, ranging between 54 and 179 bp, and 11 introns, ranging between 0.1 and 3.4 kb, were identified. The GRIK5 translation start site is comprised within the second exon (129 bp). The first and second exons are separated by a 3.4 kb intron.

Proposed Course of the Project:

1.We plan to continue the analysis of the role of glutamate receptors in oligodendrocyte development. Experiments *in vitro* will be continued on cultured cortical O-2A progenitors, to clarify the mechanism by which activation of GluRs inhibits O-2A cell proliferation and lineage progression. We also plan to elucidate, by flow cytometric analysis, whether activation of such receptors causes a G0 or a G1 arrest, or increases the length of the cell cycle. In parallel studies, we will analyze cyclin expression in O-2A cells cultured with different growth factors and determine whether GluR activation induces the expression of any cyclin-dependent kinase inhibitor in O-2A cells. A new approach will also incorporate double *in situ* hybridization and immunohistochemical studies, to determine whether AMPA- and kainate-preferring receptors are expressed by oligodendrocyte lineage cells *in vivo*.

2. We will further investigate the mechanism of GluR-induced phosphorylation of the transcription factor CREB as well as its importance in the context of GluR-induced gene transcription in O-2A progenitor cells. We will establish whether PKC directly phosphorylates CREB, or triggers a cascade of events that lead to CREB phosphorylation. We will also determine which specific amino acid residues of CREB are phosphorylated in response to GluR and muscarinic receptor activation, as well as direct PKC activation by phorbol esters. Further experiments will also elucidate the molecular mechanism of CREB activation by PKC and determine whether PKC-induced CREB phosphorylation affects its binding to CREB-response elements (CREs), or only promotes the interaction with the basal transcriptional machinery. Finally, we will extend our analysis of PKC-induced, CREB-mediated transcriptional activation to other CREs (low and high affinity).

3. The molecular and functional characterization of kainate-preferring receptors in the rat neural tube progenitors will be completed. Follow-up molecular and biochemical studies will determine whether kainate-preferring receptor subunits are co-expressed in the same neural progenitor cells both at the mRNA and protein level, and whether such receptors are functional. We will also analyze the transcriptional potential of the cloned *GRIK5* 5' flanking region in undifferentiated neural tube progenitor cells, to determine whether this region is sufficient to direct tissue-specific expression before lineage specification has occurred.

4. The molecular analysis of the GRIK5 gene promoter will be continued in order to identify DNA elements directly involved in the binding of *trans*-acting factors. *In vitro* techniques, such as gel retardation and DNAse I footprinting assays, will be used and combined to transient transfection experiments performed in CG-4 and PC12 cells. Nuclear extracts will be prepared from different neural cell types expressing GRIK5 and from CNS tissue at different

developmental stages. To determine the sequence specificity of the footprints, the effects of mutations on nuclear protein binding sites will also be examined *in vitro* as well as in transient transfection assays.

5. We will complete the analysis of the intron-exon organization of the rat gene GRIK5. The intron-exon arrangement of this gene will be compared to that of other GluR genes and will provide a framework for future studies. We plan to determine whether: i) the 5' flanking region further upstream of the previously analyzed 2Kb (comprised in a newly-isolated genomic clone) is involved in regulating GRIK5 transcription; ii) the first 3.4Kb intron comprised between the transcription start sites and the ATG codon is involved in regulating GRIK5 expression in different neural cell types. The transcriptional analysis of these DNA elements will be performed in cultured glial cells and neurons, and in transgenic mice.

Significance to Biomedical Research and Program of the Institute:

It is well established that glial cells perform a pivotal role in brain function and express a large variety of voltage-dependent and ligand-gated channels. This finding greatly increases the complexity of the cellular elements that can respond to classical neurotransmitters, because the ratio between glial cells and neurons in the mammalian central nervous system is approximately 10:1. Glutamate receptors are abundantly expressed in different glial cell types and they can mediate drastic changes in glial membrane potential. The physiological role of glutamate-gated channels in glia is not understood, but the close anatomical relationship between neurons and glia both in the developing and in the mature mammalian central nervous system indicates that also glial cells are very likely to be directly affected by glutamate in the intact brain. The appearance of glutamate receptors during early glial development and the regulation of glial proliferation and differentiation by glutamate indicate that, in addition to his role as mediator of information transfer at synaptic junctions, glutamate plays a role in shaping the development of the central nervous system. This has very important consequences for the physiology of the mature brain, in which glial cells perform crucial functions, such as myelination and metabolic support of neurons. Glutamate receptors in glial cells may also play an important role in the pathophysiology of stroke and cerebral anoxia, as already established for neurons. In addition, periventricular white matter injury, the principal variety of brain injury of the human premature infant, cause cell death of differentiating oligodendroglia and appears to involve glutamate receptors.

Publications:

Gallo V, Armstrong R . Developmental and growth factor-induced regulation of nestin in oligodendrocyte lineage cells, J Neurosci 1995;15:394-406.

Gallo V, Russell JT. Excitatory amino acid receptors in glia: different subtypes for distinct functions? J Neurosci Res (In Press).

Holtzclaw L, Gallo V, Russell JT. AMPA receptors shape Ca^{2+} responses in cortical oligodendrocyte progenitors and CG-4 cells. J Neurosci Res (In Press).

Szpirer C, Molne M, Antonacci R, Jenkins NA, Finelli P, Szpirer J, Riviere M, Rocchi M, Gilbert DJ, Copeland NG, Gallo V. The genes encoding the glutamate receptor subunits KA1 and KA2 (*GRIK4* and *GRIK5*) are located on separate chromosomes in human, mouse and rat. Proc Natl Acad Sci (USA) 1994;91:11849-11853.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01301-13 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Human Immune Response to Polysaccharide-Protein Conjugate Vaccines

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

PI: R. Schneerson Research Medical Officer LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LCDB, NIDDK (J. Shiloach); SUNY Downstate Medical Center, N.Y. (G. Schiffman); Carolinas Medical Center, Charlotte, N.C., (J.C. Parke, Jr.); National University Hospital, Iceland (J. Jonsdottir). LCI, NIAID (J.

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.8	2.3	3.5

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 surface polysaccharides of bacterial pathogens, which include capsular polysaccharides and lipopolysaccharides, serve as protective antigens. The immunologic properties of these bacterial polysaccharides, namely their age-related and T-cell independent immunogenicity, limit their use as vaccines. Covalently attachment to medically-useful proteins to form conjugates, both increases their immunogenicity and confers T-cell dependent properties to these polysaccharides. The capsular polysaccharides of Streptococcus pneumococcus type 6B, Staphylococcus aureus types 5 and 8, Group B streptococcus type 3 have been bound to several proteins and evaluated clinically. S. aureus type 5-rEPA was evaluated in end stage renal disease patients; type 5 antibodies of the three major Ig classes rose significantly though to a lesser degree than in healthy volunteers, no booster response to reinjection at 6 weeks was found. These antibodies had opsonophagocytic activities. Pn6B-TT was evaluated in patients with sickle cell disease, healthy infants at 3, 4 and 6 months of age or at 7 and 9 months of age. Type specific antibodies of the three Ig classes, with booster responses, were induced. The magnitude of these responses was lesser than of Hib-TT. GBSIII-TT was evaluated in females of child bearing age. IgG antitype III rose similarly to the response to the polysaccharide alone. Technical problems with this lot were identified. All conjugates were safe, with only minor local reaction. The LPS of shigellae was detoxified, their O-specific polysaccharides bound to bacterial toxoids and their immunogennicity in mice found to be satisfactory. In Phase 1 and Phase 2 studies, these conjugates of the O-specific polysaccharides were safe and immunogenic: LPS antibody levels elicited by the investigational conjugates were similar to those in recruits convalescent from shigellosis. In preliminary studies, a S. sonnei-rEPA conjugate protected against shigellosis caused by this pathogen. A more extensive study showed protection of 75%.

Others:	J.B. Robbins	Chief	LDMI, NICHD
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	D. Pavliakova	Visiting Associate	LDMI, NICHD
	D.A. Bryla	Statistician	LDMI, NICHD
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Cooperating Units: Bennett); LMB, NCI (D. Fitzgerald); WRAIR, Washington, D.C. (D.N. Taylor); IDF, Israel (D. Cohen); Sheba Medical Center, Israel (J. Passwell); Children's Hospital of Michigan, Detroit, MI (S. Sarnaik)

Project Description:

The surface polysaccharides of bacterial pathogens that cause systemic infections are both essential virulence factors and protective antigens. Primary pathogens cause systemic infections in otherwise healthy individuals. Their surface polysaccharides may be capsular polysaccharides or lipopolysaccharides. Most opportunistic pathogens also have surface polysaccharides that may be protective antigens. The immunologic properties of polysaccharides limit their usefulness as vaccines. For capsular polysaccharides there is: 1) Age-related serum antibody response. Immunization of infants elicits few responses of mostly IgM and of short duration. "Protective" levels start to be achieved in two year-olds and an adult response develops at ~6 - 12 years of age; 2) T-cell independent. Most polysaccharides do not elicit a booster response at any age. The situation for lipopolysaccharides (LPS) is slightly different. LPSs are composed of three domains: O-specific polysaccharide - Core - Lipid A. The latter is attached to the outer membrane and the pharmacologic properties of the bacteria are mediated by Lipid A (pyrogenic, inflammatory, vasomotor activity [shock] and effect upon WBCs and platelets). Accordingly, the lipid A alone or as part of LPS is not suitable in a vaccine; and 3) the O-specific polysaccharide is the region in contact with the host and is a protective antigen but acts as a hapten when purified.

Objectives:

Conjugates are more immunogenic than systemic infections: Vaccines more immunogenic than disease. When bound to foreign proteins, especially inactivated and purified vaccine components (tetanus and diphtheria toxoids), the immunologic properties of polysaccharides improve. One of our objectives is to improve the immunogenicity of polysaccharide-based conjugates. One important variable has been the molecular weight of the polysaccharide component. This has been controversial, with some suggesting an "optimal" size between high molecular weight, perhaps about 40,000D, and "oligosaccharides." Our laboratory and others have shown that higher molecular weight polysaccharides elicit higher levels of antibodies. There is, however, the technical problem that high molecular weight polysaccharides tend to form more insoluble and technically unsatisfactory conjugates.

Another problem is the poor immunogenicity of the polysaccharides such as the capsular polysaccharides of pneumococcus type 6 and the poly $\alpha(2\rightarrow8)$Neu*p*NAc of Group B meningococci and E. coli K1: both of these capsules are associated with important and common pathogens. As conjugates, these polysaccharides have proven to be lesser immunogens, than other polysaccharide conjugates.

Pertussis toxin will certainly be a component, if not the sole component of the new acellular pertussis vaccines. Since it is anticipated that additional carrier proteins will be required for a multivalent polysaccharide conjugate formulation, pertussis toxin should be a useful candidate. Preliminary experiments showed that polyanions, including medically useful capsular polysaccharides, combine and precipitate with pertussis toxin. Pertussis toxin itself is not soluble at pH from about four to nine making it difficult to use for conjugation. Further, the neutral polysaccharide, pneumococcus type 14, is also from a common and important pathogen for children. Conjugates formed

between pneumococcus type 14 and pertussis were synthesized and were not toxic in vitro. The type 14-pertussis toxin conjugate elicited both type 14 and pertussis neutralizing antibodies in mice. The clinical safety and efficacy of such a conjugate must now be evaluated.

A heterofunctional, fully protected tetrasaccharide was synthesized which corresponds to a complete repeating unit of the O-specific polysaccharide of S. dysenteriae type 1 and was used as a building block for the preparation of higher-order saccharides. A dimer, a trimer and tetramer of this repeating unit were assembled, corresponding to octa-, dodeca- and hexadeca-saccharide fragments of the O-specific.

Lastly, the LDMI has demonstrated good immunogenicity and preliminary efficacy of Shigella sonnei O-specific polysaccharide-protein conjugates in armed forces recruits in the Israel Defense Force. The LDMI now began evaluating these investigational vaccines in children, the age group that suffers the highest incidence, morbidity and mortality caused by shigellae and other enteric pathogens.

Methods Employed:

The capsular polysaccharides of pneumococcus type 6B and Group B streptococci were purified and characterized according to methods established in our laboratory. The LPS was extracted from Plesiomonas shigelloides (identical to S. sonnei) and S. flexneri type 2a purified by hot phenol extraction and its O-specific polysaccharide purified by acid hydrolysis. The final products were purified by gel filtration and their purity and biosafety assayed by established methods.

Clinical evaluation of conjugate vaccines at the Clinical Center, NIH, the Walter Reed Army Institute of Research and the Israel institutions of Israel Defense Force and the Sheba Medical Center proceeded after approval by the NIH, FDA, IRBs of the above institutions.

Serum antibodies to capsular polysaccharides, O-specific polysaccharides and to the carrier protein are measured by a standardized ELISA.

In order to avoid the potential toxicity of pertussis toxin as a conjugate, a non-toxic protein will be used. A genetically engineered strain of B. pertussis synthesizing a cross-reacting non-toxic mutant pertussis toxin has been cultivated and initial studies of it purification and methods for improving its solubility are underway. Since the mutation has been created in the enzymatically active site, the techniques of affinity chromatography, used successfully for the native toxin are not applicable.

Major Findings:

In a preliminary study recruits of the Israel Defense Forces, the S. sonnei-rEPA conjugate vaccine was shown to confer type-specific protection against diarrhea and fever. A more extensive placebo-controlled, randomized blinded study showed an efficacy of about 75%. A phase 2 study (safety and immunogenicity in children and then, if the results are satisfactory, in infants) has been approved and

started by the Pediatrics Department, Sheba Medical Center, Israel. Prelminary epidemiologic analysis of enteric infections has started in a rural area of Vietnam in order to provide information about Shigella types that are important but not common in the U.S. or in countries like Israel.

S. aureus type 5-rEPA injected into 17 end-stage-renal disease patients induced type 5 antibody rises in the three major Ig classes, mostly in IgG. Maximal levels at six weeks, were not boosted by an additional injection at this time. Fold increases and GM levels were about one-half those of normal volunteers, but at six months, the GM IgG levels were 10-fold higher than the preimmunization levels.

Pn6B-TT and GBS III-TT were found to be injected at about half the intended dose due to the conjugates binding to the vials. GBS III-TT was also shown to break down over a two year period. Pn6B-TT injected into adult and chronic obstructive pulmonary disease individuals induced antibodies of isotypes and opsonic activities similar to those induced by the 23 valent polysaccharide vaccine. This Pn6B conjugate lot was injected into infants at 3, 4 and 6 months of age (n=21) or at 7 and 9 months of age (n=19). In the younger infants only two achieved the estimated protective level after the primary immunization, in the older infants ten reached this level. Booster injections at 18 months induced high antibody levels (5-15-fold the protective level) in most children. This lot was also injected into 17 infants with sickle cell disease. The antibody responses of these infants was similar to those of the healthy infants.

GBS III-TT injected into child-bearing age females induced GM IgG anti-type III levels similar to the reported for the polysaccharide alone, with a decline of one-third at one year.

Proposed Course:

Methods to improve the immunogenicity of conjugate vaccines, especially of lesser immunogenic polysaccharides will be investigated. Clinical lots of Haemophilus influenzae type a and meningococcus group B will be prepared.

Significance to Biomedical Research and the Program of the Institute:

The above diseases are major causes of morbidity and mortality in the U.S. and worldwide. In particular, shigellosis is a major cause of stunted growth of children throughout the world. In addition to providing an effective vaccine for preventing these infections, the LDMI research program is attempting to confirm that vaccine-induced serum antibodies, especially of the IgG class, can be a protective mechanism against enteric infections including both bacterial and viral pathogens.

Publications:

Robbins JB, Schneerson R, Szu SC. Hypothesis: How licensed vaccine confer protective immunity. J Jpn Soc Pediatr Infect Dis 1994;7:39-50.

Robbins JB, Schneerson R, Szu SC. Perspective: Hypothesis: Serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating

the inoculum. J Infect Dis 1995;171:1387-98.

Robbins JB, Schneerson R, Vann WF, Bryla DA, Fattom A. Prevention of systemic infections caused by group B Streptococcus and Staphylococcus aureus by multivalent polysaccharide-protein conjugate vaccines. In: Williams JC, Goldenthal KL, Burns DL, Lewis BP, Jr. eds. Combined vaccines and simultaneous administration: Current issues and perspectives. New York: New York Academy of Sciences, 1995;68-82.

Claesson BA, Trollfors B, Anderson PW, Johansson J, Taranger J, Schneerson R, Robbins JB. Serum antibodies in 6-year-old children vaccinated in infancy with a Haemophilus influenzae type b-tetanus toxoid conjugate vaccine. Pediatr Infect Dis J (accepted for publication).

Welch PG, Fattom A, Moore J, Jr, Schneerson R, Shiloach J, Bryla DA, Li X, Robbins JB. Safety and immunogenicity of Staphylococcus aureus type 5 capsular polysaccharide-Pseudomonas aeruginosa recombinant exoprotein A conjugate vaccine (SA5-rEPA) in patients on hemodialysis. J Am Soc Nephrol (accepted for publication).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01308-12 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Conjugate-Induced Polysaccharide Antibodies

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

PI: S.C. Szu Research Chemist LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)* Walter Reed Army Institute of Research, Washington, D.C. (D.N. Taylor); LCDB, NIDDK (J. Shiloach); Carolinas Medical Center, Charlotte, N.C. (J.C. Parke, Jr.)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Unit on Molecular Biophysics and Biochemistry

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.4	4.2	1.2

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.)*

Serum antibodies to the surface polysaccharides of Gram-negative enteric bacteria confer protective immunity against these pathogens. Vi capsular polysaccharide of Salmonella typhi was conjugated with protein to improve its immunogenicity. Phase 1 studies confirmed the superior immunity of recombinant exoprotein A of Pseudomonas aeruginosa (rEPA) bound to Vi. Phase 2 and phase 3 studies with this investigational vaccine are planned for Vietnam, an area with a high attack rate of typhoid fever. A semi-synthetic Vi antigen using plant polysaccharide, pectin, was prepared and showed identical antigenicity as the native Vi. A conjugate, composed of a di-O-acetylated pectin as a vaccine for typhoid fever, is being prepared for clinical evaluation. Escherichia coli O157, considered as an important emerging pathogen in the U.S., causes hemolytic uremic syndrome in young children. Phase 1 study of the detoxified O-specific polysaccharide and covalently bound to rEPA elicited 100-fold antibody rise in healthy adults after four weeks and the antibodies were bactericidal. We plan a phase 2 study for therapeutic usage of hyperimmune plasma in children. Salmonella paratyphi A accounts for 10% of enteric fever in Southeast Asia. A vaccine prepare in the similar fashion as E. coli O-157 was prepared and elicited bactericidal antibodies in mice. We plan to use this conjugate as a control in our phase 2 study in Vietnam. Poly(dA) has similar charge distribution as polyα(2-8) sialic acid, the capsular polysaccharide of group B meningococci. We conjugate poly(dA) with a carrier protein and mice immunized with poly(dA)-BSA conjugate elicited anti-sialic acid antibody. We showed for this a highly charged, flexible polymer, the charge distribution is the dominant factor for specificity.

PHS 6040 (Rev. 5/92)

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	D.A. Bryla	Medical Statistician	LDMI, NICHD
	D.W. Towne	Chemist	LDMI, NICHD
	H.T. Tran	Biologist	LDMI, NICHD

Project Description:

Enteric bacterial pathogens are a major cause of mortality and morbidity, especially in infants and children, throughout the world. In the United States, enteric bacterial pathogens continues to cause outbreaks of diseases and admissions to hospitals. The LDMI has developed experimental data to show that the surface polysaccharides of these bacteria are both essential virulence factors and protective antigens. Most importantly, the LDMI has shown that vaccine-induced serum antibodies to these polysaccharides can exert protection and a mechanism by which this protection may occur has been proposed. Lastly, the LDMI has extended their expertise to develop polysaccharide-proteins conjugate vaccines which can easily be standardized and can be administered to infants as part of their routine schedule of immunization.

Objectives:

Enteric fevers are common and serious worldwide: the most important of which is typhoid fever. In Southeast Asia, more than 70% isolates are antibiotic resistant. The capsular polysaccharide (Vi) is both an essential virulence factor and a protective antigen of Salmonella typhi. Field trials in Nepal and in the Republic of South Africa showed that a single injection of Vi conferred about 70% protection in children ≥5 years-old and in adults. Its protective action is to elicit a critical level of serum antibodies. Vi was conjugated to proteins to increase its immunogenicity and make it suitable for routine immunization of infants against typhoid. A Phase 1 clinical trial in the U.S. showed that Vi conjugates elicited significantly higher levels of serum antibodies than the Vi alone. LDMI is planning to clinical trials of Vi conjugates in children and infants of endemic areas.

The Vi is a linear homopolymer of (1→4)-α-D-GalpANAc, variably O-acetylated at C_3. Synthesis of Vi conjugates posed several problems. First, the high M_r of Vi ($\bar{}2 \times 10^3$ kD) causes conjugates to be poorly soluble. Second, standardization has been hindered by a lack of a colorimetric methods for Vi. Pectin, a common polysaccharide of plants, is a copolymer composed mainly of poly (1→4)-α-D-GalpA. Treatment of pectin with acetic anhydride results in *O*-acetylation of C_2 and C_3 (OAcPec). The resultant derivative differs from the Vi only in that its C_2 is *O*- rather than *N*-acetylated. As a result, OAcPec can be measured by the carbazole reaction. Pectin does not react with Vi antiserum and it does not elicit Vi antibodies in mice. OAcPec, in contrast, precipitates with Vi antiserum. Unlike Vi, OAcPec is not immunogenic in mice probably due to its comparatively low M_r ($\bar{}400$ kD). We conjugated OAcPec to proteins according to the scheme used for Vi and other polysaccharides containing an aminohexuronic acid. OAcPec has some advantages because it can be measured by standardized calorimetric assays and because it forms more soluble conjugates with proteins than Vi. One disadvantage is that its glycosidic bond is not as stable as that of Vi. We have demonstrated in mice and guinea pigs that *O*-acetylated pectin protein conjugates antibodies against Vi. We plan to compare di-OAc pectin with Vi conjugates in infants.

In order to understand the difference between OAcPec and Vi, the conformational stability of these two molecules are compared with energy minimization calculation and molecular dynamic simulation. Polysaccharides, unlike proteins,

do not fold into globular conformation and usually are in extended helical forms. Replacing the *N*-acetyl group at the C2 position with the *O*-acetyl also remove the positive charge and the possible proton donor to form hydrogen bond with the neighboring carboxyl group. The distance and angles of the glycosidic bonds are calculated both in vacuum and in water to compare the solvent effect.

The relation between polysaccharide structure and its immunologic properties is important for designing an effective vaccine or in searching for cross-reactive antigens. The aforementioned pectin-Vi study serves as a good example. One other highly charged polysaccharide, the $\alpha(2\rightarrow8)$ linked polysialic acid is the surface antigen virulence factors of E. coli K1 and group B Neisseria meningococcus and appears on the surface of mammalian neural cells. Its immunologic properties has been found to be dependent on its carboxylic groups. For example antibodies against poly $\alpha(2\rightarrow8)$ sialic acid cross react with polyanions with similar charge distributions, such as poly(dA). To study the immunogenic cross reactivity of two polymers with similar charge distribution but different molecular structure, we prepared conjugates of poly(dA) and evaluated the serum response to these immunogens in mice.

The second most common enteric fever in Southeast Asia is *Salmonella* paratyphi A which account ~15% of all the cases. As with *S. typhi*, S. paratyphi A is a habitant of and a pathogen for humans only and can be considered as a "clone". However, unlike S. typhi, paratyphi A does not contain a capsule. We have demonstrated that antiserum against its O-specific chain of LPS is bactericidal. The importance of the *O*-acetyl to the structure and immunologic properties of the O-specific polysaccharide was studied. We feel that the bactericidal antibodies elicited by our investigational O-specific polysaccharide-protein conjugate of *S. paratyphi* A is a clinical correlate of protection and clinical trials in endemic area are planned.

Escherichia coli *O*157 is considered as an "emerging pathogen." Disease caused by this organism exerts a high morbidity and mortality despite effective antimicrobials and supportive therapy. Incomplete data suggests that antimicrobial therapy of the hemorrhagic colitis caused by E. coli O157 increases the incidence of the hemolytic uremic syndrome. The pathogenesis of this complication is unknown but it likely involves the action of the shigella-like toxins of this organism. We prepared O157 O-specific polysaccharide protein conjugate prevention of infections caused by this pathogen. Vaccines prepared with different methods were evaluated in adult volunteers at the North Carolina Medical Center. LDMI is also preparing conjugates using the shigella-like toxin as a carrier for *E. coli* O157 O-specific polysaccharide conjugates in order to prepare therapeutic globulin for treatment of hemorrhagic colitis caused by E. coli O157 and related organisms such as E. coli O124.

A related pathogen, E. coli *O*111, is also an important cause of enteric infections including newborn nurseries. *O*111 strains of varying H types and with different virulence factors cause enteritis: this O type is found rarely in healthy individuals. Prevention by active immunization and treatment by passive immunization of this pathogen is planned. O-specific polysaccharide conjugates of this pathogen have been synthesized and found to induce bactericidal antibodies in mice. Due to the acid labile nature of colitose, a dideoxysugar in the O-specific polysaccharide, a non-denaturing process has been developed to

detoxify the polysaccharide.

Lastly, this laboratory has reinvestigated vaccine preparation against endotoxic shock caused by Gram-negative bacteria. Endotoxic shock is a major cause of nosocomial infection in U.S. and has the highest mortality rate. There is still no effective means for treatment and prevention. Most Gram-negatives share similar lipid A and core regions in their lipopolysaccharide. Therefore a vaccine that induces antibodies against this region(s) would cover a broad range of Gram-negatives. Both the core and the lipid region are short and presented as poor immunogens. LDMI had prepared conjugates using hydrazine-treated LPS from E. coli J5, a rough mutant of E. coli O111. Our results showed only species-specific protection against E. coli in animals. We plan to study new methods for conjugating short polysaccharides to carrier proteins.

Methods Employed:

Carboxyls on Vi were characterized by potentiometric titration, circular dichroism and kinetic analysis of the reaction of Ca^{++} and Na^{+} salts with 2-nitro-phenylhydrazine.

Conformation energy calculations were performed with Program Random Molecular Mechanics (RAMM) and molecular dynamics simulations was done in the Consistent Valence Force Field (CVFF).

Vi and the O-specific polysaccharides were purified and derivatized by methods devised or used by the LDMI. Polysaccharides were studied by NMR, PAGE for purity, Hestrin reaction for O-acetyl and 2-nitro-phenyl hydrazine for carboxyl groups, immunodiffusion and bioassays for antigenicity. Carrier proteins were purified chromatography and their purity and safety assayed by physical chemical methods. Conjugates of these polysaccharides and proteins were synthesized by methods devised at the LDMI and prepared under good manufacturing practices to achieve conditions suitable for FDA approval and clinical use.

The new method for Vi conjugation was to derivatize the carrier protein with adipic dihydrazide and then bound to Vi in the presence of water soluble carbodiimide. The new method for paratyphi A conjugation was to activate polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) at pH 7 to 8 with or without adipic dihydrazide: the former will form a conjugate directly bound to the protein.

Evaluation of the experimental vaccines in mice used clinically relevant protocols designed by the LDMI. Serum antibody responses induced in humans was assayed by quantitative and standardized ELISA. Functional assays, including the vibriocidal assay for V. cholerae, used in vitro and in vivo methods. Determination of the safety of these conjugates used methods outlined in the U.S. CFR. Clinical studies in humans were enacted after approval by the appropriate NIH, FDA and local IRB.

Major Findings:

The O-specific polysaccharide of E. coli O157 LPS was detoxified with acetic acid or hydrazine and derivatized with adipic acid dihydrazide and bound to P.

aeruginosa recombinant exotoxin A. In collaboration with Dr. J.C. Parke, Jr., 87 healthy volunteers from the Carolinas Medical Center were injected with one of the conjugate. All three conjugates elicited serum antibodies against LPS (>100 fold rise) with bactericidal activity to the O157 LPS. The highest rise of LPS antibodies was of the IgG class. The antibody levels declined slightly (to ~80 fold rise) after six months.

The O111 O-specific polysaccharide is composed of a pentasaccharide repeat with colitoses bound to the C3 and to the C6 of glucose in a trisaccharide backbone. The O111 O-specific polysaccharide was prepared by treatment of its LPS with acetic acid (O-SP) or the organic base hydrazine (DeA-LPS). The O-SP had reduced levels of colitose. These products were derivatized with adipic acid dihydrazide (ADH) or thiolated with N-succinimidyl-3(2-pyridyldithio) propionate (SPDP). The four derivatives were covalently bound to tetanus toxoid (TT) by carbodiimide-mediated condensation or with SPDP to form conjugates. Immunization of BALB/c and general purpose mice showed that DeA-LPS-TT_{ADH}, of the four conjugates, elicited the highest level of LPS antibodies. This differential immunogenicity is best explained by the intact colitose content and by the superiority of the adipic acid hydrazide as a linker.

We improved our synthesis of Vi conjugates. The improved method uses ADH derivatized protein and elicited higher antibody levels in mice than the conjugates synthesized previously with SPDP. We are planning a phase II study with both conjugates in children and in infants in Vietnam.

Structure/function relations have been studied in order to understand the immunologic properties of Vi. The degree of *O*-acetylation is critical for the immunogenicity of Vi. *O*-acetyls blocks the binding of cations with carboxyl groups on Vi. We showed by energy minimization calculation that there is only one minimum for the glycosidic linkage, (ϕ,ψ) = (110, -86). This energy minimum is shallower for Vi than for OAcPec. However, the effect of water is stronger for OAcPec (+19.2 ev) than for Vi (3.6 ev). From molecular dynamics simulations we have found that both Vi and OAcPec are stable with low probability of conformational transition.

The use of a plant polysaccharide, pectin, provides a novel approach as an immunogen for prevention of typhoid fever. OAcPec conjugated to tetanus toxoid (TT) elicited Vi antibodies in mice and guinea pigs and reinjection elicited a booster response. Clinical studies with OAcPec are planned.

O-specific poysaccharide conjugates from *S. paratyphi* A with tetanus toxoid are synthesized by several schemes: 1) CNBr activation and ADH as a linker for both acetic acid and hydrazine-treated saccharides; 2) CDAP activation at neutral pH with or without ADH as a linker with acid or hydrazine treated saccharides. Mice immunized with these conjugates had similar anti-LPS responses for conjugates using acetic acid-treated LPS. Hydrazine-treacted polysaccharide conjugates, in contrast, did not elicit LPS antibodies. Chemical analysis showed that hydrazinolysis had removed all *O*-acetyls from the polysaccharide. ^{13}C NMR showed that the polysaccharide is *O*-acetylated at two sites rather than only the rhamnose site as reported. The essential role of *O*-acetyl in the immuogenicity explains the failure of A component of TAB vaccine to confer protection against infection with *S. paratyphi* A. The use of CDAP was more effective in retaining

O-acetyls than CNBr.

In collaboration with Dr. Y.C. Lee, Johns Hopkins University, we started various ways to purify, detoxify and conjugate oligosaccharide from mutant strains: E. coli J5 and Salmonellae. We have shown that mild hydrazinolysis detoxified the LPS and retained the diglucosamine structure of the inner core.

Proposed Course:

Clinical evaluation active immunization with the above conjugates in sequential studies in adults, children and then infants is planned. Trials of the O157 conjugates in cattle are planned in order to evaluate the potential for these vaccines to eliminate carriage of E. coli O157 in this reservoir. A phase II studied is planned with the Carolinas Medical Center for 2-years-old children. Plasmaphoresis of sera from high responders in adult volunteers are planned for therapeutic usage of this vaccine. The preparation of conjugates using non-toxic shigella-like toxins I and II, subunit B for therapeutic globulin as a treatment of hemorrhagic colitis caused by E. coli O157 is planned.

Vi conjugates will be evaluated in Vietnam. The attack rate of typhoid fever in Vietnam is about 1% and most strains (70%) are antibiotic resistant. *S. paratyphi* A is the second most common cause of enteric fever in Vietnam. Conjugates using CDAP with and without a linker will be evaluated. Both phase I and II studies of these conjugates for *Salmonella* will be performed in this high endemic area.

We demonstrated the dominance of O-acetyls in the immunogenicity of S. paratyphi A. The reported structure of the O-specific side chain of *A. paratyphi* A does not explain our immunologic findings. In the reported structure, O-acetyl is linked to the rhamnose of the backbone the which sterically is not as prominent as compared to the branch sugar, paratose. In addition, from NMR we have observed two acetyl peaks. From model building and base hydrolysis, we propose that at least one of the O-acetyl group is linked to paratose and together they form the immunodominant site.

Vaccines for endotoxic shock will be prepared from two mutant strains: J5 from E. coli and *Salmonella minnesota* R595. At least two different methods for conjugation of these oligosaccharides will be studied. One will utilize the carboxylic on the KDO residue on the core. The other will be to remove the *N*-acyl linked lipid with hydrazine at 100oC and then link residual disaccharide region of the lipid A to polyacrylamide as a backbone. Both methods should preserve the diphosphate region of the lipid A which is believed to be immunodominant.

Significance to Biomedical Research and the Program of the Institute:

Prevention of enteric bacterial infections, especially of infants and children, will be an important achievement of the NICHD. The development of immunotherapy against the complications of E. coli O157, O111 as well as other enteroinvasive pathogens, may establish a new approach for treating these serious disease caused by an emerging pathogen.

The major cause of nosocomial death is the endotoxic shock caused by Gram-negative organisms. There is yet no available treatment for this serious infection. From our earlier study and clinical trials of monoclonal antibody study in other laboratories, the antibodies against the diphosphate di-glucosamine region will be protective. Both the prevention and therapeutic approach are taken here.

The relation between the polysaccharide structure and its immunologic properties are better understood by the energy minimization calculation and molecular dynamic simulation of Vi and its analogue, the O-acetylated pectin. Through the circular dichroism and intrinsic viscousity measurement of poly α(2-8) sialic acid, it is clear that this polysaccharide is extremely flexible and its binding property with antibodies are mainly enthalpy driven. The cross-reaction of poly α(2-8) sialic acid with other negatively charged polymer was further demonstrated by animal immunized with the poly(dA)-BSA conjugate. We conclude that it is possible to generate cross-reactive antibodies from two structurally different molecules with similar charge distribution.

Publications:

Szu SC, Bystricky S, Hinojosa-Ahumada M, Egan W, Robbins JB. Synthesis and some immunologic properties of an O-acetyl pectin (poly alpha-[1->4]-D-GalpA)-protein conjugate as a vaccine for typhoid fever. Infect Immun 1994;62:5545-9.

Bystricky S, Szu SC, Gotoh M, Kovac P. Circular dichroism of the O-specific polysaccharide of Vibrio cholerae 01 and some related derivates. Carbohydr Res 1995;270:115-22.

Gupta RK, Egan W, Bryla DA, Robbins JB, Szu SC. Comparative immunogenicity of conjugates of Escherichia coli 0111 O-specific polysaccharide, prepared by treatment with acetic acid or hydrazine, bound to tetanus toxoid by two synthetic schemes. Infect Immun 1995;63:2805-10.

Liao J, Nickerson KG, Bystricky S, Robbins JB, Schneerson R, Szu SC, Kabat EA. Characterization of a human monoclonal IgM antibody (IgMBEN) specific for the Vi capsular polysaccharide of Salmonella typhi. Infect Immun (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 01311-02 LDMI

PERIOD COVERED: October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: A Polysaccharide Vaccine for Mycobacterium tuberculosis

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

PI: J.B. Robbins Chief LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*: LCDB, NIDDK (J. Shiloach); CBER, FDA (S. Morris, J. Muller); Pasteur-Mérieux Serum et Vaccins, Lyon, France (D. Schulz).

LAB/BRANCH: Laboratory of Developmental and Molecular Immunity

SECTION: Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION: NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.5	4.3	1.2

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.)*

Tuberculosis remains a serious and common disease worldwide. In the U.S. emergence of multi-antibiotic resistant strains, due to the rapid passage of Mycobacterium tuberculosis in patients with AIDS now poses a public health problem. Although the most frequently used vaccine in the world, there is no scientifically-based evidence that BCG prevents primary pulmonary tuberculosis and its protective effect in children has not been related with a protective antigen or a host immune component. Our research into a new vaccine for tuberculosis is based upon the similarity of primary infection caused by M. tuberculosis with that of capsulated bacterial respiratory pathogens, viz: 1) epidemiologic data which show that tuberculous meningitis has a similar age distribution as capsulated bacteria; 2) the presence of a capsular polysaccharide on M. tuberculosis and other mycobacteria in vitro and in vivo; 3) that BCG and protein components of this and wild-type strains, without exception, at best have prolonged but never conferred protection against challenge with wild-type M. tuberculosis in laboratory animals; and 4) published data by F. Seibert that protective immunity in rabbits was correlated with the level of serum precipitating antibodies to M. tuberculosis polysaccharide II proposed to be a linear homopolymer of poly α(1→2)-D-Glu. This α(1→2) glucose moiety is identified in extracts of M. tuberculosis, but not in BCG, by its reactivity with pneumococcal type 12F typing antisera. The reactivity in mycobacteria extracted previously with saline and then with 1% Triton. The residual organisms are extracted by the hot phenol method used for LPS of Gram-negatives. The resultant product precipitates with pneumococcal type 12F antisera giving an identity reaction with dextran 1299 which has kojibiose residues. Animal and human antisera, reactive with pneumococcus type 12 and dextran 1299 elicit complement-dependent killing of M. tuberculosis strains Erdmann and wild-type M123. The cidal reaction is inhibited by a synthetic kojipentose but not by an α(1→4)-D-Gal trimer.

PHS 6040 (Rev. 5/92)

Others:	R. Schneerson	Research Medical Officer	LDMI, NICHD
	V. Pozsgay	Visiting Scientist	LDMI, NICHD
	Z. Dai	Visiting Fellow	LDMI, NICHD
	E. Dubois	Visiting Fellow	LDMI, NICHD
	N.W. Tolson	Biologist	LDMI, NICHD

Project Description:

Despite its discovery over one century ago, there is yet no scientifically-based evidence for BCG or any other vaccine for prevention of primary pulmonary tuberculosis. Tuberculosis continues to be a major cause of morbidity and mortality throughout the world and, in the U.S., multi antibiotic resistant strains of M. tuberculosis pose an important public health problem.

There is good evidence that administration of BCG in infants prevents tuberculous meningitis in young children and its administration into the bladder is an effective therapy for bladder carcinoma. But the protective antigen and the host immune response elicited by BCG are not characterized. The skin test for tuberculosis infection, detected by PPD or a comparable product, is mediated by T-cells but, is not related to protective immunity to tuberculosis.

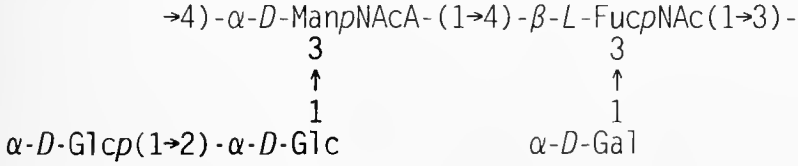
There is published experimental evidence that specific serum antibodies should be reinvestigated as a protective immune mechanism to tuberculosis, viz: 1) the age incidence of tuberculous meningitis, as an example of a systemic complication of a respiratory infection, is similar to other capsulated bacterial pathogens; 2) similar to other capsulated pathogens, polymorphonuclear white blood cells are the first to gather at the site of an inoculum of M. tuberculosis. Although WBC engulf and kill some organisms, these cells are replaced with mononuclear cells after 24 hours (please note that serum capsular polysaccharide antibodies facilitate opsonophagocytosis and intracellular killing of pneumococci); 3) M. tuberculosis and other pathoenic mycobacteria are surrounded by a clear zone, referred to as the "ETZ", shown to be a polysaccharide; and 4) preliminary evidence that serum antibodies, induced in some rabbits by multiple intravenous injections of inactivated mycobacteria, were correlated with protein against challenge. One polysaccharide, identified as Polysaccharide II by its discoverer, Dr. Florence Seibert, seems to be a reasonable candidate to investigate as a protective antigen.

Objectives:

To establish an in vitro sopsonphagocytosis and killing assay for M. tubercuosis with human antibodies, complement and white blood cells. To isolate the "Polysaccharide II" fraction of M. tuberculosis and to confirm its structre by modern techniques. To synthesize oligosaccharides of α(1→2)-D-glucose and to characterize their immunochemical and biological properties upon opsonphagocytosis of M. tuberculosis.

Methods Employed:

Pneumococcal type 12F polysaccharide, a component of the 23 valent vaccine contains an α(1→2)-D-glucobiose moiety:



Streptococcus pneumoniae type 12F (Leontein, et al., 1981).

Typing antisera to this pneumococcal type was used to identify this unusual glucan in dextrans: one dextran denoted as 1299, in particular, has been studied extensively because of its unusual reactivity with type 12 pneumococcal antiserum. Either rabbit or horse pneumococcal type 12F antiserum has been used to detect the α(1→2)-D-glucobiose moiety by double immunodiffusion. Curiously, two courses of multiple intravenous injections of formalin-treated M. tuberculosis, strain H37R, failed to elicit antibodies to pneumococcus type 12F or to dextran 1299. Immune sera from these animals, however, precipitated with another polysacchride from M. tuberculosis known as lipoarabinomannan.

We established an in vitro assay for M. tuberculosis copied after that system used for assaying bactericidal antibodies to Group B streptococci. Animal typing antisera, murine monoclonal antibodies reactive with dextrans and those elicited by immunization of mice with tubercle bacilli were evaluated for their reactivity with the α(1→2)-D-glucose moiety.

Electron microscopy of M. tuberculosis incubated with pneumococcal type 12F antisera showed intense surface strain no observed with murine antibodies to a surface protein.

Human antibodies to pneumococcal type 12 were purified from the plasmapheresis of an adult vaccination previously with a pneumococcal tpe 12F-diphtheria toxoid conjugate. Purified human antibodies were prepared by affinity chromatography with a type 12F polysaccharide-4B amino-hexyl Sepharose matrix.

Major Findings:

A saccharide-like structure, reactive with the anti-α(1→2)-D-glucose moiety of rabbit typing, was purified from M. tuberculosis strain M123 from a patient diagnosed with pulmonary tuberculosis. The preliminary isolation was done with strains grown on Long's media at CBER, FDA and at the Pasteur-Mérieux Institut. Extraction was accomplished by the hot-phenol method for preparing LPS from enterbacteriae. The α(1→2)-D-glucose was more definitely established by gas-chromatography and mass spectroscopy of methylated derivative of the saccharide.

Dextran 1299, conjugated to chicken serum abumin, even when injected along with complete Freund's adjuvant in mice, did not elicit anti-α(1→2)-D-glucose. Mice so immunized elicited precipitating antibodies to the α(1→4) and α(1→3)-D-glucose bonds of other dextrans.

Rabbit, horse and human anti-pneumococcus type 12 antibodies killed M. tuberculosis strains Erdmann and M123 in the presence of complement and human WBCs.

The antibody mediated, complement-dependent killing was inhibited by a kojipentaose but, not with an α(1→4)-D-Gal-trimer.

Significance to Biomedical Research and the Program of the Institute:

The development of a vaccine based upon induction of serum antibodies to a

surface polysaccharide of M. tuberculosis would provide an important new public health measure as well as a conceptual tool for understanding immunity to mycobacteria.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01312-01 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Isolation and Purification of Subunit B of Shiga Toxin

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

PI:	V. Pozsgay	Visiting Scientist	LDMI, NICHD
Others:	J.B. Robbins	Chief	LDMI, NICHD
	L.B. Trinh	Chemist	LDMI, NICHD

COOPERATING UNITS *(if any)*

LCDB, NIDDK (J. Shiloach); Tufts University School of Veterinary Medicine, N. Grafton, MA (A. Donahue-Rolfe); Massachusetts General Hospital, Boston, MA (S.B. Calderwood)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.3	0.1	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 Shiga toxins are multimeric proteins consisting of a single enzymatic subunit (subunit A, mw 32 kDa) and a symmetrically arranged, pentameric subunit B (mw 7.7 kDa). The latter mediates the binding of the holotoxin to cell-surface glycolipids as the first step of its endocytosis. Galabiose is the minimum binding receptor that recognizes the toxin, placed either terminally or internally in glycolipids. The binding is stronger with P blood group determinant trisaccharides, such as the P_k antigen and the P_1 antigen. Specific adsorbents of Shiga (Shiga-like) toxins may aid the development of serologic tests for rapid diagnosis of Escherichia coli infections, and receptor-analog oligosaccharides that inhibit the binding of these toxins to their cell-surface receptors can be candidates for therapeutics against such diseases. The non-toxic subunit B may be a candidate as a carrier in the synthesis of polysaccharide-protein conjugates. Based on the reported carbohydrate-specificities of subunit B, we developed a simple and efficient method for the preparative scale isolation of this toxin fragment, using a receptor-analog affinity sorbent. Specifically, we synthesized the P_1 antigenic trisaccharide equipped with an anchor at its reducing end. The P_1 trisaccharide was covalently attached to a solid support through this anchor. The resulting affinity material was used over twenty cycles to isolate subunit B of Shiga toxin from the fermentation fluid of a non-virulet Vibrio cholera strain that containd the gene for this protein, in a semi-automated fashion.

Project Description:

The proper selection of the carrier protein in the design of carbohydrate-protein conjugates is crucially important and the search for proteins that can be alternative to those already established continues. Non-toxic subunits of bacterial toxins may have the potential of being immunogenic carriers and subunit B of the Shiga and Shiga-like family of secreted toxins can be a candidate for this purpose. The gene of this subunit has been expressed in a Vibrio cholera strain which lacks the gene for subunit A that is responsible for the toxicity of the holotoxin. The carbohydrate specificity of subunit B has been established and a procedure was reported for the isolation of this subunit using receptor-analog affinity chromatography. The very low amount of the carbohydrate hapten in the glycoprotein from the hydatid fluid that was used in the published protocol precludes scale-up. Also, the method may be objectionable as the subunit to be purified is intended for human and animal experiments. Our goal was to design a method for the isolation and purification of subunit B of Shiga toxin.

Objectives:

To synthesize a receptor-analog affinity sorbent for the preparative scale isolation of subunit B of Shiga toxin and to use it in a semi-automated fashion to recover this protein from the fermentation fluid of V. cholera (Strain 0395-N1).

Methods Employed:

The P_1 trisaccharide containing D-galactose and N-acetyl-D-glucosamine residues

α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc

was assembled from suitably protected monosaccharides in stereoselective manner, using thioglycosides and 2-(trimethylsilyl)ethyl glycosides as major intermediates. The trisaccharide was attached to a heterobifunctional spacer that contained a reactive, aldehyde group at its terminus. Through this group the hapten-spacer assembly was coupled to a hydrazino group-containing solid support, using the method of reductive amination.

Subunit B of Shiga toxin was produced by Dr. J. Shiloach (LCDB, NIDDK) by fermentation of a non-virulent V. cholera strain (0395-N1) obtained from Drs. A. Donahue-Rolfe and S.B. Calderwood (Tufts University School of Veterinary Medicine and Massachusetts General Hospital). The periplasmic extract was loaded directly on the affinity column from which subunit B could be eluted selectively in high purity. In each cycle approximately 10 mg of pure subunit B was obtained. Until now the column was used over twenty cycles with unchanged capacity. The identity and purity of subunit B so prepared was assessed by SDS-PAGE electrophoresis and its antigenicity was demonstrated by its reaction with a monoclonal antibody directed against subunit B.

Major Findings:

We established that subunit B of Shiga toxin can be isolated from the

fermentation fluid of a non-virulent V. cholera strain containing the gene for subunit B in high purity using a synthetic trisaccharide-based affinity material.

Proposed Course:

The potentials of subunit B of Shiga toxin as a carrier in polysaccharide-protein conjugates will be evaluated.

Significance to Biomedical Research and the Program of the Institute:

The availability of novel protein carriers is important for the development of carbohydrate-protein conjugate vaccines of improved efficacy.

Publications:

Pozsgay V, Trinh L, Shiloach J, Robbins JB, Donahue-Rolfe A, Calderwood S. Purification of subunit B of Shiga toxin using a synthetic trisaccharide-based affinity matrix. Bioconjug Chem (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01313-01 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Synthetic Vaccine Against Shigellosis

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

PI: V. Pozsgay Visiting Scientist LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LMC, NIDDK (C.P.J. Glaudemans); National Institute of Standards and Technology, Gaithersburg, MD (B. Coxon)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF 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.)*

Shigellosis caused by Shigella dysenteriae type 1 continues to be a major enteric disease worldwide. Because of the multiple-antibiotic resistance of many strains of Shigellae, the treatment of shigellosis is difficult. Although the need for vaccines to control this disease has been documented by the WHO, there is no licenced vaccine against shigellosis. Following the discovery by Robbins and coworkers that serum antibodies against the O-specific polysaccharide (OSP) of S. dysenteriae type 1 confer protective immunity in humans we hypothesized that extended fragments of the OSP may also be suitable for the induction of protective antibodies when coupled to immunogenic proteins, provided that the conformational ensemble of such saccharides approaches that of the conformational determinant of the native polysaccharide. The use of synthetic saccharides of defined structure instead of polysaccharides of complex architecture is likely to offer advantages including enhanced uniformity of conjugates and elimination of the analytical difficulties associated with the established, polysaccharide-protein vaccines. Based on this hypothesis we are developing synthetic oligo- and poly-saccharide-based immunogens of well-defined characteristics. We designed a strategy to prepare fragments of the OSP of S. dysenteriae type 1. The OSP consists of a tetrasaccharide repeating unit that is composed of D-galactose, N-acetyl-D-glucosamine, and L-rhamnose. Starting from monosaccharide building blocks that carry orthogonal protecting and activating groups, a tetrasaccharide donor/acceptor molecule was assembled. Iterative combination of this building block afforded di-, tri- and tetramers of the repeating unit corresponding to octa-, dodeca- and hexadeca-saccharides. Nuclear magnetic resonance spectroscopy indicated that the dodeca- and the hexadeca-saccharides exhibit a high degree of conformational similarity to the native O-SP which we believe is a prerequisite for protective antibody induction. In vitro experiments showed that the synthetic saccharides inhibit the binding of the OSP to homologous monoclonal antibodies.

PHS 6040 (Rev. 5/92)

Others:	J.B. Robbins	Chief	LDMI, NICHD
	R. Schneerson	Research Medical Officer	LDMI, NICHD
	A.B. Karpas	Microbiologist	LDMI, NICHD
	C.W. Broome	Chemist	LDMI, NICHD
	H. Ao	Adjunct Technician	LDMI, NICHD

Project Description:

Shigella dysenteriae type 1 is the causative organism of epidemic and endemic diarrhea and dysentery in may parts of the world with high mortality and morbidity. It is dysentery (fever, mucous and blood in the stool) which is the leading cause of growth retardation in the word. An approach to combat this disease can be vaccine development. In spite of the fact, that the causative organisms have been identified a century ago, there is no licenced vaccine against shigellosis. The need for such vaccines is documented by a World Health Organization document which accords priority to the development of anti-shigellosis vaccines. Although shigellosis can be induced in animals, the animal model for shigellosis has limitations which is highlighted by the fact, that the challenge dose for monkeys is 10^{11} organisms which is 10^{8} times higher than the dose required to induce shigellosis in healthy humans. An essential virulence factor of Shigellae strains is the O-specific polysaccharide component of their respective lipopolysaccharides. Only smooth strains, having fully expressed O-SPs are virulent, whereas rough strains that are devoid of these structures, are not. The essential role of the O-specific polysaccharides of Shigellae and also other enteropathogenic bacteria led to the hypothesis of Robbins and co-workers, that serum IgG antibodies to the OSPs confer protective immunity to the host. The validity of this hypothesis was substantiated by experiments with conjugate vaccines containing purified, O-specific polysaccharides covalently linked to an immunogenic protein. Evidence indicates, that anti-O-specific polysaccharide antibodies in vivo interact with the lipopolysaccharides on the bacterial cell surface. However, little is known about the mechanism of such interactions and about the underlying factors at the molecular level, such as the importance of the hapten geometry, and hapten density in our current conjugate vaccines, concerning either antibody production or antibody recognition.

Objectives:

We surmise that extended fragments of the O-specific polysaccharides may be suitable for the induction of protective antibodies when coupled to immunogenic proteins, provided that the conformational ensemble of such saccharides approaches that of the conformational determinant of the native polysaccharide. Therefore we have been studying chemical synthetic approaches to such saccharides. We are also studying the conformational properties of the synthetic oligosaccharides in relation to the conformation of the native O-specific polysaccharide. A scheme has been devised to covalently attach the saccharides to proteins. The evaluation of the immunologic properties of the synthetic oligosaccharides up to a hexadecasaccharide is currently under study.

Methods Employed:

The O-polysaccharide of S. dysenteriae type 1 consists of the linear tetrasaccharide repeating unit, containing α-linked N-acetyl-D-glucosamine, D-galactose and L-rhamnose as the monosaccharide constituents.

$$[3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n$$

Our plan was to construct a repeating unit block that can serve both as a

glycosyl donor and a glycosyl acceptor in a stepwise fashion from monosaccharide synthons and use it in an iterative manner. A key tetrasaccharide intermediate was selected for this purpose. A major feature of this building block is that after its initial coupling with the aglycon under mild conditions that do not effect the functional/protecting groups, the site of the chain elongation can be unmasked by a minimal protecting group manipulation involving the chemoselective removal of one only protecting group. The monosaccharide synthons were prepared from D-galactose, D-mannose and L-rhamnose, using the methods of synthetic organic chemistry and were combined in stereoselective, stepwise manner. The structures of all the synthetic intermediates and the target saccharides **1-4** were confirmed by analytical and spectroscopic methods including various mass spectroscopic protocols and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

[3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-(1→]$_n$-O$C_{10}H_{23}$

1	n=1
2	n=2
3	n=3
4	n=4

Particular support was provided by the ^{13}C NMR spectra of the dodeca-(**3**) and hexadeca-saccharides (**4**) in the latter of which the anomeric resonances for the four Gal residues, 3 and 4 Rha residues, and 3 GlcNAc residues, respectively, coincide with the corresponding resonances of the native, O-polysaccharide of *S. dysenteriae* 1. This coincidence not only establishes the stereochemical integrity of the interglycosidic linkages but also indicates that compounds **3** and **4** express a high degree of conformational similarity to the native polysaccharide.

Major Findings:

We established that preparative-scale quantities of extended fragments of the native O-specific polysaccharide of *S. dysenteriae* type 1 can be synthesized by iterative combination of a tetrasaccharide building block. Nuclear magnetic resonance studies indicate that an internal portion of the octasaccharide fragment, corresponding to two contiguous repeating units, already expresses conformational similarity to the native O-specific polysaccharide. A higher degree of conformational similarity is expressed by the dodeca- and the hexadeca-saccharides which correspond to three and four repeating units, respectively. Preliminary experiments indicate that the synthetic saccharides inhibit the binding of O-specific polysaccharide specific monoclonal antibodies raised against heat-killed *S. dysenteriae* type 1.

Proposed Course:

The antigenic properties of the saccharide fragments of the O-specific polysaccharide of *S. dysenteriae* type 1 will be evaluated using several O-specific polysaccharide-specific monoclonal antibodies. Covalent conjugates of the synthetic saccharides and immunogenic proteins will be prepared and their efficacy to induce anti O-specific polysaccharide antibodies will be studied. The protective effect of these antibodies will be evaluated. The capacity of the synthetic conjugates to induce antibody will be correlated to their molecular

characteristics including hapten size and hapten density.

Significance to Biomedical Research and the Program of the Institute:

Shigellosis is a continuing public health problem in both the developing and industrialized countries and the development of an effective vaccine will be an important step toward eliminating this disease.

Publications:

Pozsgay V. Synthesis of a hexadecasaccharide fragment of the O-polysaccharide of Shigella dysenteriae type 1. J Am Chem Soc 1995;117:6673-81.

Pozsgay V, Coxon B. Stereoselective preparation of alkyl glycosides of 2-acetamido-2-deoxy-α-D-glucopyranose by non-classical halide-ion catalysis and synthesis and NMR spectroscopy of α-D-Galp-(1→3)-α-D-GlcpNAc-OMe. Carbohydr Res (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01314-01 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Analysis and Synthesis of Carbohydrate Antigens of Mycobacterium tuberculosis

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

PI: V. Pozsgay Visiting Scientist LDMI, NICHD

See Attached

COOPERATING UNITS *(if any)*

LAC, NIDDK (N. Whittaker); National Institute of Standards and Technology, Gaithersburg, MD (B. Coxon)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	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.)*

Mycobacterium tuberculosis, the causative organism of tuberculosis is responsible for approximately three million death cases annually and the mortality rate of infections caused by multiple drug-resistant strains of this bacterium exceed 80%. M. tuberculosis is coated by a number of polysaccharides of unusual structure. A unique component termed polysaccharide II is composed of alpha, 1,2-linked D-glucose residues. Schemes were designed to isolate this polysaccharide from polysaccharide-containing extracts of M. tuberculosis. We demonstrated that the major monosaccharide components in soluble polysaccharide fractions from Mycobacteria are arabinose, mannose and galactose. A polysaccharide fraction was also identified which contains mannose and glucose in approximately equal amounts. We demonstrated the presence of a glycogen-like polysaccharide as one of the extracellular polysaccharides of Mycobacteria. We developed a method for the chemical synthesis of oligosaccharides corresponding to the putative polysaccharide II. We showed that for such compounds the stepwise synthesis is the method of choice.

PHS 6040 (Rev. 5/92)

Others:	J.B. Robbins	Chief	LDMI, NICHD
	R. Schneerson	Research Medical Officer	LDMI, NICHD
	E. Dubois	Visiting Fellow	LDMI, NICHD
	C.W. Broome	Chemist	LDMI, NICHD

Project Description:

Tuberculosis caused by Mycobacterium tuberculosis continues to be a major cause of death worldwide and the fatility rate of this disease when caused by multiple antibiotic-resistant strains in immunocompromised patients is higher than 80%. There is increasing evidence that antibodies against mycobacterial surface antigens may play a role in protection.

The major component of the extracellular fraction of M. tuberculosis termed polysaccharide II by Siebert was shown to be a linear of α1,2-linked D-glucose residues. Experiments were designed to isolate and identify this unique polysaccharide for studies of its possible role as a protective antigen. As an alternative approach to this polysaccharide, chemical synthetic approaches were also planned.

Objectives:

To identify glucose-containing polysaccharide(s) among the carbohydrate fractions of the cell-surface polysaccharides of M. tuberculosis. To synthesize oligosaccharides containing α1,2-linked D-glucose residues, corresponding to polysaccharide II and to covalently attach them to high molecular weight carriers.

Methods Employed:

(a) Polysaccharide fractions of M. tuberculosis were analyzed by a combination of chemical and instrumental methods. These included chemical conversion of the saccharides into (partially methylated) alditol acetates which were analyzed by gas chromatography combined with mass spectroscopy. The intact saccharide fractions were also analyzed by high-resolution nuclear magnetic resonance spectroscopy.

(b) The putative glucan antigen of M. tuberculosis termed Polysaccharide II is a linear polysaccharide containing α-(1→2)-linked D-glucopyranose residues. Retrosynthetic analysis of this polysaccharide indicated that a single building block, equipped with a temporary, non-participating group at O-2 may be sufficient for its construction. We have chosen the glucosyl donor 3,4,6-tri-O-acetyl-2-O-benzyl-β-D-glucopyranosyl bromide for this purpose which was obtained stereoselectively from the corresponding, α-phenylthio glucopyranoside. The donor afforded α-linked glucosides upon solvolysis with primary alcohols in a completely stereoselective manner. Subsequent, iterative chain extension involving chemoselective liberation of the elongation site followed by silver salt-catalyzed glucosidation with the donor/acceptor building block afforded fully protected kojibiose to kojipentaose derivatives from which the protecting groups were removed to provide di- to penta-saccharide analogs of the elusive Polysaccharide II. Because of the limitations of the monosaccharide donor-based chain extension we also investigated several donor/acceptor derivatives of the disaccharide kojibiose. The rationale behind this approach is that if successful, the chain is increased by two residues after each glycosidation. Reaction of these donors with partially protected glucosides having their HO-2 free afforded kojitriose derivatives in high stereoselectivity. On the other hand, attempted coupling of the kojibiose donors with kojibiose or

kojitriose derivatives having a free HO-2 at their non-reducing end termini failed. Major products in these reactions included cyclo-kojibiose and trehalose-type tetrasaccharides.

Major Findings:

(a) We demonstrated that the prevalent monosaccharide components in polysaccharide fractions from Mycobacteria are arabinose, galactose and mannose. We identified a polysaccharide fraction which contains an mannose and glucose in approximately equal amounts together with a smaller proportion of arabinose. We also demonstrated the presence of a glycogen-like polysaccharide as one of the extracellular polysaccharides of Mycobacteria.

(b) We developed a method for the chemical synthesis of koji-oligosaccharides. We showed that the stepwise synthesis is the method of choice for the synthesis of such compounds. The failure to use [2+n (n>1)] block-synthetic approaches indicate extensive clashes between the acceptor and the donor. The immunochemical properties of the synthetic kojipentaose have been studied as described in Dr. John B. Robbins' report (Z01 HD 01311-02).

Proposed Course:

(a) The structure of the glucose-enriched fraction of M. tuberculosis will be studied using specific degradation instrumental analytical methods.

(b) Higher-order oligosaccharides corresponding to Polysaccharide II will be synthesized in a form that allows their covalent attachment to macromolecules. The conformation of the synthetic koji-oligosaccharides will be studied. These studies may reveal a conformational determinant of Polysaccharide II and may provide a better understanding of the molecular basis of the unique properties of this polysaccharide.

Publications:

Pozsgay V, Robbins JB. Synthesis of a pentasaccharide fragment of polysaccharide II of Mycobacterium tuberculosis. Carbohydr Res (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01315-01 LDMI

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Modulation of Protein and Cell Functions by Heparin/Heparan Sulfates

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

PI: A.L. Stone Chemist LDMI, NICHD

COOPERATING UNITS *(if any)*

LCM, NIMH (G.E. Deibler); Harvard Medical School, Cambridge, MA (R.P. Junghans); LDDRD, NCI (J.B. McMahon); BEIP, NCRR (M.S. Lewis)

LAB/BRANCH

Laboratory of Developmental and Molecular Immunity

SECTION

Section on Bacterial Disease Pathogenesis and Immunity

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland, 20892-2720

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.87	1.0	0.87

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.)*

Heparin\heparan sulfate and heparin-mimetic sulfated oligosaccharides (S-oligoS) were studied in vitro to elucidate the structural basis of their specific modulations of protein and cell membrane functions, using physi-cal, biochemical, and biological methods. In one such study, strategy was based on our view that heparin-like molecules play a role in the molecular mechanisms underlying the infectivity of human immunodeficiency virus (HIV-1): If the known inhibition of the cytotoxic (CT) and syncy-tium-formimng (SF) infectiousness of HIV-1 by S-oligoS were governed by a structural specificity, then rationale for clinical usefulness of a putative S-oligoS agent against AIDS would be enhanced.

Here first, structural specificity in the inhibition of HIV-1 by S-oligoS was demonstrated, using an HIV-inhibitory anticoagulant pharmaceu-tical which comprised a mixture of S-oligoS components(Cps) from sulfated xylan. Highly active (HA) Cp were then purified and this specificity was separable from anticoagulantly active molecules.

Now 1) The minimum-sized HA-Cp, CpF, (EC50 vs SF and CT= 100 and 200 ng/ml) was purified. Molecular mass of Cps was determined by S-E ultra-centrifugation of monodansyl derivatives. Minimum mass of anti-HIV-1 S-oligoS was ~4500 and a smaller S-oligoS, HA vs CT but not SF, had mass of ~nonomer. 2) Cp contain S-α 1,2 D-GlcA monomers on the S-xylose chain. Molecular modeling revealed that such GlcA could impart local structures resembling those in heparin. Analysis for GlcA by o-nitrophenylhydrazide reaction revealed unexpectedly that Cps had multiple GlcA, supporting a view that their heparin-mimetic action redounds from GlcA-containing structures. 3) Anti-HIV-1 activity appears to reside in ~ a tetradecaglu-curonoxyloside containing 2 or 3 GlcA. 4) Methods upscaling preparation of CpF were developed and are being modified for clinical application.

Project Description:

This project studies the structure/function relations (st/fn) of heparins and heparan sulfates (H/HS) (glycosamino polysaccharides of cells and the extracellular matrix) to elucidate the molecular basis of their capacity to bind to and modulate the biological activity of proteins and cell membranes (e.g., in development, blood coagulation, cytokine regulation, tumor growth, lymphocyte homing, and viral infectivity) [Z01 MH 013605-12 LNB]. The methodological approaches in these studies are physical, biochemical and biological. Except for Rosenberg's elucidation of the structural specificity of H/HS in anticoagulation, st/fn of H/HS remain undetermined. We provided a model of structural elements in H that may be applicable to the diverse specificities of H/HS and heparin-mimetic oligosaccharides (S-oligoS). Current studies include H/HS and S-oligoS in vitro inhibition of HIV-1, as ligands for CD4 lymphocytes, and conformational modulation of human basic fibroblast growth factor (bFGF). Understanding such structural specificity would enhance the development of new H/HS-mimetic pharmceuticals as well as generate understanding of the basic structural modes of action of the H/HS class of biopolymers. Other collaborative projects study structural aspects of the biology of the soluble T-activation antigen chain of IL2R and myelin basic protein.

Objectives:

1. AL Stone with JB McMahon, FCRDC, and MC Lewis, NCRR.

To study specificity in st/fn of heparin-mimetic S-oligoxylans, S-oligoS components (Cps)of SP54 were purified by our analytical-scale methods (LPLC through 195 cm, i.d. 0.6 cm, BioGel P10 in 0.5 M NH_4HCO_3, using quantitative spectroscopy for detection and quantitation. Cps were bioassayed for activity vs cytoxicity (anti-CT) (soluble formazan method) and vs the syncytium-forming infectivity of HIV-1 (anti-SF) (by quantitation of fusion between infected and uninfected CD4 cells) [Z01 MH 02593 02-3 LBG]. We found that antiviral capacities of the S-oligoS were governed by a structural specificity (evidenced by their differential and biphasic potencies). This enhanced a rationale for potential usefulness of an S-oligoS against AIDS and offered an explanation for previous clinical failures using mixtures. Molecular modeling suggested that the heparin-mimetic capacity of such S-oligoxylans might obtain by virtue of monomeric a-linked D-glucuronic acid branches which occur randomly on the average of one in 10 xyloses in the native xylan chain. Three types of anti-AIDS virus Cps were identified among 19 which ranged from ~30,000 to <2000 in apparent molecular mass: 1) CpFC, relatively high in molecular mass, ~ an eicoS, highly active (HA) against CT and SF (EC_{50}=50-100 ng/ml) and anticoagulantly active in vitro (up to 25% the potency of heparin); 2) CpF, a HA ~tetradecS having EC_{50}=250 vs CT and 50 ng/ml vs SF, but low activity vs thrombin; and 3) CpC, an ~nonoS, HA vs CT but not vs SF and anticoagulantly inactive [1994 project report].

Antiviral Cps were characterized by: 1) refinement of the analytical-scale LPLC system to analytical use in identifying CpFC, CpF and CpC, based on elution volume; 2) quantitation of maximum metachromatic reactivity of Cp-methylene blue (MB) complexes (based on minimum extinction coefficient obtained at the a absorption band of MB ($E_{min\ a}$), which yielded characteristic $E_{min\ a}$ among Cp; 3)

establishment of a quantitative colorimetric analysis of GlcA content for these sulfated oligoS through application of the o-nitrophenyl hydrazide method of determination of activated uronic acids [GlcA standard curve was linear in the ug range; sample response was linear in the range 5 to 40 ug GlcA]; 4) determination of molecular mass by sedimentation-equilibrium analysis of uv-absorbing, monodansylated S-oligoS using absorption optics, our calculated **n** (0.475) and an equation we derived to correct the $mass_{observed}$ by an "effective charge" factor; 5) quantitation of antiviral potency as above; and 6) measurement of APTT by an automated clinical assay and evaluated relative to heparin.

Characteristics of CpF showed that this Cp contained the minimum-sized HA structure vs HIV-1. Moreover, the structure was ~S-tetradecaS, containing 2 or 3 GlcA. These data showed that the heparin-mimetic activity of sulfated xylan was due to glucuronoxyloside degradation products rather than associated with a "sulfated pentosan". Since CpF might yield an agent suitable for testing against the AIDS virus <u>in vivo</u>, we next developed up-scaled preparations of CpF.

a) We reasoned that CpFC region through 190 cm x 2.3 cm BioGel P6 in 0.5 M NH_4HCO_3 would be separated near the void peak, while the CpF region would be better fractionated. Using reference Cp as chromatographic markers and lambda-carrageenan to determine the void volume, three 150-200-fold scaled-up preparations yielded the CPFC, CpF, and CpC regions. Yield of CpF was ~10 percent. This product was further purified. Under modified good manufacturing practices, the CpF (~600 mg) was further purified by the modified methods (through 176 cm x 2.3 cm), yielding three fractions of which aliquots were prepared for anti-CT and anti-SF bioassays and vs HIV-1 in the hollow fiber assay in mice.

b) A 25-fold, direct upscale of a modified analytical system is in progess; and

c) Multiple 40-fold upscaled preparations using the modified P6 system are underway as a means towards obtaining sufficient CpF for potential clinical application. CpF regions have been identified for ongoing combinations and procedures.

The findings provide a basis for: 1) potential development of an inexpensive adjunct to AIDS therapy (CpF); 2) study of molecular mechanisms involving S-oligoS in the effects of HIV-1 that kill immune cells; and 3) improvement of clinical usefulness of S-oligoS in anticoagulation therapy, in addition to elucidating st/fn of H/HS and S-oligoS. Studies will be continued. A manuscript has been submitted. A manuscript, "Differential inhibitory potency of sulfated xylan oligosaccharides in the inhibition of HIV-1 cytotoxicity and syncytium-forming infectivity <u>in vitro</u>", by AL Stone, DJ Melton, and JB Mc Mahon, is in progress.

2) AL Stone, RP Junghans collaboration with MS Lewis.

To determine the molecular form of the soluble T activation antigen (Tac) chain of the interleukin 2 receptor(IL2R), a purified recombinant Tac was examined by size exclusion chromatography, velocity-sedimentation and sedimenation-

equilibrium ultracentrifugation analysis and low ultraviolet circular dichroism spectroscopy. High affinity binding of the cytokine, IL-2, to IL2R during lymphocyte activation requires cooperation among three known receptor polypeptide chains. One of these, Tac, is absent in resting lymphocytes and is strongly upregulated upon cell activation to generate the high affinity IL2R. After the acivation, Tac is released from the membrane in a soluble form. There was controversy about whether this highly regulated chain was dimeric or monomeric in the native form, which clouded all proposed molecular mechanisms of action. Seiving HPLC indicated oligomeric, mixed or dimeric structure, while immunological and electrophoretic evidence indicated a monomeric form. We now show that HPLC chromatography led to artifacts probably induced by solute-column interactions and that the Lewis ultracentrigfugal methods are critically superior. Spectroscopy showed that variation in forms due to raising or lowering the solvent salt concentration were not seen absent chromatographic matrix. Moreover, molecular mass determined by sedimentation ultracentrifugal analysis was that of a monomeric form. Studies of renal filtration and a one-epitope stoichiometry of antibody-antigen titrations indicated that Tac was monomer. These findings resolve the contoversy (above) and point out that the biologically relevant form is a momomeric protein. Knowledge of the molecular form of soluble Tac is essential to understanding the regulation and process of lymphocyte activation in vivo. A manuscript is in preparation.

3) AL Stone with MR Ranson, Christie Hospital, Manchester, UK.

To study the molecular basis of the modulation of bFGF function by H/HS (low affinity bFGF receptor) and suramin (S) (an anti-cancer, growth factor antagonist), the structure of human recombinant bFGF was examined by low uv circular dichroism (CD) and by static fluorescence (F) and anisotropy spectroscopy in the absence and presence of the modulators (work completed previously [Z01 MH 02593-02-3 LBG]). In brief, we showed that S was bound within 0.9nm of bFGF cell-receptor site W. H (from CD analysis) also altered the peptide conformations around the W in the cell-receptor and concomitantly led to purturbation of the W and oligomerization of bFGF (by anisotropy of F analysis). Findings were important for understanding molecular events that subserve modulation of bFGF function by H/HS; they support our idea that heparin modulates protein functions by exerting a "conformational catalysis" which enables a specific transition to a new stable state. A manuscript this year was revised for resubmission.

4) AL Stone, GE Deibler collaboration, LCM, NIMH.

Myelin basic protein (MBP)is the encephalitogenic antigen in the experimental autoimmune model for study of multiple sclerosis. Brain myelin contains several isoforms, in particular a phosphorylated form which appears to be coupled to a kinase and phosphatase in the membrane and to have a rapid rate of phosphate turnover. The role(s) of MBP isoforms in myelin is unclear. We proposed that MBP has a conformational adaptability which might be subserved by the isoforms. To study this model in human MBP, threonine-phosphorylated 18.5 kDa isoform (PT96) and nonphosphorylated isoforms were purified by Deibler's new method of selective thrombic digestion and structural properties of isoforms were compared by CD spectroscopy [1994 project report]. Results showed that in vivo phosphorylation to PT96 produced an increase in stable folding of 18.5 kDa hMBP

up to ~30% (similar to that predicted from its amino acid sequence), which was likely transition to a class B type II beta turn and beta-structure. These findings supported our suggestion that conformational adaptability associated with isoforms of hMBP may subserve a structural integrity of myelin.

Significance to Biomedical Research and the Program of the Institute:

Discovery of biologically active components among the polymeric sulfated saccharides has potential for providing probes for cell-surface HIV-1 interactions as well as for other human proteins such as basic myelin protein. Clinically acceptable fragments of sulfated inulin are being evaluated for their activity in HIV-1 infected CD4 human lymphocytes.

Publications:

Stone AL, McMahon JB, Melton DJ, Lewis MS. Sulfated glucuronoxylyloligosaccharides as potent inhibitors of the cytotoxocity and syncytium-forming infectivity of the human immunodeficiency virus (HIV-1) in vitro. Glycoconjug J 1995;12:586.

Deibler GE, Burlin TV, Stone AL. Three isoforms of human myelin basic protein: Purification and structure. J Neurosci Res (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00047-26 LDN

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Biochemical Studies of Neurons and Other Cell Types

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

PI:	D. Brenneman	Head	LDN,NICHD
Others:	J. Hill	Expert	LDN,NICHD
	G. Glazner	IRTA Fellow	LDN,NICHD
	G. Gibney	IRTA Fellow	LDN,NICHD
	I. Gozes,R. Alvero	Guest Researcher	LDN,NICHD
	T. Dibbern, J. Wu	Guest Researcher	LDN,NICHD
	D. Warren	Bio. Lab. Tech.	LDN,NICHD

COOPERATING UNITS *(if any)*

Dept. Clin. Biochem. Tel Aviv Univ., Israel (I. Gozes); Dept. Organic Chem., Weizmann Inst. (M. Fridkin); Dept. Neurol., Univ. Kansas (B. Festoff); Dept. of Immunol. George Washington Univ. (T. Phillips); Univ. Queensland, Australia (Alice Cavanaugh)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Developmental and Molecular Pharmacology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
8.1	5.3	2.8

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.)*

Structure-activity studies of a peptide derived from Activity Dependent Neurotrophic Factor (ADNF), a novel protein released from glial cells by vasoactive intestinal peptide (VIP), revealed that complete survival-promoting activity was retained with a nine amino acid peptide (ADNF-9). A panel of thirty test peptides was utilized to establish the loci and identity of critical amino acid residues. In dissociated cell cultures from the rat cerebral cortex, ADNF-9 was found to exhibit neuroprotective actions to prevent neuronal cell death associated with excitotoxicity and gp120, the envelope protein from the human immunodeficiency virus. Further structural studies on peptides from protease digests of intact ADNF indicated close homology to a recognized intracellular stress protein.

Radiolabeled gp120 administered to pregnant rats was recovered in the brains of fetuses. Both intact gp120 and its neurotoxic fragments were found to preferentially accumulate in fetal brain two days after injection. Treatment of whole cultured mouse embryos with gp120 produced a dose-dependent inhibition of growth as measured by somite number and image analysis of whole body size. The growth deficits produced by gp120 were prevented by co-treatment with either VIP or a VIP analogue, peptide T.

A novel VIP agonist (SNV) was synthesized and shown to be neuroprotective against the neuronal cell killing action of beta amyloid peptide, a toxic substance associated with Alzheimer's disease. SNV was shown to preferentially interact with VIP binding sites that were insensitive to GTP in the developing rodent brain and that were independent of cAMP action. SNV, like VIP, produced significant increases in growth in early post-implantation embryos. VIP-mediated increases in mitosis in embryos was associated with increased cyclin A expression as measured by reverse transcriptase polymerase chain reaction.

Project Description:

Objectives:

1) To investigate the structure and functions of activity dependent neurotrophic factor (ADNF), a survival-promoting substance that is released by vasoactive intestinal peptide (VIP).

2) To study the relationship between VIP and glia-derived cytokines.

3) To extend the investigation of VIP, VIP analogues and ADNF neurotrophism to embryonic development *in utero*.

4) To develop and characterize new agonists and antagonists to VIP receptors in the central nervous system (CNS).

5) To study the neurotoxicity of the HIV envelope protein in developing animals and to devise VIP-related protective agents against HIV-related viral products.

Methods Employed:

Dissociated cultures derived from the central nervous system of fetal rodent were used for studies of VIP, cytokines and growth factors that increase neuronal survival. In particular, the development of cerebral cortical cultures from term rats was emphasized. This preparation allowed for a system homologous to that used for the isolation of ADNF. Computerized image analysis, calcein uptake and ouabain binding were used to assess neuronal populations in culture. For specific cellular phenotype assessment, immunocytochemical methods for neuron specific enolase, glial fibrillary acid protein, methionine-enkephalin and VIP were performed. Quantitative Northern blot hybridizations and RT-PCR were conducted for the analysis of neuropeptide gene expression. The following biochemical techniques were used: radioimmunoassay for peptides and cyclic nucleotides, capillary electrophoresis combined with luminescence-based ELISA assays for cytokines, one- and two-dimensional polyacrylamide gel electrophoresis, assays for choline acetyltransferase and glutamic acid decarboxylase and radioligand binding. Standard chromatographic (anion exchange, sizing, and reverse phase) separations were conducted to purify ADNF and to measure VIP degradation products. Autoradiography of VIP receptor binding was conducted on frozen thin sections of brain as was *in situ* hybridization. Whole embryo cultures and embryos obtained *in utero* were used to study the growth promoting actions of VIP and related drugs. E9.5 mouse embryos were incubated for up to 24 hours in the presence of fresh rat serum. Embryos were evaluated by embryonic volume, protein and DNA content, receptor distribution and by mitotic assay of bromodeoxyuridine incorporation into nuclei as detected by immunocytochemistry. For analysis of dendritic arbor, Golgi impregnation was utilized with quantitative image analyses.

Major Findings:

1. Structure-activity studies of thirty peptides indicated that the survival-promoting action of ADNF (Activity Dependent Neurotrophic Factor) could be retained in a nine amino acid

peptide (ADNF-9). This finding of a peptide that mimics an intact growth factor is unprecedented.

2. In cerebral cortical cultures, ADNF-9 was found to be neuroprotective against the neuronal cell death produced by beta amyloid peptide, a substance associated with neural degeneration in Alzheimer's disease.

3. Neuronal cell death produced by the envelope protein of the human immunodeficiency virus was prevented by treatment with ADNF-9 at femtomolar concentrations in cerebral cortical cultures.

4. The amino acid sequence of five additional peptides derived from protease digests of ADNF were determined. Four of the five peptides had sequence homology to a recognized intracellular stress protein.

5. Radiolabeled and biologically active gp120 was recovered in the brains of mouse fetuses after systemic administration of gp120 to the maternal circulation. Toxic fragments of gp120 were also found in fetal brain as assessed by cerebral cortical cultures treated with brain homogenates.

6. Inhibition of somite growth was observed in whole mouse embryo cultures treated with gp120. The growth deficits produced by gp120 were prevented by VIP or the VIP analogue, peptide T.

7. A lipophilic VIP analogue (stearyl, norleucine$_{17}$VIP, SNV), discovered and synthesized by our Israeli collaborators (Drs. Gozes and Fridkin) was found to increase somite growth of early post-implantation embryos.

8. *In vitro* autoradiographic studies revealed that SNV displaced VIP binding from those sites that were insensitive to GTP and not linked to cAMP accumulation.

9. SNV provided neuroprotection from neuronal cell death produced by beta amyloid peptide in dissociated cerebral cortical cultures.

10. PACAP38, a peptide that shares 50% homology to VIP, was shown to increase the release of cytokines from astroglia, albeit 60-80% less than that observed with VIP. Peptide T, cytoprotective VIP analogue currently in phase two clinical trials for the treatment of NeuroAIDS, also increased cytokine secretion, but was 3-5 fold less efficacious than VIP.

11. Stimulation of microglia with 0.1 nM VIP produced no detectable release of the eight cytokines observed with astrocytes. However, micromolar amounts of VIP did produce significant release of interleukin-1 beta.

Significance to Biomedical Research and the Program of the Institute.

Investigations of the neurotrophic properties of VIP have now been extended to drug development. The fundamental concept which provides the rationale for this extension is that

molecules important to regulation of neuronal survival during development may also be important in the regulation of repair mechanisms after nervous system injury. Having established that VIP exhibits potent neuronal-survival promoting activity in cell culture, the demonstration that VIP and VIP analogues are neuroprotective against agents associated with human neuro-degenerative diseases sustains the development/injury association. The development of lead compounds for the treatment of Alzheimer's disease is certainly suggested by our results which indicate that our lipophilic VIP analogue is effective in preventing cell death associated with the beta amyloid peptide. Similarly, our studies with the protective properties of VIP on gp120-induced neurotoxicity and growth retardation are strongly suggestive as useful strategies to treat the problems associated with growth retardation and neurological impairment associated with infection with the human immunodeficiency virus during pregnancy. With the discovery that a nine amino acid peptide that exhibits neuroprotection at femtomolar concentrations, the therapeutic potential of ADNF is greatly increased. One of the major dilemmas in the use of growth factors in the treatment of neurodegenerative diseases lies in the challenges of getting these proteins into the brain and protecting against potential autoimmune effects. With the discovery that ADNF can be mimicked by a nine amino acid peptide, both CNS entry and drug development may be realized for the treatment of the neurological symptoms associated with HIV infection, Alzheimer's disease and potentially other prevalent neurodegenerative diseases.

Proposed Course:

Based on the discovery of ADNF-9, peptidemimetics will be developed and tested for neuroprotection. Investigations of ADNF-9 and related analogues will also be conducted for their ability to cross the blood-brain barrier.

Antisera against ADNF will be tested for effects on the development of mammalian embryos. If the anti-serum produces growth retardation, detailed neuroanatomical and biochemical studies will be conducted. Investigations of developmental milestones and learning behavior will also be tested in neonates from mothers treated with anti-ADNF.

In collaboration with our colleagues in Israel, the molecular cloning of ADNF will continue to be pursued. Both our lab and Dr. Gozes's group will employ separate tactics to obtain the cDNA of ADNF. Expression cloning methods will be undertaken with our newly-acquired anti-ADNF-15; in addition, strategies involving PCR cloning will be conducted. More amino acid sequence of ADNF digests may also be required.

The relationships between VIP, ADNF and chaparonin-10 will also be explored during mid-gestational pregnancy. Collaborations with Alice Cavanaugh (Univ. of Queensland, Australia) will focus on the role of early pregnancy factor and its potential role in regulating embryonic development.

Effort will intensify on developing a biochemical/immunological assay for ADNF. With the production of large amounts of rabbit anti-ADNF and the procurement of capillary electrophoresis equipment, this goal should see progress.

The lipophilic analogues of VIP will be used to further investigate the relationship between VIP and cytokines release from astrocytes.

Protocols:

Animal

93-025	Brenneman	The role of vasoactive intestinal peptide and related peptides in neurobehavior development and the morphology and neurochemistry of the developing nervous system
93-026	Brenneman	The role of vasoactive intestinal peptide and related peptides on behavior and the morphology and neurochemistry of the brain
94-021	Brenneman	Glial-derived trophic factors
95-003	Brenneman	Influence of VIP and antagonists on developing neuroepithelium in mouse embryos

Publications:

Arimura A, Somogyvari-Vigh A, Weill C, Fiore C, Tatsuno I, Bay V, Brenneman DE. PACAP functions as a neurotrophic factor. NY Acad Sci 1994;739:228-43.

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Gozes I, Fridkin M, Westphal H, Glowa J, Hill JM, Reshef A, Zhukovsky S, Waner T, Niska A, Rubinrout S, Lilling G, Davidson A, Glazer-Steiner R, Moody TW, Rostene W, Brenneman DE. Neuronal VIP: from gene to sexual behavior, memory and clinical applications. In: Proceedings

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Lilling G, Wollman Y, Goldstein MN, Rubinraut S, Fridkin M, Brenneman DE, Gozes I. Inhibition of human neuroblastoma growth by a specific VIP antagonist. J Mol Neurosci. In press.

McCune SK, Hill JM. Ontogenic expression of two alpha-1 adrenergic receptor subtypes in the rat brain. J Mol Neurosci. In press.

Olah Z, Lehel C, Anderson WB, Brenneman DE, Agoston DV. Subnanomolar concentration of VIP induces the nuclear translocation of protein kinase C in neonatal rat cortical astrocytes. J Neurosci Res 1994;39:355-63.

Sone M, Smith M, Gozes I, Brenneman DE, Fridkin M, Ghatei MA, Bloom SR. Pituitary adenylate cyclase-activating polypeptide (PACAP)/vasoactive intestinal peptide (VIP) receptor subtypes in rat tissues: investigation of receptor binding and molecular identification by chemical cross linking. Biomed Res 1994;15:145-53.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00056-20 LDN

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Biosynthesis, processing & secretion of neuropeptides & pituitary peptide hormones

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

PI:	Y. Peng Loh	Section Chief	LDN, NICHD				
Others:	Le-Ping Pu	Visiting Fellow	LDN, NICHD	ChunFa Zhang	Courtesy Associate	LDN, NICHD	
	Theodore Friedman	Med Staff Fellow	LDN, NICHD	Vicki Olsen	Guest Researcher	LDN, NICHD	
	AnaMaria Bamberger	Visiting Fellow	LDN, NICHD				
	David Cool	IRTA	LDN, NICHD				
	Niamh Cawley	Pre-IRTA	LDN, NICHD				
	Diane Louie	Pre-IRTA	LDN, NICHD				

COOPERATING UNITS *(if any)*

ERRB(H-C Chen); U of Auckland, New Zealand (N. Birch); Brown U (E. Nillni, I. Jackson);Sandoz Inst. Med Res. London (C. Snell);NCI (A. Bird);NIDDK (L. Parnell)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Cellular Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.9	3.9	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 sorting signal motif for targeting pro-opiomelanocortin (POMC, pro-ACTH/endorphin) to the regulated secretory pathway has been identified as a 13 amino acid amphipathic loop located at the N-terminus of POMC. An amphipathic loop sorting signal has also been identified for pro-enkephalin, indicating the generality of these motifs as sorting signals for the regulated secretory pathway. Recently, a sorting receptor has been identified in bovine pituitary Golgi and secretory granule membranes. It is a ~47 kD protein and is highly specific for the N-POMC sorting signal. The POMC sorting signal bound to this receptor optimally at pH 5.5-6.5, consistent with the pH of the trans Golgi network where sorting begins. Our findings support a receptor-mediated mechanism for sorting prohormones to the regulated secretory pathway. A novel class of prohormone processing aspartic proteases was studied. One of these, yeast aspartic protease 3 (YAP3), has been purified and shown to cleave prohormones at a pair of basic residues; and a pair or mono-basic residue with an additional basic residue upstream or downstream from the cleavage site. The Km and kcat of YAP3 for various prohormone substrates showed that the catalytic efficiency was enhanced by increasing the number of basic residues flanking the cleavage site. The specificity and pH optimum of YAP3 were similar to the ~70 kD mammalian aspartic protease, pro-opiomelanocortin converting enzyme (PCE). YAP3 antibody cross-reacted with PCE on Western blots, suggesting that YAP3 and PCE are homologues. Western blot analysis showed the presence of two YAP3-like proteins, ~70 kD and ~90 kD in mouse brain and pituitary. Immunocytochemistry using anti-YAP3 in combination with *in situ* hybridization for neuropeptide cDNAs revealed colocalization of YAP3-like immunoreactivity with CCK mRNA in hippocampal neurons and vasopressin mRNA in supraoptic nucleus neurons. Thus YAP3-related processing enzymes likely play a role in pro-hormone processing in the CNS. YAP3-related cDNA clones from a mouse brain library are currently being analyzed.

Project Description

Objectives:

The ACTH-endorphin family of peptides play many physiological roles (e.g. in stress, fetal development, osmoregulation and adrenal regeneration). These peptides are derived from a single multivalent prohormone (pro-opiomelanocortin, POMC). This prohormone is proteolytically processed at paired and tetrabasic residue sites to yield various active peptide products (α-MSH, ACTH, β-endorphin, β-LPH, N-POMC$_{1-49}$, γ_3-MSH) which, in turn, are modified at the C- and/or N-terminus. Differential processing occurs in different brain and pituitary regions. Proteolytic processing of POMC occurs in specific cellular compartments, beginning in the trans-Golgi network and continues within the secretory granule (vesicle). A highly specific set of proteases are involved in the various cleavages of this prohormone. The prohormone is differentially processed in various brain and pituitary regions, giving rise to a large diversity of secreted end-products with different biological actions. Moreover, the processing also appears to be modifiable by physiological inputs to the tissue. Our long-term goal is to understand the molecular mechanisms involved in the intracellular trafficking and sorting of POMC to the secretory granule for processing and the regulation of tissue- and stimulus-specific processing of this prohormone by different processing enzymes. Such cellular mechanisms uncovered for POMC will likely be extendable to other prohormones and pro-neuropeptides. Elucidating the molecular mechanisms which regulate the intracellular trafficking, biosynthesis and secretion of the POMC-derived peptides will facilitate the overall understanding of the regulation of the hypothalamic-pituitary-adrenal (HPA) axis in normal and disease states. We have chosen yeast cells, the frog and mouse brain, anterior and intermediate pituitary and bovine pituitary secretory vesicles as models and biological preparations, which are especially useful for addressing these issues. Our immediate major objectives are (i) to investigate the signals and mechanisms involved in the intracellular targeting and sorting of POMC, into the regulated secretory pathway of endocrine and neuronal cells; (ii) to identify, purify, characterize and clone the enzymes involved in the processing of pro-opiomelanocortin to their respective hormones and to determine how these enzymes regulate tissue-specific processing; (iii) to study the regulation of biosynthesis (at the transcriptional, translational and post-translational level), turnover and secretion of peptides that regulate the HPA axis such as ACTH, α-MSH, endorphin, vasopressin and pro-TRH-derived peptides, in the pituitary and CNS, during various types of pertubations (e.g. stress, salt-loading) and clinical paradigms.

Methods Employed:

Acid-urea and SDS gel electrophoresis, high performance, and fast performance liquid chromatography (HPLC, FPLC), radioimmunoassays (RIA), immunochemistry, enzyme assays, column chromatography, antibody production, gene cloning techniques, *in situ* hybridization, electron microscopy and tissue culture.

Major findings:

Identification of prohormone sorting signals for the regulated secretory pathway.

The search for molecular sorting signals to the intracellular regulated secretory pathway has been a major challenge in cell biology. Dr. David Cool and Winnie Tam have continued to investigate the signal and mechanisms involved in the targeting and sorting of POMC to the regulated secretory pathway. Previous studies used different POMC/CAT fusion genes containing different fragments of the POMC cDNA fused to the CAT gene, transfected into AtT-20 cells, to define the molecular domain of POMC that is sufficient and necessary for targeting the prohormone into secretory granules. Those studies indicated that the targeting signal for the regulated pathway resides in the first 26 amino acids of POMC. This region of POMC is highly conserved across species and contains a unique structural motif of a hairpin loop stabilized by two disulfide bridges located at Cys_2-Cys_{24}, and Cys_8-Cys_{20}.

To further define the sorting signal motif, two sets of mutations were made to the N-terminal region of full-length POMC. In the first set, three deletion mutations were made; i) amino acids 2-26 were deleted (Δ2-26); ii) amino acids 8-20 were deleted to remove the loop (Cys8-Cys20 Del); iii) 78 amino acids from Lys_{24} to Arg_{101} were deleted (78 aa DEL). A second set of three mutations were made to disrupt one or both disulfide bridges by substituting a serine residue for a cysteine residue at positions 2, 8 or both 2 and 8 (CS2; CS8; CS2,8, respectively), to determine whether the disulfide bridges were necessary for sorting, and if so, which bridge was more important. The constructs were transfected into the mouse neuroblastoma cell line, Neuro-2a, which does not synthesize endogenous POMC, but does have both a regulated and a constitutive secretory pathway. Wild type POMC and the CS2 and 78 aaDEL mutants, when expressed in these cells, showed punctate ACTH immunopositive secretory granules and stimulated secretion. However, when Δ2-26, Cys8-Cys20 Del, CS8 or CS2,8 mutants were expressed in the Neuro-2a cells, the $ACTH_i$ was not found in punctate granules but rather in the Golgi and perinuclear region. Only constitutive secretion of unprocessed POMC was found for Neuro-2a cells expressing these mutants. This data showed that a motif containing residues 8-20 and one disulfide bridge (Cys_8-Cys_{20}) was not only sufficient but necessary for the sorting of POMC to the regulated secretory pathway. In collaboration with Dr. Christopher Snell (Sandoz Institute, London), this hairpin loop region of N-POMC (Cys_8 to Cys_{20}) was modeled and identified as an amphipathic loop with two lobes containing four amino acid residues (Asp_{10} Leu_{11} Glu_{14} Leu_{18}) which are highly conserved across species. This, 13 amino acid amphipathic loop structure is stabilized by the disulfide bridge, Cys_8-Cys_{20}. This model has recently been confirmed by NMR studies with the N-$POMC_{1-26}$ peptide, done in collaboration with Dr. Andrew Bird (NCI) and Dr. Lewis Parnell (NIDDK).

We proposed the hypothesis that the mechanism of sorting POMC to the regulated secretory pathway involves the binding of the sorting signal to a membrane receptor at the trans Golgi network which then pinches off to form a secretory granule. Dr. Cool assayed for a sorting receptor using iodinated N-$POMC_{1-26}$ which contains the sorting signal as a ligand, and membranes from lysed Golgi and secretory granules derived bovine intermediate lobe as a source of sorting receptors. He found pH-dependant binding of N-$POMC_{1-26}$ to granule membranes which was optimal between pH 5.5-6.5 and

was trypsin-sensitive. Unlabeled N-POMC$_{1-26}$ inhibited the binding to secretory granule membranes with an IC$_{50}$=65 μM at pH 5.5. The Kd was 130 μM and Bmax was 550 μmoles/mg protein. Ca^{++}, Na^{+}, K^{+}, Mg^{2+} and EDTA had no effect on binding. Recombinant full length [^{35}S] POMC expressed using the baculovirus/sf9 cell system by Dr. ChunFa Zhang also bound the secretory granule membranes optimally at an acidic pH of 6.5 and the binding was inhibited by N-POMC$_{1-26}$. In contrast, recombinant [^{35}S] labeled Δ2-26-POMC lacking the sorting signal showed essentially no binding to the same membranes. The specificity of the binding of ^{125}I N-POMC$_{1-26}$ was further confirmed by the lack of inhibition by other parts of the POMC molecule such as ACTH and β-endorphin. In addition [^{125}I] N-POMC$_{1-26}$ did not bind to intact secretory granules showing that the receptor is localized only on the luminal and not to the cytoplasmic side of the granules. Finally, cross-linking experiments revealed binding of a N-POMC$_{1-26}$ to a ~64 kD protein in lysed secretory granule membranes which was inhibited by unlabeled N-POMC$_{1-26}$. Thus all the data indicate the existence of a specific sorting receptor for the N-POMC$_{1-26}$ sorting signal, supporting a receptor-mediated mechanism for sorting POMC to the regulated secretory pathway. Work is now in progress to purify and clone the sorting receptor.

To determine the generality of an amphipathic loop motif as a sorting signal for the regulated secretory pathway in other prohormones, Dr. Bamberger investigated the sorting of pro-enkephalin (pro-ENK). Molecular modeling studies in collaboration with Dr. Snell revealed an amphipathic loop motif in the N-terminal 31 amino acids of this prohormone. Expression of pro-ENK$_{1-31}$-CAT fusion protein in Neuro2a cells showed that the pro-ENK$_{1-31}$ motif is sufficient to act as a sorting signal to target the CAT bacterial protein to the regulated secretory pathway. Thus an amphipathic loop sorting signal motif for the regulated secretory pathway appears to be more general. Dr. Snell has now modeled numerous prohormones using the POMC sorting signal as a template and found similar putative amphipathic loop sorting signals in those molecules.

Characterization of the Yeast Aspartic Protease 3 Proprotein Processing Enzyme

Niamh Cawley has continued to study prohormone converting enzymes focusing on the novel aspartic protease family which has specificity for mono- and paired basic residues. He purified and characterized the only member of this family that has been cloned, the yeast aspartic protease 3 (YAP3p) which processes pro-α-mating factor in KEX-2 deficient mutants. YAP3p is a membrane-associated enzyme which is attached to the membrane via a glycosyl phosphatidyl inositol (GPI) anchoring site at the C-terminus of the protein. Truncation of the YAP3 gene to remove the GPI binding site resulted in secretion of YAP3p. YAP3p has a pH optimum and an isoelectric point of 4.0-4.5. Analysis of the temperature stability of YAP3p showed that the enzyme retained 100% of its activity at 37°C, while at 50°C there was 80% loss of activity after 1h. The dependence of activity on temperature demonstrated a calculated Q_{10} of 1.95. Secreted, purified YAP3p was found in two forms of ~180 and ~90 kD due to differential glycosylation. Removal of the sugars resulted in one apparent band of ~65 kD on SDS gels. However, upon N-terminal amino acid sequencing (in collaboration with Dr. H-C Chen), two forms of YAP3 were found. One sequence had an N-terminal beginning with Ala^{68} and another with Asp^{145}. These two sequences led to the discovery that YAP3p is synthesized as a pro-enzyme and is cleaved on the carboxyl side of the Lys^{66}-Arg^{67} pair to remove the pro-region. Subsequently this active form is cleaved between Asn^{144}-Asp^{145} to yield two subunits

which presumably associate to form a heterodimer, similar to the Cathepsin D aspartic protease. Dr. Zhang has expressed full length YAP3p and a form with the pro-region deleted using the baculovirus expression system. Full length YAP3p containing the proregion was inactive but upon incubating the enzyme at pH 4.0, the pro-region was autocatalytically cleaved to yield an active enzyme. However, the expressed YAP3p enzyme lacking the pro-region was not active at pH 4.0. This indicates that the pro-region is essential for the proper folding of the protein so as to yield an active enzyme. The specificity of YAP3p for 15 different potential paired/monobasic cleavage sites were examined in various substrates, including ACTH, anglerfish pro-somatostatin I and II, pro-insulin, dynorphin, amidorphin and cholescytokinin 33 (CCK 33). YAP3p recognized three specific motifs. One of these is the classical paired basic residue motif. The other two are either a paired or a mono-basic residue site with an additional basic residue upstream at P4-P6, or downstream at P4'-P6' relative to the cleavage site at the P1 position. The Km and kcat values were determined for the cleavages. Km values were between 10^{-4}-10^{-5}M for the various substrates with the highest affinity exhibited for the tetrabasic site of $ACTH^{1-39}$ (1.8×10^{-5}M). The relative catalytic efficiency of YAP3p indicates that the tetra-basic residue site of $ACTH^{1-39}$ was cleaved with the highest relative efficiency (kcat/Km = 1.6×10^{8} $M^{-1}s^{-1}$) while that of the mono-basic site of CCK 13-33 and the paired basic site of pro-insulin B-C junction were cleaved less efficiently at 4.2×10^{5} $M^{-1}s^{-1}$ and 1.7×10^{5} $M^{-1}s^{-1}$ respectively. Comparison of the primary sequence around the cleavage site of the substrates with the relative efficiency of cleavage also suggested that the greater the number of basic residues in the P4-P6 and P2'-P6' positions, the more efficient the cleavage. Denaturation of pro-insulin decreased the efficiency of cleavage of the B-C junction indicating that in addition to the primary sequence, the efficiency is influenced by the secondary and tertiary structure of the prohormone.

Large scale production of recombinant, C-terminally truncated, (minus the GPI binding site) secreted YAP3p, with the pro-region intact (inactive), and the activated form, are underway for crystallization and X-ray diffraction studies to be done in collaboration with Dr. T. Blundell (England). Knowledge of the specificity and the best substrates for this model enzyme in conjunction with the X-ray crystal structure will lead to the understanding of the mechanism of action of basic-residue specific aspartic proteases.

The specificity of YAP3p also indicates that it will be a very useful enzyme commercially for cleaving a large number of recombinant proproteins including prohormones, pro-neuropeptides, growth factors and serum proteins.

Mammalian homologues of yeast aspartic protease 3

Pro-opiomelanocortin converting enzyme (PCE) is an aspartic protease which we have purified from bovine pituitary secretory granules and characterized as a paired basic residue specific prohormone processing enzyme. Recently, Dr. Niamh Cawley showed by Western blotting that an antibody against YAP3 cross-reacted with one 70 kDa protein, the size of PCE, from secretory vesicle extracts, as well as a purified preparation of PCE from bovine intermediate lobe. In addition, Western blots of mouse anterior pituitary showed a ~70 kD and a ~90 kD YAP3p-like immunoreactive band, while mouse hypothalamus contained only the ~90 kD form. Preimmune antiserum did not show any immunostaining.

Immunocytochemistry was performed by Dr. L.-P. Pu on mouse brain sections using the YAP3 antibody. The results showed immunoreactive YAP3p staining specifically in the granular cell layer of the dentate gyrus and the pyramidal cells in the hippocampus, both neuropeptide-rich regions. Immunostaining was also observed in the arcuate and paraventricular nuclei where POMC and vasopressin are synthesized, respectively. In addition, the YAP3p antibody immunostained cells in the bovine pituitary intermediate lobe and a subset of cells characteristic of the distribution of corticotrophs in the bovine anterior pituitary. No staining was observed with preimmune antiserum in all studies.

Preliminary results from immunocytochemistry using anti-YAP3 in combination with *in situ* hybridization for various neuropeptide cDNAs revealed colocalization of the YAP3-like immunoreactivity with CCK mRNA in hippocampal neurons and vasopressin mRNA in supraoptic neurons. These data suggest that YAP3-related processing enzymes are colocalized with peptidergic neurons and may play a role in pro-hormone processing in the CNS.

Previous studies showing the similarity in specificity, pH optimum and immunological identity of PCE with YAP3p indicates that PCE is a mammalian homologue of YAP3p. Moreover, there appears to be two forms of YAP3-related mammalian homologues in brain and pituitary. We have recently screened a mouse brain λgt10 library using the full length YAP3 sequence as a probe. Two positive clones have been characterized with respect to restriction sites. We now have partial sequences for both clones. Expression libraries are also being screened with the antibody against YAP3p to try and clone the family of mammalian homologues of YAP3p.

Processing of pro-TRH by prohormone convertases PC1 and PC2.

Pro-TRH is a 26 kD precursor molecule which in the rat contains 5 copies of TRH (pGlu-His-Pro-NH_2) as well as other cryptic peptides. One of the cryptic peptides, proTRH$_{178-199}$, has been found to be a corticotropin releasing inhibitory factor (CRIF), and plays a role in the regulation of the HPA axis. The enzymes that process proTRH have not been identified. Dr. Friedman, in collaboration with Drs. Eduardo Nillni and Ivor Jackson at Brown University, studied the processing of pro-TRH by the subtilisin-like prohormone processing enzymes, PC1 and PC2. Recombinant PC1 (provided by Dr. N. Birch, New Zealand) and intermediate lobe secretory vesicle membranes which contain PC1 and PC2 were used. Purified, ^{3}H-radiolabeled pro-TRH was incubated with recombinant PC1 or intermediate lobe secretory vesicle membranes and antibodies specific for various cryptic peptides of pro-TRH were used for immunoprecipitation to identify the cleavage products. Recombinant PC1 was found to cleave labeled pro-TRH at either position 128-129 or 134-135 to generate the 15 kD N-terminal (pro-TRH$_{1-127}$) and the 10 kD C-terminal (pro-TRH$_{136-231}$) products. The 15 kD N-terminal product was then cleaved to a 6 kD (pro-TRH$_{1-50}$) and a 3.8 kD (pro-TRH$_{59-88}$) product. The 6 kD product was further cleaved to a 4 kD product (pro-TRH$_{1-25}$). The 10 kD C-terminal product was cleaved to a 5.4 kD (pro-TRH$_{184-231}$) product. These cleavages were inhibited by Zn^{++} and had a pH optimum of 5.5 consistent with the characteristics of these subtilisin-like enzymes.

The vesicle membrane preparation cleaved pro-TRH at basic residues in a similar manner to recombinant PC1. In addition a 16.5 kD C-terminal product was generated. The pH profile for the

appearance of both C-terminal and N-terminal propeptides showed a bimodal distribution with optima at both 5.5 and 7.5. The cleavage of pro-TRH was enhanced by Ca^{++} and partially inhibited by Zn^{++}. Immunodepletion studies with antisera specific for PC1 or PC2 demonstrated that both these enzymes contribute to the processing of pro-TRH by the membrane preparation. However, the difference between PC1 and PC2 is the generation of the 16.5 kD C-terminal (pro-TRH_{91-231}) product by PC2 only, while PC1 generated the 6 kD (pro-TRH_{1-50}), 4 kD (pro-TRH_{1-25}) and 3.8 kD (pro-TRH_{59-88}) N-terminal products. Thus, both PC1 and PC2 can process pro-TRH and there appears to be enzyme-specific processing of this precursor.

In order to determine if these two enzymes are candidates for pro-TRH processing *in vivo*, co-localization studies were carried out in rat brain by Dr. Pu. Double *in situ* hybridization using radiolabeled riboprobes for PC1 and PC2 mRNAs and digoxigenin-labeled riboprobes for pro-TRH mRNA were used in this study to determine the cellular co-localization. Analysis of rat brain showed overlapping, as well as differential co-localization of PC1 and PC2 mRNA with pro-TRH mRNA in various brain regions, supporting the role of PC1 and PC2 in pro-TRH processing *in vivo*. Many neurons in the hypothalamus were double labeled for mRNAs of pro-TRH and PC1 and PC2, particularly in the paraventricular nucleus. In contrast, pro-TRH neurons in the amygdala, glomerular layer and external plexiform layer of the olfactory bulb and the basal lateral hypothalamus showed co-existence with PC2 mRNA, but not PC1 mRNA. Cellular co-localization of mRNAs for pro-TRH and PC1 was evident in the neurons of the tenia tecta but PC2 mRNA was absent. However, in the thalamic reticular nucleus, pro-TRH mRNA containing neurons did not contain PC1 or PC2 mRNA. The differential distribution of PC1 and PC2 within TRH neurons may account for the differential post-translational processing of pro-TRH in various brain regions reported. Interestingly, in the thalamic reticular nucleus where there was a lack of co-expression of pro-TRH mRNA with PC1 and PC2, large processed forms of TRH but no TRH tri-peptide were found. It is possible that prohormone convertases other than PC1 and PC2 may participate in the processing of pro-TRH in this brain region.

Another line of evidence which can support a role of PC1 and PC2 in processing pro-TRH *in vivo* is the demonstration of co-ordinate regulation of synthesis of these enzymes with pro-TRH. Pro-TRH mRNA has been shown to increase in the dentate gyrus of rats after electrical kindling, which results in seizure. Dr. Pu examined the expression of PC1, PC2 and pro-TRH mRNAs in electrically kindled rats using quantitative *in situ* hybridization. A statistically significant increase of PC1 (1.1 fold), PC2 (1.4 fold), and pro-TRH (3 fold) mRNAs was observed in the granule cells of the dentate gyrus 4h following seizure; compared to non-kindled sham controls. This coordinate regulation of PC1 and PC2 mRNA with pro-TRH mRNA in the dentate gyrus provides further evidence that PC1 and PC2 play a role in the processing of this prohormone *in vivo*. Interestingly, the increase in PC2 mRNA expression was greater than PC1, suggesting that there may be changes in the pattern of pro-TRH processing in the dentate gyrus after kindled seizure, since PC1 and PC2 process pro-TRH in a differential manner.

Significance of Biomedical Research and the Program of the Institute:

The pro-opiomelanocortin family of peptides (ACTH, α-MSH and β-endorphin) which are synthesized both in brain and the pituitary, have been implicated in higher brain functions (e.g., avoidance learning, analgesia and stress response) and the regulation of the hypothalamo-pituitary-adrenal axis. In addition α-MSH has been shown to have a role in neural regeneration, neuronal differentiation and osmoregulation. Studies on the regulation of the biosynthesis, intracellular transport post-translational modification and secretion of these pro-opiomelanocortin-related peptides in the nervous system of adult and developing animal models will ultimately provide insights into the integrative and regulatory roles these peptidergic neurons may play during development, as well as genetic diseases associated with defects in these cellular processes.

Proposed Course:

In the coming year, Dr. Cool will purify and attempt to clone the sorting receptor for the regulated secretory pathway. A new postdoctoral fellow will join the Section in October to study the role of intermolecular association of POMC as part of the mechanism involved in sorting pro-opiomelanocortin to the regulated secretory pathway. Winnie Tam will study the role of the four highly conserved amino acids ($D_{10}L_{11}E_{14}L_{18}$) in the POMC sorting signal motif using site-directed mutagenesis. Dr. Cawley will focus on the expression and characterization of the YAP3-related mammalian cDNA clones and the enzymes they encode. This will be facilitated by Dr. Pu who will study the distribution of these enzymes in different tissues and brain and their co-expression with various prohormones and proneuropeptides using *in situ* hybridization and immunocytochemistry. Vicki Olsen, a graduate student from the University of Copenhagen will study the *in vivo* biosynthesis and processing of YAP3p using various yeast genetic mutants that are defective in certain intracellular transport steps.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00064-19 LDN

PERIOD COVERED

October 1, 1994 through September 30, 1995

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

Neurobiologic Studies of Neurons and Glia in Cell Culture

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

PI:	P.G. Nelson	Chief	LDN,NICHD
Others:	R. Davenport	NRC Fellow	LDN,NICHD
	M. Li	Snr NRC Fellow	LDN,NICHD
	M. Jia	Visiting Associate	LDN,NICHD
	P. Latham	IPA	LDN,NICHD
	D. Bisant	Adj Post Doc	LDN,NICHD
	V. Dunlap	Biol Lab Tech	LDN,NICHD
	E. Thies	Technician	LDN,NICHD
	A. Parfitt	Expert	LDN,NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
6.9	4.9	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.)*

We have previously shown that chronic (>24 hr.) stimulation of sensory neurons *in vitro* produces an alteration in growth cone sensitivity to stimulation and a down regulation of voltage-sensitive Ca++ channels (VSCC). We now show specificity in this effect both with regard to the type of VSCC affected and for different patterns of stimulation. The low voltage activated VSCC (T current) is extremely sensitive to stimulation; single stimuli at a steady rate of 0.5 Hz completely abolishes the T current while not affecting the high voltage activated (N and L) VSCC. The same number of impulses organized into different patterns of tetanic ('bursty') or steady stimulations have differential effects on the N and L currents. Binding of a radio-labelled VSCC ligand, PN200-110 is also down regulated by chronic stimulation confirming that the number of Ca++ channels is affected by the stimulation. Activation of cultured skeletal muscle cells with acetylcholine produces an increase in prothrombin mRNA, which is consistent with the role we have proposed for thrombin in the process of activity dependent synapse elimination. We fail to demonstrate a modulation of the serine protease inhibitor Protease Nexin 1 in muscle, however. If this endogenous inhibitor does play a role in synapse elimination it may be because of its presence (and modulation) in some other cell type, either nerve or glia. Evidence for PN 1's presence and modulation in glia cells has indeed been obtained by Brenneman's group in the LDN.

To investigate the cellular localization of activity-independent guidance cues in the developing visual system, we have established retinal and tectal cultures in modified 3-compartment chambers. Using embryonic chick we have localized a previously identified aversive component to tectal neurons. We have discovered a possibly novel, positive guidance component on radial glia. Both of these guidance mechanisms are restricted to one portion of the tectum during development.

PHS 6040 (Rev. 5/92)

Project Description

Objectives

We continue our interest in the role of electrical activity in regulating development of the nervous system, in particular its synaptic circuitry. We are also studying those molecular and cellular cues which are involved in neuronal interactions involved in synaptogenesis which may be independent of electrical activity. The relationship between these classes of phenomena should be important and we are using vertebrate cell culture systems to allow cellular and molecular understanding of them.

Methods Employed

A variety of dissociated cell culture preparations from the mammalian central nervous system are utilized, with emphasis on the fetal mouse spinal cord and sensory neuron culture system. The electrophysiological techniques utilized include intracellular and patch electrode recording and voltage-clamping. Pharmacological agents are applied by local perfusion or bath application.

Calcium imaging was performed with calcium-sensitive dyes and a video/computer analytic system.

A culture system is being utilized which incorporates three chambers separated from one another by barriers which allow the growth of axons between chambers, but retain neuronal cell bodies within the chambers in which they are plated. Various markers or stains can be used to demonstrate axons that project from one chamber to the other, and the development of synaptic interactions between specific cell types can be followed morphologically and physiologically.

To assess developmental guidance mechanisms in the optic pathway of embryonic chick and mammals, we have dissociated retinal and tectal cells from animals at embryonic ages (E6-11 in chick; E13-16 in mouse) when retinal axons are just innervating their target nuclei *in vivo*. Target cells are dispersed usually in the center compartment and retinal cells or explant strips are placed in the side compartments of a redesigned Campenot chamber. We have replaced the teflon chamber barriers which separate compartments with glass coverslips to reduce the distance retinal axons must extend to reach the center compartment by approximately 10-fold. Cells are kept in culture for 2-10 days and assessed with time-lapse video microscopy or whole cell patch pipette recording.

Semiquantitative and quantitative polymerase chain reaction technology is used to measure a variety of mRNAs of interest.

Major Findings

Our previous findings had indicated that chronic (>24 hours) electrical stimulation of sensory neurons produced a down regulation of the voltage-sensitive calcium channels expressed by these cells. We have examined the recovery time course following termination of stimulation,

and the observation that 24 hours or more is required for full recovery indicates that probably more than functional alteration of the Ca^{++} channels is involved in the response to chronic activation. Further analysis has shown that the consequence of activation of these cells shows specificity, both in terms of the type of Ca^{++} channel affected and in the stimulus pattern specificity of the effect. The low voltage activated Ca^{++} current (T current) is substantially the most sensitive species of channel, being essentially eliminated by single pulse stimulation at 0.5 Hz. Its recovery had not begun at 24 hours after cessation of stimulation, but was complete at 120 hours. The peak and steady high voltage activated currents (N and L currents) were somewhat less sensitive to stimulation and showed some differential responsiveness to different patterns of chronic stimulation. Thirty Hz. stimulation delivered for 2 sec every 10 sec produced less response than 10 Hz stimulation delivered for 0.5 sec every two sec, even though the total number of stimuli delivered was the same in each case. Steady stimulation at 0.5 Hz produced effects intermediate between the two types of 'burst' stimulation. The electrophysiological experiments were confirmed by binding studies using a radio-labelled Ca^{++} channel ligand, PN200-110. Specific binding was decreased by about 50% by chronic stimulation of the sensory neurons, with a time course very similar to that demonstrated in the electrophysiological experiments.

Previous results had indicated that activity-dependent synapse elimination(ADSE) in a tissue culture model of the cholinergic neuron to muscle connection was mediated, at least in part by the serine protease, thrombin. The process can be completely blocked by an endogenous serine protease inhibitor, Protease Nexin 1 (PN 1), and we hypothesized that a proper balance between protease activity and PN 1 might be crucial for optimal development and stabilization of synapses. Thrombin secretion and prothrombin mRNA are modulated by cholinergic stimulation of cultured skeletal muscle, but we find no significant effect of activation on PN 1 message levels. If PN 1 modulation by activity plays a role in ADSE, then some cell other than muscle cells must be involved. Indeed, Dr. Brenneman's group has shown that stimulation of glial cells can increase the output of PN 1. Such a differential cellular disposition of protease and inhibitor provides a basis for a model of selective elimination and stabilization of synapses during development. That thrombin may be involved in central nervous system development is suggested by preliminary data indicating that hirudin, a specific blocker of thrombin, increases neuronal survival under certain conditions in CNS cultures.

During development of the nervous system both activity-dependent and -independent mechanisms are utilized to bring about the ordered connections. Our focus has been on projections in the PNS and the critical role electrical activity serves. We have begun to investigate model systems in embryonic chick and mouse based on the retinal projection to its target nuclei which will enable us to evaluate both activity-dependent and -independent mechanisms during CNS development. The map formed by retinal ganglion cells on their target nuclei has served widely as a model system to investigate mechanisms underlying the highly precise and stereotypic connectivity of the nervous system. Activity-independent guidance components are known to initially affect rudimentary map formation; activity-dependent events refine these maps. While it is clear that activity-dependent mechanisms must involve post-synaptic target neurons, the substratum for activity-independent guidance remains unknown. To investigate cellular localization of activity-independent guidance cues in the developing retinotectal system, we modified our standard (Campenot) 3-compartment chamber and created areas where cultured embryonic retinal and tectal cells could encounter one another and

guidance behavior be readily assessed and electrical activity imposed. Preservation of relevant guidance information on living cell populations enabled separate evaluation of retinal growth cone behavior upon encounter with tectal neurons and radial glia. Using embryonic chick, we localized a previously defined, aversive guidance component to neurons and discovered a possibly novel guidance component on radial glia, both restricted to cells from one region of the optic tecta during development. Together these differential interactions suggested a novel hypothesis for the development of retinotectal topography that more clearly defines how development of ordered connections critically depends on cell-specific cues distributed selectively on target neurons and glia.

We have learned that retinal ganglion axons are electrically excitable under our culture conditions. Intracellular calcium levels rise with imposed activity using a redesigned compartmentalized chamber. Furthermore, their cocultured target cells are not only electrically excitable, but possess a full complement of voltage-dependent channels. These channels seem to be expressed with a time course in culture that requires several days, thus allowing examination of the functional consequences of voltage-dependent channel expression. Spontaneous synaptic activity occurs in these cultures, but also may require several days to develop.

Based on our previous experiments which suggest a functional role for protease/protease inhibitors in synaptic plasticity and remodeling of the neuromuscular junction in culture, we are beginning to investigate whether these same molecules serve a similar role *in vivo* in the CNS. In the developing chick, a transient ipsilateral projection occurs from retinae to tecta and subsequently lost spontaneously and completely. Initial experiments performed in collaboration with Dr. S McLoon (Univ Minn) suggest that hirudin and possibly the less specific protease inhibitor leupeptin can reduce the loss of the ipsilateral projection *in vivo*. Though preliminary, these results suggests that protease inhibitors may serve a similar role in natural CNS synaptic reduction as we have been studying at the neuromuscular junction.

Significance to Biomedical Research and the Program of the Institute

Assembling appropriate and functionally effective neural circuits is a critical developmental process of fundamental importance to the successful adaptation of the organism. Understanding the mechanisms involved in the process is clearly important for neuroscience and the NICHD, which has a lead role in dealing with problems of mental retardation and more subtle aspects of inadequate cognitive development.

Proposed Course

Further studies of the mechanism of the ADSE will be carried out in the nerve-muscle preparation. A peptide is available that partially mimics a receptor mediated component of thrombin's activity. We will test if this peptide can produce synapse loss as does thrombin itself. If it does not it would suggest that thrombin is acting through some other target. We need to examine a variety of stimulus patterns to see if synapse elimination shows any specificity in this regard. The tetanic stimuli used to date produce a non-Hebbian loss of both stimulated and non-stimulated connections, and it may be that other stimulus configurations may have different effects. Possible retrograde signals coupling postsynaptic function have been postulated and we

will exploit our system in looking for such factors. The nitric oxide and arachidonic acid systems are attractive candidates for such studies. Anatomical and physiological studies of the acute and chronic sequelae of synapse activation will be continued.

We will further investigate the localization and characterization of the activity-independent guidance components differentially distributed across retinal target nuclei in both the embryonic chick and mouse. We will examine the spontaneous and evoked calcium responses of target cells to evaluate activity-dependent regulation of the topographic development of the retinotectal projection. We will further characterize the development of voltage-dependent channels in tectal cells and the electrical, synaptic input from projecting retinal axons. We intend to follow up the preliminary studies on the effect of protease/protease inhibitors in embryonic chick CNS projection *in vivo* and to further evaluate the natural abundance and timing of these molecules during development.

Protocols:

Animals:

94-013 Nelson Developmental neurobiology in culture systems

Publications:

Liu Y, Fields RD, Festoff BW, Nelson PG. Proteolytic action of thrombin is required for electrical activity-dependent synapse reduction. Proc Natl Acad Sci 1994;91:10300-04.

Nelson PG, Fields RD. Developmental plasticity of the brain: problems and solutions in dynamic imaging of the synaptic bouton. Proceedings of 2nd Hamamatsu Intl Symposium on Biomolecular Mechanisms and Photonics: Cell-Cell Communications 1995 (in press).

Nelson PG, Fields RD, Liu Y. Neural activity, neuron-glia relations and synapse development. Persp Dev Neurobiol 1995;2:399-407.

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Sheng HZ, Lin P-X, Nelson PG. Analysis of multiple heterogeneous mRNAs in single cells. Analytical Biochem 1994;222:123-130.

Sheng HZ, Lin P-X, Nelson PG. Combinatorial expression of immediate early genes in single neurons. Mol Brain Res 1995;30:196-202.

Singer HS, Chiu AY, Meiri KF, Morell P, Nelson PG, Tennekoon G. Advances in understanding the development of the nervous system. Curr Opin Neurol Neurosurg 1994;7:153-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00094-25 LDN

PERIOD COVERED

October 1, 1994 - September 30, 1995

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

Pineal Regulation: Environmental and Physiology Factors

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

PI:	David C. Klein	Head	LDN, NICHD
Others:	Steven Coon	NRC Fellow	LDN, NICHD

COOPERATING UNITS *(if any)*

Georgetown University (M.A.A. Namboodiri); University of Geneva (N. Schaad); University of Giessen (H. Korf)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Neuroendocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF 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 regulation of the pineal gland by environmental and physiological factors is analyzed, exclusive of transmembrane and intracellular regulatory mechanisms (see Z01 HD 00095-25 LDN). The pineal gland is part of the melatonin rhythm generating system, a neural circuit which includes a circadian clock in the suprachiasmatic nucleus (SCN); the SCN is reset and entrained by light acting through the eye. An area of special current interest is proteins involved in transmembrane signal processing. The most exciting finding is that there is a daily rhythm in the abundance of mRNA encoding the α-1B-adrenergic receptor. This increase seems to be driven by the SCN, and is blocked by light acting through the eyes. Studies in organ culture indicate the receptor is regulated by cAMP. This second messenger is controlled by several receptors, two of which have been found to mediate increases in the abundance of mRNA encoding the α-1B-adrenergic receptor. This work is important because it provides an interesting mechanism through which one receptor can regulate another.

Project Description

Objectives:

To determine how the pineal gland is influenced by physiological, and pharmacological factors, and to study the mechanisms involved in the development and regulation of neural stimulation of the pineal gland.

Methods Employed:

The function of the pineal gland is studied by measuring the production of melatonin. Melatonin is measured directly by high performance liquid chromatography-electrochemical detection. In addition, the major metabolite of melatonin, 6-hydroxymelatonin, is measured in urine using a mass chromatography method. Pineal enzymes are measured by radiochemical techniques. Proteins are resolved by polyacrylamide electrophoresis and electroblotted to membranes for immunological analysis (see Z01 HD 00095-25 LDN for further details). PCR technology, *in situ* hybridization and Northern blot technology are used to analyze mRNA expression.

Major Findings:

The major new finding in the program was the discovery that there is a high abundance of mRNA encoding the α_{1B}-adrenergic receptor subtype, and that this appears to be a primary member of the α_1-adrenergic receptor family which is expressed in the pineal gland. Other studies established that the abundance of mRNA encoding this receptor is higher in the pineal gland than in other tissues, and that there is a 2- to 4-fold daily rhythm in the abundance of this receptor. Organ culture studies have indicated that this receptor is regulated by cAMP and agonists which elevate cAMP.

Developmental studies have indicated that the major developmental increase in the abundance of this receptor occurs during the first few days of life in the rat.

Significance to Biomedical Research and the Program of the Institute:

The α_{1B}-adrenergic receptor plays a key role in signal processing regulating the production of the hormone of the pineal gland, melatonin. Accordingly, studies on this receptor in the pineal gland provide valuable information in two areas:

Control of melatonin: The program yields new information about the control of melatonin production. This compound is thought to influence reproduction and mood in humans. Therefore, any advance made by this program could eventually have direct influence in clinical practice. In addition, pioneering work done within this program may lead to tests of pineal function in humans.

Neural processing and development: The program yields new information about neural processing and development. This provides investigators an opportunity to learn how a single neural system develops and how it functions during development. In addition, it might provide some insight into how environmental factors alter development.

Proposed Course:

The relationship of changes in mRNA encoding the α_{1B}-adrenergic receptor and in total receptor number will be studied using agents which elevate the receptor without interacting with it directly.

Protocols:

93-016	Klein	Neuroendocrine regulation: Rat pineal gland

Publications:

Coon, S.L., McCune, S.K., Sugden, D. and Klein, D.C.: Day/night rhythm in pineal α_{1B}-adrenergic receptor mRNA: Regulation by a β-adrenergic → cyclic AMP mechanism Endocrinology; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZO1 HD 00095-25 LDN

PERIOD COVERED

October 1, 1994 - September 30, 1995

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

Pineal Regulation: Transsynaptic and Intracellular Mechanisms

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

PI:	D.C. Klein	Head	LDN, NICHD
Others:	J.L. Weller	Chemist	LDN, NICHD
	P. Roseboom	Staff Fellow	LDN, NICHD
	S. Coon	IRTA Fellow	LDN, NICHD
	R. Baler	Biotechnology Fellow	LDN, NICHD
	M. Bernard	Visiting Fellow	LDN, NICHD

COOPERATING UNITS *(if any)*

Georgetown Univ (M.A.A. Namboodiri); Univ of Giessen (H. Korf); NIMH (D. Jacobowitz); LDN, NICHD (J. Russell)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Neuroendocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS	PROFESSIONAL	OTHER
8.0	6.9	1.1

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)*

Mechanisms involved in the transduction of neural signals and the control of tissue specific gene expression are studied. The pineal and pituitary glands are used as model systems. The details of the chemical and ionic components of transmembrane signal processing and of neural and tissue specific regulation of gene expression are analyzed. Signal transduction in the pineal gland is of special interest because cAMP and cGMP are regulated by dual receptor mechanisms which appear to interact at the level of regulation of adenylyl and guanylyl cyclases. One leg of these pathways activates these enzymes via GTP binding regulatory proteins, similar to Gsα.

In the area of the neural and developmental control of gene expression, a major advance has been made by the cloning of the gene encoding serotonin N-acetyltransferase. This will make it possible to study how neural signal regulate expression of this gene in vertebrates and how the activity of this enzyme is controlled.

The major hormonal product of the pineal gland is melatonin. Melatonin has been found to block GnRH induced increase in [Ca++]i and to block GnRH-induced depolarization. Melatonin appears to act on a subpopulation of GnRH-sensitive cells. IT is clear from associated studies that melatonin acts through an action on the calcium, not cylcic AMP, and that the effect of melatonin on calcium is secodary to an effect on membrane potential.

Project Description

Objectives:

To describe the transsynaptic and intracellular mechanisms through which neuronal activity regulates the biochemical activity of a neural tissue, the pineal gland.

Methods Employed:

General: Tissue is obtained from normal animals or surgically prepared animals. Cultures of pineal tissue are used to study biochemical activity under highly defined conditions. Pineal glands and dispersed suspension cultures of pineal cells are used. Tissues are treated with chemicals of interest and the activity of enzymes and concentrations of cell chemicals are measured by a variety of techniques. To study development, pineal glands are removed from rats at sequential stages of development and enzyme and metabolic chemicals are measured. Enzymes are purified by a variety of methods. Antisera are produced in rabbits; antisera are used to identify proteins. cDNA libraries are prepared using a λgt11 vector and screened with synthetic oligonucleotides and cDNA probes.

Pineal tissue was obtained from normal animals or surgically operated animals. Pineal enzymes are measured by radiochemical techniques. Proteins are resolved by polyacrylamide gel electrophoresis and electroblotted to membranes for immunological analysis. Total RNA is isolated by a single step method using acid guanidinium thiocyanate-phenol-chloroform extraction; resolved on an agarose gel and transferred onto positively charged membranes for message level analysis. In addition, polymerase chain reaction technology is used to confirm the results obtained by Northern analysis.

Cyclic nucleotide regulation: Pineal glands from rats are cultured, and treated with various agents to stimulate cAMP and cGMP. Cyclic nucleotides from both glands and culture medium are measured by radioimmunoassay (RIA). Guanylate cyclase activity was measured by *in vitro* reaction at 37°C in the presence of Mn^{++}, GTP, and phosphodiesterase inhibitor, then subsequent RIA for cGMP. Proteins are analyzed by SDS-PAGE and Western blot. Immunoprecipitations are performed using antisera against Gsα.

Melatonin mechanism of action: The mechanism of intracellular transduction of melatonin effect is investigated using primary cell culture from neonatal rat pituitary glands. LH-release is determined by radioimmunoassay, and intracellular Ca^{++} concentrations are determined from fluorescence of a suspension of cells loaded with calcium sensitive dye Fluo-3. A similar approach is used to measure membrane potential. In addition, single cell analysis of neonatal pituitary cells has been initiated.

Transcription factors: Analysis is through use of highly specific antiserum, combined with labelled oligonucleotide probes in gel shift assays. In addition, PCR is used to detect mRNA transcripts.

Major findings:

Guanylate cyclase and cGMP: The role of NO in the regulation of guanylyl cyclase was examined using several approaches. First, it was found that NO synthase, the enzyme which generates NO and citrulline from arginine is present in the pineal gland. Second, it was found that this enzyme is calcium and calmodulin dependent. Third, it was found that inhibitors of this enzyme inhibited the norepinephrine stimulation of cGMP production. Fourth, it was found that norepinephrine stimulation of cGMP was accompanied by an increase in the production of citrulline and of a diffusible factor with NO-like characteristics. These findings indicate that NO plays a key role in the adrenergic stimulation of cGMP in the pineal gland. In addition, it was found that the sensitivity of the cGMP system to NO is regulated by a $G_s\alpha$ mechanism, providing clear indication that interaction of receptor input can occur at this level.

An important effect of cGMP on pineal signal processing was discovered. It was found that cGMP activates influx of calcium, and that this reflects the interaction of cGMP with a cGMP gated channel, which was found to be identical to the channel found in the retina, accordingly to polymerase chain reaction (PCR) analysis. The presence of this channel is important because is appears to mediate effects of vasoactive intestinal peptide (VIP) on calcium.

HIOMT: It was found that HIOMT is expressed at very low levels in the retina. In addition, it was found that HIOMT levels in Y-79 cells are strongly dependent upon a serum factor, and that removal of serum results in a rapid loss of enzyme activity and mRNA encoding HIOMT. Other studies indicate that HIOMT can be regulated by retinoic acid, suggesting that this might be involved in the regulation of HIOMT.

Melatonin mechanism of action: Studies using a single cell analysis technique indicated that melatonin acts on a subpopulation of GnRH sensitive gonadotrophs. Melatonin appears to act by causing hyperpolarization which then alters calcium currents through voltage sensitive channels. Melatonin acts through a G-protein linked receptor mechanism which is antagonized by pertussis toxin. Preliminary studies suggest that this action involves a phosphatase and that the melatonin receptor is a member of a family of receptors which act on membrane potential through a pertussis toxin sensitive mechanism involving regulation of the activity of a phosphatase.

N-Acetyltransferase: An expresison cloning technique was used to identify a clone encoding serotonin N-aceltyltransferase. The identity of the clone was verified substrate specificity analysis. This was extended by the cloning of cDNA encoding the human , rat and chicken forms of the enzyme. cDNA probes were used to determine there was a large increase in mRNA encoding this enzyme in the rat pineal gland, , a smaller increase in the chicken and not increase in sheep. Tissue distribution studies revealed that mRNA encoding this enzyme is not detectable in any species in any endocrine or internal organ but is consistently detectable in retina. In addition, it is detectable in sheep brain regions.

Adrenergically induced proteins: The most prominent adrenergically-stimulated protein in norepinephrine-treated glands, as judged by incorporation of ^{35}S-methionine incorporation into proteins has a molecular weight of about 37 kDa and a pI of about 6. This protein was partially purified and resolved by two-dimensional gel electrophoresis. It was identified by microsequencing to be an isoform of malate dehydrogenase. This was confirmed by immunochemical techniques, using antisera raised against synthetic malate dehydrogenase peptides. It was also determined that the appearance of this protein reflects two processes, new synthesis of protein and cotranslational phosphorylation.

Transcription factors: The cAMP-responsive factor CREB has been identified in the pineal gland and it has been found that it is phosphorylated following norepinephrine stimulation. The time course and dose dependency of this response has been characterized.

The presence in the pineal gland of the newly identified inducible inhibitory transcription factor ICE-CREM has been confirmed by PCR. The regulation and expression of this factor is being analyzed. Studies on c-*fos* revealed that norepinephrine does not alter c-*fos* expression significantly, but does cause massive induction of a 42/46 kDa *fos* related antigen (fra). This has been documented both by Western blot and Northern blot analysis. The protein exhibits a marked circadian rhythm, which appears to be due to neural stimulation of the gland by the suprachiasmatic nucleus. The gene has been cloned and the coding region has been fully sequenced, revealing this protein to be the rat homolog of 42/46 kDa fra.

Significance to Biomedical Research and the Program of the Institute:

The pineal gland offers an attractive model system for the study of the neurochemical transduction from the receptor to the gene. This is a fundamental aspect of brain function and further understanding will lead to better treatment of neurological and mental diseases, and a better understanding of problems of mental retardation.

Proposed Course:

N-acetyltransferase: The promoter will be identified and the factors regulating expression of this gene will be determined, including transcription factors. Special attention will be paid to the role of CREB, ICER and Fra-2.

Factors regulating the activity of the enzyme will be analyzed using expression systems, with special attention paid to the role of phosphorylation of two putative protein kinase A sites.

Antiserum is being raised against expressed protein, against three peptides and against two phosphopeptides. These will be used to monitor the total amount of enzyme protein and the phosphorylation state of the protein, and to determine how this relates to enzyme activity.

HIOMT: Rat HIOMT: Upon determination of the DNA sequence of the rat pineal protein hydroxyindole-O-methyltransferase, PCR will be used to determine the expression of this gene at various stages in the light/dark cycle, after pharmacological manipulation and at various stages of development. In addition, oligonucleotides will be used as probes for Northern blot analysis and *in situ* hybridization studies to characterize the tissue distribution in the rat.

Human HIOMT: Isolation of the promoter region of the human HIOMT gene - The 5′-flanking region of the HIOMT gene will be isolated and subcloned in a vector that contains a reporter gene, i.e. chloramphenicol acetyltransferase (CAT). The inserted DNA will then undergo nested deletions from the 5′ end. CAT activity will be assayed following transfection of Y-79 cells (retinoblastoma cell line). Y-79 cells have been shown to express HIOMT activity and synthesize melatonin in a cAMP-dependent manner. By assaying for CAT activity, regions of DNA important for transcription will be identified. The sequence of the region that activates transcription will then be analyzed by computer to determine whether known promoter and/or enhancer sequences are present.

The three mRNA splice variants will be expressed and the expressed protein will be analyzed for enzyme activity and immunogenicity.

Y-79 HIOMT: The factor regulation HIOMT activity in Y-79 cells will be purified and identified.

Protocols:

93-016 Klein Neuroendocrine regulation: Rat pineal gland

Publications:

Baler, R. and Klein, D.C. Physiological regulation of oncogene FRA-2: Circadian → adrenergic → cyclic AMP control in the rat pineal gland . J Biol Chem. (in press).

Bernard, M., Donohue, S.J., and Klein, D.C. Hydroxyindole-*o*-methyltransferase in the human pineal gland and retina and in Y79 retinoblastoma cells. Brain Res. (in press).

Detera-Wadleigh, S.D., Hsieh, W-T, Berrettini, W.H., Goldin, L.R., Rollins, D.Y., Muniec, D., Grewal, R., Guroff, J.J., Turner, G., Coffman, Barrick, J., Mills, K., Murray, J., Donohue, S.J., Klein, D.C., et al. Genetic linkage mapping for a susceptibility locus to bipolar illness: Chromosomes 2,3,4,7,9,10p, 11p, 22 and Xpter. Am J Med Genetics 1994; 54:206-218.

Lin, A.M.-Y., Schaad, N.C., Schulz, P.E. Coon, S.L., and Klein, D.C. Pineal nitric oxide synthase: Characteristics, adrenergic regulation and function. Brain Research 1994;651:160-168.

Roseboom, P.H., Weller, J.L., Babila, T., Aiken, A., Sellers, L.A., Moffett, J.R., Namboodiri, M.A.A. and Klein, D.C. Cloning and characterization of the ε and ζ isoforms of the rat 14-3-3 proteins. DNA and Cell Biology. 1994;13:629-640.

Roseboom, P.H. and Klein, D.C. Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: Primary role of a β-adrenergic receptor/cyclic AMP mechanism. Molecular Pharmacology 1995; l47:439-449.

Schaad, N.C., Vanecek, J., Rodriguez, I.R., Klein, D.C., Holtzclaw, L., and Russell, J.T. Vasoactive intestinal peptide elevates pinealocyte intracellular calcium concentrations by enhancing influx: Evidence for involvement of a cyclic GMP dependent mechanism. Molecular Pharmacology 1995; 47:923-933.

Vanecek, J. and Klein, D.C. Mechanism of melatonin action on neonatal rat gonadotrophs. In Advances in Pineal Research, Møller, M., Pévet, P. and Reiter, R.J. (eds), John Libbey (London), 1994; Vol. 8, pp. 363-370.

Vanecek, J. and Klein, D.C. Melatonin inhibition of GnRH-induced LH release from neonatal rat gonadotroph: involvement of Ca^{++} not cAMP. Am. J. Physiol. 1995;269:E85-E90.

White, B. and Klein, D.C. Stimulation of cyclic GMP accumulation by sodium nitroprusside is potentiated via a G_s mechanism in intact pinealocytes. Journal of Neurochemistry 1995; 64:711-717.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 00704-11 LDN

PERIOD COVERED
October 1, 1994 - September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Tetanus Toxin Effects and Localization in Neurons

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

PI:	E.A. Neale	Head	LDN, NICHD
	L.C. Williamson	Sr. Staff Fellow	LDN, NICHD
Others:	L.M. Bowers	Biologist	LDN, NICHD
	S.C. Fitzgerald	Biologist	LDN, NICHD
	V. Dunlap	Biol. Lab. Tech.	LDN, NICHD

COOPERATING UNITS *(if any)*
Div.Bacterial Prod., Ctr Biol. Eval. Res., FDA (JH Halpern, WH Habig); NIDR (SH Leppla); U.S. Army Med. Res. Inst. of Infectious Diseases (JE Brown); Dipartimento di Science, Univ. of Padova (C. Montecucco); Dept. of Biology, Georgetown U. (M.A.A. Namboodiri)

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Section on Cell Biology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.4	0.7

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.)*

Blockade of evoked neurotransmitter release by tetanus and botulinum neurotoxins is correlated with proteolysis of their respective intracellular substrates, VAMP/synaptobrevin, SNAP-25 and syntaxin in intact neurons. Botulinum neurotoxin C cleaves syntaxin and in addition, acts directly or indirectly on SNAP-25. BoNT C is the only clostridial neurotoxin that acts on two of the three synaptic proteins that form the core complex for vesicle fusion and is cytotoxic for both young and mature neurons. Toxin blockade of synaptic vesicle exocytosis is coincident with a block in synaptic vesicle membrane retrieval, except with botulinum neurotoxin A. Synaptic vesicle endocytosis, visualized by activity-dependent uptake of FM1-43 or of horseradish peroxidase, occurs with stimulation even when vesicular release is blocked totally by botulinum neurotoxin A.

The rank order of potency of the seven serotypes of botulinum in blocking neurotransmitter release in spinal cord cell cultures is D>A>B>C>G>E>F. Competitive binding studies indicate that A, B and E have different high affinity receptors.

A chimeric protein consisting of the transmembrane domain of anthrax toxin and the catalytic domain of tetanus toxin inhibits (in the presence of the anthrax binding factor) endocytosis and exocytosis in nonneuronal cells, suggesting that this chimeric protein affects multiple membrane trafficking pathways.

Project Description:

Objectives:

To analyze the presynaptic action of the clostridial neurotoxins at the biochemical and ultrastructural levels.

Methods Employed:

Primary neuronal cell culture; morphologic techniques including light, video, fluorescence, and electron microscopy; immunohistochemistry and immunoelectron microscopy; computer-assisted image analysis and morphometry; biochemical techniques including scintillation spectroscopy, thin-layer chromatography, and gel electrophoresis and immunoblot.

Major Findings:

Clostridial neurotoxins (CNTs) include tetanus toxin (TeNT) and the seven serotypes of botulinum neurotoxin (BoNT). The CNTs are zinc endopeptidases which block neurotransmission and have been shown to cleave, in vitro, specific proteins involved in synaptic vesicle docking and/or fusion. TeNT and BoNT B, D, F and G cleave VAMP/synaptobrevin. SNAP-25 is cleaved by BoNT A and E, and syntaxin is cleaved by BoNT C. We have examined the alterations in toxin substrates, as visualized by immunohistochemistry and immunoblotting, in intact neurons under conditions of toxin-induced blockade of neurotransmitter release. In TeNT-exposed spinal cord cell cultures, VAMP is no longer detected in synaptic terminals identified by synaptophysin immunoreactivity. BoNT A and C have no effect on VAMP staining. In BoNT C-exposed cultures, although syntaxin remains detectable by immunohistochemistry, immunoblots demonstrate syntaxin cleavage but not total degradation. In BoNT A-treated cultures, staining of the carboxyl terminus of SNAP-25 is abolished, consistent with its proteolytic action at this site. Unexpectedly, immunostaining of the carboxyl terminus of SNAP-25 in cultures blocked by either TeNT or BoNT C differs from controls. When VAMP is cleaved by TeNT, staining of the carboxyl terminus of SNAP-25 is enhanced suggesting that VAMP binds within the region of SNAP-25 that is cleaved by BoNT A. In contrast, in BoNT C-treated cultures, SNAP-25 (COOH-terminus) immunoreactivity is greatly diminished and immunoblots using antibodies against the amino terminus of SNAP-25 show two reactive bands. Thus, in addition to cleavage of syntaxin, BoNT C has a secondary action on the carboxyl terminus of SNAP-25. The loss of SNAP-25 immunoreactivity in BoNT C-exposed cultures occurs over a slower time course than syntaxin cleavage. The loss of the carboxyl terminus of SNAP-25 is not likely due to contamination of BoNT C with BoNT A since immunoblots of each toxin show no reactivity with antibodies against the other toxin. Furthermore, the action of BoNT C was blocked totally by preincubation with antibodies against BoNT C, whereas preincubation with antibodies against BoNT A had no effect on BoNT C cleavage of syntaxin or on the loss of the carboxyl terminus of SNAP-25. In addition, the cleavage sites for BoNTs A and C on SNAP-25 appear slightly different. Whereas, BoNT A cleaves the last 9 amino acids from the carboxyl terminus of SNAP-25, we believe BoNT C cleaves the last 5 or 6 amino acids. This study is the first to demonstrate in

vivo that the physiologic response to these toxins is correlated with the proteolysis of their respective substrates. Furthermore, we demonstrate for the first time that BoNT C also acts, directly or indirectly, to cleave SNAP-25. BoNT C is the only CNT that acts on two of the three synaptic proteins that form the core complex for vesicle fusion. A paper describing these findings has been submitted.

Unlike the other CNTs, we have discovered that BoNT C is severely cytotoxic for both young and mature neurons. In newly-plated spinal cord cultures, neurons do not survive beyond five days following treatment with BoNT C. In mature cultures, synaptic terminals become enlarged within 24 hours of toxin exposure. During the next four days, axons degenerate and then dendrites and cell bodies are lost. Electron microscopy after two days of BoNT C exposure reveals synaptic terminals packed with vesicular elements (100-200 nm) and membranous dense bodies indicative of degeneration. Synaptic release sites remain relatively intact with synaptic vesicles accumulated at most active zones. This neurotoxicity is unique to BoNT C. All the clostridial neurotoxins block neurotransmitter release but only BoNT C causes overt neuronal degeneration. These observations suggest that syntaxin, alone or in combination with SNAP-25, may be necessary for neuronal survival. A manuscript describing BoNT C neurotoxicity is in preparation.

We have correlated further the synaptic blockade induced by either TeNT or BoNT A with immuno-histochemistry of their respective protein substrates, with fine structure of the synaptic release site, with activity-dependent uptake of the dye FM1-43, and with stimulated uptake of horseradish peroxidase (HRP) into newly recycled synaptic vesicles. Potassium-evoked release of both an inhibitory (glycine) and an excitatory (glutamate) neurotransmitter is blocked completely in toxin-treated cultures. As mentioned above, immunoreactivity for VAMP and for SNAP-25 is abolished in cultures exposed to TeNT or BoNT A, respectively. Electron microscopy shows that approximately twice the number of synaptic vesicles are "docked" (located within 10 nm of the membrane) at the active zones of toxin-blocked cultures as are seen in either spontaneously active or Kcl-stimulated control cultures. Consistent with the block in synaptic activity, TeNT- (and BoNT B-, C-, or D-) treated cultures show no activity-dependent uptake of FM1-43. In contrast, cultures treated with BoNT A (up to 300 ng/ml) continue to show FM1-43 loading, indicating the persistence of synaptic vesicle endocytosis in the absence of exocytosis. These findings are confirmed by Kcl stimulation in the presence of HRP. TeNT-blocked synapses show HRP-labeling of only occasional synaptic vesicles, whereas a large proportion of synaptic vesicles are labeled in BoNT A-blocked terminals. The labeled vesicles appear to form via conventional clathrin-coated intermediate structures. These observations suggest that BoNT A may be used to uncouple the cycle of triggered exocytosis-endocytosis that occurs at the synaptic terminal, allowing discrete analysis of the endocytosis component. They further suggest that VAMP (or another as yet unidentified substrate of tetanus toxin) participates in the process of synaptic vesicle membrane retrieval. This work has been presented in abstract form and a manuscript is in preparation.

While the proteolytic action of the clostridial neurotoxins on their respective substrates has been characterized, very little is known about their receptors or internalization. We have shown that spinal cord neurons in cell culture display a differential sensitivity to the action of the various serotypes on inhibitory and excitatory neurotransmitter release. The rank

order of potency is D>A>B>C>G>E >F. BoNT D is as potent as TeNT. Differential sensitivities of these cultures to BoNTs may reflect differences in receptor binding and internalization. Competitive inhibition of [125I] BoNT A binding was examined using 100-fold excess of unlabeled BoNTs B or E. BoNT B offers no competitive inhibition whereas BoNT E is 70% as effective a unlabeled BoNT A, suggesting some homology in the binding domains of these two toxins. However, BoNT E does not inhibit the action of BoNT A in blocking neurotransmitter release. These data indicate that BoNT E may bind to the same low affinity ganglioside receptors as BoNT A, but does not bind to the same high affinity (functional) receptors. Additionally, BoNT B does not bind to the same low or high affinity receptors as BoNT A. These data further suggest that BoNTs A, B, and E have different high affinity receptors. These studies provide the first comparative analysis of all seven serotypes of BoNTs in an intact cell culture system and demonstrate the potential of this system for addressing questions of toxin binding, internalization and action. This work has been presented in abstract form and a manuscript is in preparation.

In collaboration with Drs. Naveen Arora and Stephen Leppla (National Institute of Dental Research) and Dr. Jane Halpern (Food and Drug Administration), we have characterized a chimeric protein consisting of anthrax lethal factor and TeNT light chain (LF-LC) (Arora et al., 1994). The light chain of TeNT inhibits neurotransmitter release by cleavage of VAMP. A homologue of VAMP, cellubrevin, has been identified in nonneuronal cells and may be important in membrane trafficking and vesicle fusion. The function of cellubrevin in nonneuronal cells (which lack receptors for tetanus toxin) was examined using LF-LC, which allows the uptake of TeNT light chain into cells by receptor-mediated endocytosis via anthrax toxin receptor. We have demonstrated previously that LF-LC is cytotoxic to several nonneuronal cell lines. In order to elucidate the mechanism of this cytotoxic effect, we have studied the effects of LF-LC on cellubrevin levels and vesicle trafficking in Chinese Hamster Ovary (CHO) cells. At a concentration of LF-LC that completely inhibits cell growth, there is no consistent detectable cleavage of cellubrevin as measured by immunoblot or immunofluorescence. At the same concentration of LF-LC, transferrin receptor recycling and fluid phase pinocytosis are significantly inhibited. These data suggest that LF-LC can affect multiple membrane trafficking pathways. We are further characterizing LF-LC action under a variety of conditions and in CHO cell variants to determine if its cytotoxicity results from cellubrevin cleavage. This work has been presented in abstract form and a manuscript is in preparation.

With our collaborators, Drs. Mark Dertzbaugh and Michael West (U.S. Army Medical Research Institute of Infectious Diseases), we have examined BoNT A for segments that, when injected into mice, are effective in stimulating the production of protective antibodies. PCR was used to amplify and clone fragments of the BoNT A gene, resulting in ten overlapping fragments. The peptides encoded by these fragments were expressed in E. coli and purified. The ability of each peptide to induce an antibody response and to protect against a lethal challenge of BoNT A was assessed in mice and in murine spinal cord cell cultures. All of the truncated peptides were immunogenic except one segment that encoded amino acid residues H1078-1220 of BoNT A. Only two of the peptides were shown to confer protection against BoNT A intoxication in mice. Both of these peptides are derived from segments of the heavy chain, H455-661 and H1150-1289, of BoNT A. Antibodies against each of these peptides were premixed with BoNT A and applied to spinal cord cell cultures.

Blockade of evoked neurotransmitter release by BoNT A was inhibited only with antibodies against the peptide derived from segment H455-661. These data suggest that the segment H455-661 may be a useful candidate for incorporation into a vaccine for BoNT A. A manuscript describing these findings has been submitted.

We have collaborated with Dr. M.A.A. Namboodiri (Georgetown University) in assisting his graduate student, V. Sung, with studies on the ultrastructural localization of the excitotoxin quinolate in human peripheral blood monocytes/macrophages. A manuscript describing these findings has been submitted.

Significance to Biomedical Research and the Program of the Institute:

Tetanus is a leading cause of neonatal death worldwide and studies of its effects on young neurons and on the possibility of recovery might have clinical implications. Botulinum neurotoxins are used therapeutically to alleviate certain pain and to correct an increasing number of spasmodic disorders including torticollis, spinal dystonia, and facial, voice and limb disorders. Characterization of similarities and differences among the BoNTs provides significant support to clinicians in terms of their understanding and controlling toxin treatment for these disorders. We recently demonstrated that BoNT C acts on two substrates and is cytotoxic to neurons. If corroborated by in vivo studies, these findings are critical since it may or may not be desirable to totally eliminate a population of neurons with toxin therapy.

Proposed Course

We will continue to use the clostridial neurotoxins to analyze the molecular mechanisms responsible for vesicle targeting, fusion and recycling. We will further characterize BoNT C neurotoxicity. We will take advantage of botulinum neurotoxin A for uncoupling synaptic vesicle endocytosis from exocytosis to study the regulation of synaptic vesicle membrane retrieval and the limitations of the nerve terminal plasmalemma to provide synaptic vesicle membrane. We will continue studies of the internalization, transport, and translocation of tetanus toxin from the endosome into the cytoplasm. Additionally, we plan to compare, biochemically and morphologically, the internalization and transport of tetanus toxin and botulinum neurotoxin in ventral horn-muscle co-cultures in Campenot multicompartment chambers. These latter studies promise to provide basic information on the cell biology of protein targeting, much as the toxins have contributed to our understanding of membrane trafficking.

We will continue collaborative efforts to characterize the proteolytic action of clostridial neurotoxins in nonneuronal cells using fusion proteins of anthrax lethal factor and toxin light chains. Studies have been initiated to characterize the action of a chimeric protein consisting of BoNT A light chain and anthrax lethal factor in neuronal and nonneuronal cells.

Protocol:

94-012 Cell culture studies of neuronal development

Publications:

Arora N, Williamson LC, Leppla SH, Halpern JL. Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells, J Biol Chem 1994;269:26165-71.

Halpern JL, Neale EA. Neurospecific binding, internalization, and retrograde axonal transport. In: Montecucco C, ed. Clostridial neurotoxins. Curr Top Microbiol Immunol. Berlin: Springer-Verlag, 1995, in press.

Williamson LC, Neale EA. Bafilomycin A1 inhibits the action of tetanus toxin in spinal cord neurons in cell culture, J Neurochem 1994;63:2342-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 00708-11 LDN

PERIOD COVERED
October 1, 1994 through September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between borders.)*
Morphological Studies of Neuronal and Non-neuronal cells in CNS Cell Cultures

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

PI:	E.A. Neale	Head	LDN,NICHD
Others:	A.G. Parfitt	Expert	LDN,NICHD
	L.C. Williamson	Snr Staff Fellow	LDN,NICHD
	L.M. Bowers	Biologist	LDN,NICHD

COOPERATING UNITS *(if any)*
Lab. of Neurophysiol., NINDS, NIH (T.J. Smith, Jr.); Div. of Bacterial Products, FDA(W.H. Habig, J.L. Halpern); Lab. of Cell Biology, NIMH (L. Eiden); Dept of Biochem., USUHS (S. Chung); Dept Physiol. & Biophysics, Case Western Reserve Univ. (M. McEnery).

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Section on Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	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.)*

Instrumentation is in place to capture images for morphometry of neurons developing in the absence of electrical activity. Redesign of multicompartment chambers for the study of activity effects on synapse stabilization/elimination improves optical resolution and axonal growth. Antibodies against the vesicular acetylcholine transporter label a subpopulation of neurons and synaptic terminals, and may allow the immunohistochemical identification of cholinergic neurons as early as one week after plating. Antibodies against the homeobox gene (Howa7) product label neuronal and not glial nuclei.

PHS 6040 (Rev. 5/92)

Project Description:

Objectives:

To identify classes of cells, in cultures of nervous tissue, based on cell type specific proteins, neurotransmitters, neuropeptides, or specific receptors, in order to understand their interrelationships during development and to examine the morphologic development of neurons and phenomena related to neurite outgrowth and formation of early contacts with target cells.

Methods Employed:

Primary neuronal cell cultures; extracellular electrical stimulation; intracellular injections; morphologic techniques including light, video, fluorescence, and electron microscopy; computer-assisted image processing and analysis; immunohistochemistry; radioautography.

Major Findings:

Preliminary studies of early neuronal development indicated that, in the absence of electrical activity, axon outgrowth and dendrite branching patterns appear normal after one week in culture, but that the number of synapses formed is almost twice that found in control, electrically active preparations. Progress on this study was limited by the image quality obtained with the available video camera. Dr. Parfitt has obtained a high sensitivity cooled CCD camera with a dynamic range that will accommodate wide variations in fluorescence intensity without loss of spatial detail. This camera will allow us to capture images showing delicate, and dimly stained, structural detail, and will be used for experiments involving either immunohistochemistry or intracellular injection.

In order to visualize clearly fluorescently labeled components of the neuromuscular junction in living cells, we have had to develop culture conditions to meet the needs of cultures grown on glass coverslips. We have therefore continued to experiment with surface treatments for glass to identify substrates that are capable of supporting the growth of both neurons and muscle cells. The most successful treatment to date comprises the sequential application of PEDA (a proprietary amine containing silane from SAIC Corporation) and L-laminin. PEDA can be replaced with poly-L-lysine with acceptable efficacy although contracting myotubes are not as well retained, possibly because poly-L-lysine is adsorbed rather than covalently linked to the surface of the glass and is thus less able to withstand the mechanical stress of muscle contraction. The Campenot chamber also has been redefined to facilitate a more luxuriant growth of axons into the field of myotubes. The compartment sizes have been reduced and the teflon barrier section between the side wells and the center slot has been replaced by a removable glass barrier. Substantial experimentation established that an effective barrier comprises two thin sections of glass, each having their lower edge ground optically flat, separated by a thin spacer. This arrangement prevents cell leakage between compartments without blocking the outgrowth of neurites from ventral horn cells into the field of myotubes when the barrier is removed. Since fluorescence visualization will be achieved using the newly acquired 1K by 1K cooled CCD camera, appropriate microscope

and computer display modifications have been made to allow full chip image display on a high resolution monitor.

Immunohistochemistry for ChAT (choline acetyltransferase) and GFAP (glial fibrillary acidic protein) in spinal cord cultures prepared from the trisomic 16 mouse (accepted as a model for Down's syndrome) has revealed specific deficiencies in the number of cholinergic neurons and in the number of non-neuronal cells which stain for GFAP. This system is described by Dr. Nelson in Project Z01 HD 00064-18.

Collaborative studies (with Dr. Lee Eiden) have characterized an antibody against the vesicular acetylcholine transporter (a-VACh) using immunohistochemistry for comparison with antibodies against choline acetyltransferase. In mature spinal cord cell cultures, staining patterns with both antibodies are similar. However, it appears that VACh can be detected several weeks prior to the appearance of ChAT. VACh immunoreactivity is present in neuronal cell bodies as early as six days in culture. By two weeks in vitro, VACh staining is found in varicosities presumed to be synaptic terminals, as well as in cell bodies. After three weeks, the cultures exhibit extensive networks of immunostained varicosities. Thus, a-VACh might provide us with an additional means of identifying cholinergic neurons and terminals. Anti-VACh staining in spinal cord cultures during development has been included in a review chapter submitted by Dr. Eiden.

In a collaborative study with Dr. Su Chung, we demonstrated that antibodies against the homeobox gene, Hoxa7, stains the nuclei of neurons and not of glia in mixed cultures. This is a question not answered readily in tissue sections. The staining persists for several weeks after plating. A manuscript which includes this finding has been submitted.

In collaborative studies with Dr. Maureen McEnery, we are in the process of assessing antibodies against a number of neuron-specific proteins. Antibodies against sodium-potassium ATPase specifically stained neurons in spinal cord cultures; immunolabel was localized throughout the neuronal plasma membrane. Antibodies against w-conotoxin receptor promises to be more valuable. Omega conotoxin GVIA binds to voltage-gated N-type calcium channels. Whereas we could demonstrate substantial specific binding of w-conotoxin in the spinal cord cultures, immunohistochemistry to localize the N-type channel has been hampered by the lack of an affinity-purified antibody.

Significance to Biomedical Research and the Program of the Institute:

Ongoing electrical activity is critical for neuronal survival and for the formation and maintenance of appropriate neuronal circuits during early development. Activity effects are likely mediated by alterations in synaptic function. These studies, which allow the control of activity levels and the identification of synaptic structures, should increase our understanding of the relationship between neuronal structure and function, and provide insight into developmental disorders which result in loss of nervous function.

Proposed Course:

The quantitative analysis of neuronal structures as affected by electrical activity will be

carried out using images recorded by the high resolution CCD camera. We will further examine electrically inactive preparations with the electron microscope, with particular attention to the fine structure of synaptic terminals that have never been active. We also will monitor preparations treated chemically to induce an increase in electrical activity. Finally, we will repeat these studies using cultures prepared from ventral horn instead of whole spinal cord, since previous data indicate that cholinergic neurons are particularly sensitive to activity blockade early in development.

We will apply quantitative methodologies to the analysis of synaptic terminals in cultures incubated with protease inhibitors. These experiments relate to the phenomenon of synapse elimination and are described in detail under Dr. Nelson's Project # Z01 HD 00064-18.

In order to define the role of the N-type calcium channel in synaptic function, we will examine the effect of w-conotoxin on neurotransmitter release. Further, we will affinity-purify antibodies against the w-conotoxin receptor in order to localize this calcium channel in relation to other synaptic proteins by double-label immunohistochemistry.

We will continue to monitor morphologic changes that accompany synapse elimination from multiply innervated muscle fibers. At the light microscope level, this will involve monitoring four-color fluorescence. Innervating neurites originating from opposite sides of the modified Campenot chamber will be stained with DiI and DiO. This will be accomplished either by adsorbing microcrystals onto cotton or glass fibers which will be placed gently into contact with emergent neurites, or by allowing dye crystals to settle onto the neurites that pass through the fluid compartment between each of the glass coverslips that comprise the removable chamber barrier. Post-synaptic cholinergic receptors will be labelled with Lucifer Yellow-bungarotoxin. Presynaptic activity in neurites labelled with DiI will be monitored using FM1-43. Synapse elimination from multiply innervated muscle fibers will be elicited by unilateral electrical stimulation of neurites entering the field of myotubes. Stimulation frequency and duration have yet to be optimized. Morphologic analysis will focus initially on the appearance of the neuromuscular junction at two time points only, before and after the experimentally elicited loss of functional bilateral innervation. We hope eventually to label other postsynaptic markers (e.g., utrophin) and components of the synaptic cleft (e.g., s-laminin, agrin) to characterize further the process of junctional dissolution. An important extension of our understanding both of the dynamics of synapse elimination and of the factors which elicit it will come from analysis of time-lapse video taken during the process of elimination.

We presently are exploring the co-localization of VACh with CHAT or with synaptophysin by double-label immunohistochemistry. Further studies are in progress to examine, under a number of experimental circumstances, the co-expression of VACh and ChAT.

Protocol:

94-012 Cell culture studies of neuronal development

Publications:

Erickson J, Weihe E, Shäefer MK-M, Neale E, Williamson L, Bonner TI, Tao-Cheng SJ-H, Eiden LE. The VACh/ChAT "cholinergic gene locus": new aspects of genetic and vesicular regulation of cholinergic function. In: Cuello CA, ed. Progr Brain Res, Amsterdam: Elsevier, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 00710-07 LDN

PERIOD COVERED
October 1, 1994 to September 30, 1995

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Characterization of Glutamate Receptor Expression in Brain

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

PI:	A. Buonanno	Head	LDN,NICHD
Others:	M. Ozaki	IRTA Fellow	LDN,NICHD
	M. Sasner	IRTA Fellow	LDN,NICHD
	J. Cheng	Biologist	LDN,NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Unit on Molecular Neurobiology

INSTITUTE AND LOCATION
NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.8	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.)*

Activity-dependent neural plasticity during development and in the adult is largely mediated by N-methyl-D-aspartate receptor (NR) activation; the natural ligand for this excitatory amino acid receptor is glutamate. The effects of NMDA receptor activation on neural plasticity are largely mediated by the calcium influx, which needs to be regulated since excessive Ca^{2+} entry kills neurons. For this reason, the regulation of NR channel kinetic properties has important consequences on the signals transduced by these receptors. There are changes in NR function during development in response to activity. We have found that the expression patterns of NR 2B repression and NR 2C activation closely follow the spatial-temporal pattern of granule cell innervation. The down-regulation of NR 2B mRNAs, which occurs after granule cells have migrated into the IGL where they receive afferent inputs, may result from synaptic activity. Thus, different NR subunits may not only function to distinctly modulate synaptic connections in response to activity, but their expression patterns may also be responsive to epigenetic factors. Transcriptional control is a common mechanism directing cell-type specific expression of genes during development and in the adult. In order to begin understanding the complex mechanisms that direct regional-specific transcription of NR genes during neurogenesis and modulate their levels in response to synaptic activity, we have begun to investigate the mechanisms that control expression of the NR2 subunits at the transcriptional level. We have found that transcription of NR 2B transcripts is initiated from different sites, however differential promoter use cannot account for the patterns of NR 2B expression during development since the patterns of initiation were the same in cerebellum and forebrain. Analysis of regulatory regions in transgenic mice reveal that distinct DNA regulatory sequences are required for the neural-specific and developmental regulation of the gene. We have identified an 800bp upstream region that directs neural-specific transcription in transgenic mice but fails to impart the proper developmental down-regulation of the gene in cerebellum. Additional cis-acting sequences residing downstream of the major initiation sites, possibly located within the first intron, were shown to be necessary to repress NR 2B expression during cerebellar development.

Project Description

The changes in synapse efficacy are dependent on NR activity, and are accompanied by changes in the electrophysiological and pharmacological properties of the receptor. The molecular basis for these changes are presently unknown. However, there has been considerable progress in understanding the basis for changes in receptor properties in the cerebellum. During development, there is a switch in the expression of NR subunits where the 2B subunit is expressed early in migrating granule cells but is repressed, as the cells mature, to be replaced by the 2C subunit. This switch in subunit expression is followed by changes in the single channel properties of the receptor. Interestingly, in the forebrain expression of the 2B gene peaks at P14 during maximal synaptogenesis but continues to be expressed thereafter. Since the receptor composition can influence the amount of calcium entering the cell and the downstream signaling that leads to neural plasticity, we are interested in understanding the molecular mechanisms that regulate the expression of the 2B and 2C subunits during cerebellar development. In particular, we are interested in the effects of innervation (and activity) on 2B down-regulation specifically in cerebellum and the up-regulation of the 2C subunit. As described below, we found that the 5′ non-coding region of the NR 2B gene is composed of two exons with transcription starting at multiple sites. The down-regulation of the 2B gene can not be accounted by differential transcription from distinct promoters, since similar utilization was observed in forebrain and cerebellum of 7-day-old mice. Using transgenic mice, we have mapped two different sites in the 2B gene. An upstream 800 nucleotide region, including the first exon, is sufficient to direct neural-specific expression in the brain. However, additional sequences residing in the first intron or second exon are necessary to direct the proper developmental repression of the gene in the cerebellum.

Objectives:

1) The aim has been to isolate and map the promoter regions of the mouse NR 2B gene, in order to determine if the transcription initiation sites are differentially used in distinct regions of the CNS.

2) To characterize the regulatory regions that confer neural and developmental-specific transcription to the NR 2B gene. A special emphasis will be directed at identifying the sequences that may differentially direct forebrain Vs cerebellum transcription during development. In order to perform delineation analysis of the NR 2B gene regulatory regions we have used transgenic mice.

3) To isolate and characterize the regulatory regions of the NR 2C gene to identify the regulatory sequences that confine its expression to granule cells during cerebellar maturation.

4) To inactivate the mouse NR 2C gene locus by homologous recombination to determine the functional role of this receptor during cerebellar development.

Methods:

In situ hybridization histochemistry: Newborn and post-natal rats were used for the experiments. The animals were sacrificed, and brain and cerebella were rapidly dissected and freeze-embedded in Tissue-Tek media using dry ice. Sections on gelatin-coated slides were fixed in paraformaldehyde, acetylated in acidified triethanolamine, and delipidated in an ethanol series and chloroform. Oligonucleotides were end-tailed with terminal transferase using standard

methods. One hundred microliters of the probe (1.5x106 cpm) were pipetted onto 6-8 sections per slide, covered with parafilm strips and incubated in humidified dishes at 37°C for 18-20 hours. Slides were washed at high stringency, dried, exposed to film for one week, and then dipped in emulsion and stored in the dark for 4-5 weeks at 4°C. They were then processed, stained in 0.2% methyl green, dehydrated and cover slipped.

Cloning and characterization of NMDA receptor genes: Cloning of the mouse NR 2B gene were described in last year's report. The NR 2C gene was isolated from the same l DASH-1 library. The genomic inserts were mapped on Southern blots using specific oligonucleotides. DNA sequence of subcloned fragments was obtained by the Sanger dideoxy-termination technique using oligonucleotides as primers. The technique of Rapid Amplification of cDNA Ends, designated RACE, and RNase protection assays were used to map the transcription initiation sites of the 2B gene. RNase protection was performed on forebrain and cerebellar RNA isolated from 129/SV mice of different ages to map transcription initiation sites and to quantitate the relative contributions of the different initiation sites. Because the results of RACE cloning suggested that NR 2B gene transcription initiates at multiple sites over a large area, two probes were used to identify start sites. Probes were transcribed with T7 RNA polymerase and α-32P UTP (800Ci/mmol, NEN). Conditions were optimized and checked for linearity of assay by varying amount of RNA used, concentration of RNase(s), and comparing RNase A+T1 mix with RNase T1 alone using unleveled sense riboprobes as positive controls. Quantitation was performed on a Molecular Dynamics phosphorimager system.

Generation of transgenic mice and analyses: Transgenic mice were generated by injection of (C57BL/6 X SJL)F1 or FVB/N pronuclei with linearized DNA containing mouse 2B and 2C gene upstream sequences linked either to the CAT or ß-gal reporter genes. The construct 0.8-CAT is a 806bp region extending from the HindIII to the SacII site which contains the first exon and upstream sequence. 0.8int-CAT harbors a fragment between the same HindIII site and the end of the second exon. The region -552 to +255 (0.8-CAT) and the region -552 to +1631 (0.8int-CAT) were cloned into the HindIII-XbaI sites upstream of the CAT gene in the pCAT-Basic vector (Promega). Three CAT-expressing lines (#6184, 6188, and 6190) for the 0.8-CAT construct and two CAT-expressing lines (#6944, 6948) for the 0.8int-CAT construct were characterized. F1 animals were tested for incorporation of the transgene by slot blotting DNA prepared from tail clips. Slot blots were also used to determine transgene copy number. The tissue-specificity conferred by the regulatory sequences was determined by assaying for CAT activity in extracts made from different tissues or staining for ß-gal activity using X-gal as the chromophore. A time course was performed to show that these conditions are in the linear range of the assay. Quantitation was performed on a Molecular Dynamics phosphorimager system.

Major Findings

Although there has been considerable progress in understanding the molecular mechanisms regulating tissue and developmental expression of genes in non-neuronal cells, this advancement has not been significantly evident in the nervous system. The bewildering complexity of neural and glial cell types in the nervous system, the intricate connections between cells and the added disadvantage that genetic material is difficult to introduce into the post-mitotic neurons, greatly

accounts for the lack of systems suitable for studying genetic regulation in neurons. The cloning of cDNAs coding for NR subunits represented the first step in understanding the molecular composition of native receptors. Recent studies strongly support the notion that hetero-oligomerization, between the widely expressed NMDAR1 subunits and members of the NR 2A-D family, is necessary to form receptors with appropriate electrophysiological properties. The composition of native receptors largely depends on the regional and temporal expression of the NR2 subunits. The restricted expression of NR2 subunits may contribute to the changes in receptor function associated with modulation of neural function during development and in the adult. Considering that the various combinations of NR2 subunits impart different channel properties and modulate the extent of magnesium block to the receptor, comprehending how NR2 subunit expression is developmentally regulated would contribute to our understanding of the molecular mechanisms underlying neural plasticity. Our goal is to elucidate the molecular mechanisms regulating NR2 gene expression.

Regulation of NR channel properties during development may play an important role in modulating downstream events that occur after channel activation and the entry of Ca^{2+} into the cell. Thus, the kinetic properties of the channel and its regulation play an important functional role during development and in the adult. The duration of NR-mediated currents have been shown to decrease during development in the superior colliculus and layer IV neurons in the visual cortex. Dark-rearing interferes with the time-course of receptor change, indicating that activity signals these changes. However, in these cases the molecular basis for the changes in receptor properties have not yet been identified, but could include post-translational modifications, differential splicing of subunits or the differential control of genes coding for NR subunits. Given that these changes in receptor properties are long-lived, the changes in gene transcription would seem a plausible mechanism. Perhaps the best case supporting changes in subunit gene expression can be made for the developmental switch that occurs in cerebellar granule cells. The kinetic properties of NRs in cerebellar granule cells change during development; migrating granule cells (prior to innervation) express receptors that have higher conductance, longer mean open channel times and burst lengths than the post-migratory granule cells residing in the IGL of P22 rats (which probably have received afferent inputs). Granule cells present in the EGL and those that have migrated into the IGL express NR1 and NR 2B, whereas by P14 most granule have begun to express the NR 2C transcripts and down-regulate NR 2B expression. The single channel properties of the pre-migratory and maturing granule cells closely ressemble those of in vitro synthesize dimeric receptors NR1/NR 2B and NR1/NR 2C, respectively . As in the case of the visual system, the NR-mediated currents in the pre-migratory receptors on granule cells are longer. Assuming that the properties of these receptors are manifested at synapses, the earlier receptors could function to accomodate inputs that have less coincident activity. A switch to receptors with lower conductance and/or mean open channel times during development would presumably lower Ca^{2+} influx. The change in NR subunit expression is reminiscent of the τ to ϑ switch that occurs at the neuromuscular junction. In this case, activity plays an important role in down-regulating transcription of the τ subunit gene. As discussed below, we and others have evidence that the NR 2B gene is also down-regulated by innervation. Our work is presently focused in identifying the regulatory interactions and DNA sequences that mediate the NR 2B to NR 2C switch in the developing cerebellum, and that lead to the changes in NMDA receptor function.

Gradients of NMDA receptor expression in developing cerebellar granule cells. In situ hybridization histochemistry was used by Dr. Buonanno to analyze the developmental regulation of NR2 subunits in the developing cerebellum. This analysis reveals that the expression of NR 2B and 2C transcripts during development differs markedly in the external and internal granule cells layers (EGL and IGL, respectively). Expression of 2B transcripts is observed in pre-migratory granule cells in the EGL and after migration into the IGL. In contrast, 2C expression is restricted to the IGL. Furthermore, the NR 2C gene is expressed in a gradient during development. Begin at P7 expression progresses from the posterior to the anterior lobes, and within a lobe transcripts accumulate in a medial to lateral pattern. This gradient cannot be simply accounted by the migratory pattern of granule cells, since the expression of NR1 and 2B in the IGL significantly precedes 2C expression. Similar to the acetylcholine receptor τ to subunit switch that occurs during skeletal muscle development, our results suggest that the initial NR 2B expression in granule neurons is cell-autonomous whereas innervation may provide the signals that activate NR 2C expression and repress NR 2B. In order to decipher the molecular mechanisms directing the 2B to 2C switch, we have generated transgenic mouse lines harboring different regulatory regions of these genes. The results are described below.

Multiple transcription start sites are used by the NR 2B gene. A transcriptionally regulatory mechanism frequently utilized for the differential expression of a gene in various tissues or at distinct times in development, is to initiate transcription from different promoters. Since expression of the NR 2B gene is differentially regulated in the brain cortex and cerebellum of 1-week-old and adult rodents, we were interested in mapping transcription initiation sites using the two independent methods of RNase protection and RACE . The cDNA products obtained by RACE originate from using an "anchored primer" located downstream of the translation initiation site and extending to the 5'-most end of the RNA. Dr. Mike Sasner found that discrete products of different sizes were obtained by RACE and that these originated from NR 2B transcripts of different lengths. He used RNase protection as a second independent method to map the transcription initiation sites. Two probes were used for the RNase protection assays, hybridization products from both probes show multiple bands, indicative of multiple transcription start sites. Caution was taken to circumvent conditions that may result in the generation of false RNase protected bands due to "probe breathing", frequently seen with probes containing stretches of adenosines and thymidines, which could be misinterpreted as representing initiation sites.

Genes containing multiple transcription sites frequently alternate their use during in different tissues or during development. However, we found no evidence for the differential use of promoters in the NR 2B gene during development, since the patterns of protected products were similar when forebrain or cerebellar RNAs from 1-week-old and adult mice were used. From the calculated sizes of the protected products, transcription start sites were mapped to the genomic sequence. The sites obtained by RNase protection and RACE mapped to similar positions in the gene. No consensus TATA or CAAT boxes are evident upstream of the transcription start sites, but consensus initiator elements are present. Given that there are neither multiple genes nor differential use of promoters that can account for the distinct expression of NR 2B mRNAs in the cerebellum and forebrain during development, next we investigated if the cloned genomic DNA had the potential to properly regulate transcription of the bacterial reporter gene chloramphenicol acetyl transferase (CAT) when driven by NR 2B upstream sequences.

A 0.8kB upstream region is sufficient to confer tissue-specificity to a reporter construct.
Two CAT reporter constructs with identical 5' ends were used to assay transcriptional regulation. The first, 0.8-CAT, harbors a fragment that extends from 550 bp upstream of the 5'-most start site down to the first non-coding exon (see Methods). The second, 0.8int-CAT, originates from the same upstream site and extends to the second non-coding exon; it was used to test the potential role of the first intron and second exon in regulating transcription. Both constructs conferred reporter activity specifically in the brain and failed to be expressed in the other 5 non-neural tissues tested, including excitable tissues such as cardiac and skeletal muscle. Analysis of the same tissues from one-week old mice also showed that CAT expression was restricted specifically to the brain. Non-transgenic littermates displayed no detectable reporter levels in any of the tissues tested. Interestingly, expression driven by the upstream promoter region alone was much higher than that driven by constructs containing sequences downstream of the first exon; a situation that may result from the presence of repressor sequences in the 0.8int-CAT construct. The number of integrated copies did not correlate with the levels of CAT expression. These results indicate that regulatory cis-acting elements residing in the upstream 550 bp or in the first exon are sufficient to specifically direct NR 2B expression in the brain.

Downstream sequence is necessary to confer developmental down-regulation of the NR 2B gene in the cerebellum. Dr. Sasner quantitated the levels of CAT activity in extracts made from the cortex and cerebellum of transgenic mice of different ages, to test if these sequences were also sufficient to confer proper developmental regulation. Interestingly, he found that although mice harboring the 0.8 construct expressed CAT specifically in the brain, these sequences failed to repress expression of the reporter in the cerebellum of adult mice. Moreover, levels of CAT activity in mice from 3 independent 0.8-CAT lines were higher in the 1-week-old and adult cerebellum than in the cortex. In stark contrast, expression driven by the 0.8int-CAT construct was down-regulated in the cerebellum after 1 week of age. Both of the 0.8int-CAT lines had negligible levels of CAT expression in the cerebellum as adults, although there was significant cerebellar expression at one week of age, as occurs with the endogenous NR 2B gene. To test the time course of cerebellar down-regulation of the reporter, tissue from 10-day old and 2-week old animals were assayed. Cerebellar expression in the 10-day old was decreased relative to 7-day and was lost in 2-week old animals; again, this is similar to the developmental down-regulation of the endogenous gene. These results demonstrate that sequences in the first intron and/or exon are necessary to recapitulate the proper pattern of NR 2B expression. The fact that CAT reporter levels in mice harboring the 0.8int-CAT construct are considerably lower than those obtained with the intron-less construct suggest that this region may contain important repressive elements.

In summary, we have identified a genomic region sufficient to confer tissue specific expression to a reporter gene in transgenic mice, and a distinct intronic region that is responsible for developmental down-regulation in the cerebellum. Sequence elements have been identified in these regions that have previously been shown to bind brain-specific transcription factors. Expression of the reporter gene driven by the larger of the constructs described shows the same temporal and spatial expression as the endogenous gene. These constructs will allow a detailed investigation of the molecular mechanisms that control both the tissue-specificity of the gene and the developmental down-regulation of the NR 2B gene in the cerebellum. This will include analysis of the potential role synaptogenesis and/or neuronal activity may play in the control of subunit composition of the receptor during development. (A manuscript describing this work

has been submitted for publication).

Characterization of the NMDA receptor NR 2C gene. As disucussed above, there is a NR 2B to NR 2C switch during maturation of granule cells as they receive afferent inputs from ascending mossy fibers. We are interested in developing molecular markers to analyze how the NR 2B gene is repressed by granule cell innervation, and replaced by expression of the NR 2C. To this end, Dr. Miwako Ozaki, who recently joined the laboratory, has cloned and characterized the mouse NR 2C gene. The upstrem region of the NR 2C gene is extremely complex. Initial results by Dr. Sasner had shown that splicing at the 5' noncoding sequence of the NR 2C mRNA was complex; recent results show that there are four 5' non-coding exons that can be differentially spliced. Transgenic lines harboring ß-gal constructs driven by different 5'-falnking sequences have been generated. Preliminary resutls demonstrate that more than the 5'-flanking six kilobases are required for the expression of the NR 2C gene in cerebellum. Experiments are in progress to identify the sequences that direct the cerebellar expression of the NR 2C gene during granule cell maturation.

Significance to Biomedical Research and the Program of the Institute:

Glutamate receptors are known to mediate excitatory postsynaptic transmission in the brain, and are the most abundant excitatory receptors in the vertebrate CNS. Glutamate and its analogs are potent neurotoxins. In addition, experimental animal studies have suggested that GluRs maybe implicated in some degenerative phenomena in the CNS, such as Huntington's disease. In fact, intrastriatal injections of kainate produce a pattern of neuronal degeneration and biochemical changes similar to those observed in postmortem tissue from Huntington's disease patients. An important step in understanding the basis of neurotoxicity, and the expression of GluRs during development, is to identify the genes coding for these receptors. The analysis of how GluRs are expressed and regulated during cerebellar development at a genetic level will set some of the foundations for the understanding of the developmental biology of glutamatergic synapses; sites for the action of several psychoactive drugs.

On the other hand, the delineation of regulatory sequences that temporally and regionally direct gene expression in the brain is of extreme importance for their future use in gene therapy. Presently, there is a notable absence of characterized promoters directing neural expression in specific regions of the brain. Current experiments on the use of gene therapy in the CNS generally utilize expression constructs that are driven by viral promoters; these promoters have the disadvantage that they lack specificity. The present approaches available to introduce the expression constructs into the CNS rely on the stereotaxic delivery of recombinant viral vectors (i.e. herpes simplex virus or adenovirus) or genetically modified neuronal cells harboring the expression constructs; the former strategy has the advantage of avoiding cellular immunological responses. However, the level of specificity provided stereotaxic injections of either viruses or cells is minimal. The use of developmental- and regional-specific neural promoters in the expression constructs would greatly improve the specificity of these approaches. An obvious requirement in the use of neural-specific promoters to target expression is that the viral vectors have a size limitation. For this reason, it will be imperative to delineate the shortest DNA regulatory sequences providing specificity and quantitative expression. Our work on the characterization of NR regulatory sequences provide an initial step toward this goal.

Proposed Course:

Dr. Sasner will continue to analyze the regulatory sequences that impart neural- and developmental-specific expression of the NR 2B gene in cultured cells and transgenic mice. We are presently generating transgenic lines that harbor constructs containing deletions in the NR 2B gene in order to locate the cis-elements conferring down-regulation of the gene during development. In collaboration with Dr. P. Nelson's group we will determine if NR transcription is regulated by electrical activity, if so, we will begin mapping the cis-acting elements conferring activity-dependence.

Drs. Ozaki , Buonanno and Ms. Jen Chung are working to understand what aspects of granule cell maturation are necessary for the down-regulation of NR 2B and the up-regulation of the NR 2C gene. Dr. Ozaki will continue to identify the sequences that activate the NR 2C gene during innervation.

Protocols:

Animal:

93-029	Buonanno	Targeted DNA delivery into tissues
93-030	Buonanno	Production of transgenic mice

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 00711-06 LDN

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Transcriptional Regulation of Skeletal Muscle-Specific Genes by Electrical Activity

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

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	J. Stauffer	Adjunct Scientist\IRTA	LDN, NICHD
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COOPERATING UNITS *(if any)*

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NICHD, NIH, Bethesda, Maryland 20982

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.6	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.)*

Skeletal muscle diversity and plasticity are dramatically influenced by motoneuron depolarization; nerve-derived electrical activity regulates the contractile properties of slow- and fast-twitch muscles by regulating transcription of specific genes. We have used transgenic mice to identify regulatory sequences that direct the fiber-specific transcription of the slow and fast troponin I (TnI) genes which are specifically regulated by selective depolarization frequencies. Mice harboring different chloramphenicol acetyltransferase (CAT) reporter constructs driven by progressive deletions of the rat TnI slow gene revealed that a 128 bp enhancer was sufficient to direct specific expression in slow muscle when linked to the 500bp promoter sequence; mice harboring just the 500bp TnI slow promoter region failed to show transcriptional activity in any tissue. A 144 bp enhancer from the quail TnI fast gene was found to confer transcription specifically in fast muscles when linked to the same 500bp promoter from the TnI slow gene. Interestingly, the rat TnI slow and quail fast fiber-specific enhancers share similar core elements. These results suggest that related trans-acting factors, or the formation of higher-order transcriptional complexes, are used to respond to selective patterns of muscle depolarization. On the other hand, we have identified sequences in the myogenin promoter that cause transcriptional repression in response to activity. Analyses in transgenic mice and muscles injected with myoblasts engineered to express the CAT reporter have revealed a region of approximately 0.5kb involved in the down-regulation of myogenin expression in response to innervation. Factors expressed specifically in denervated muscle were found to bind a 30bp element residing in the 0.5kb fragment. Ets factors have been implicated in coupling extrinsic signals to transcription. A novel ets-related transcription factor, PEF, was cloned and characterized. We found that PEF interacts synergistically with MyoD and myogenin to enhance transcription of muscle genes. Cotransfection with MEF2A showed no cooperativity with PEF. Our results suggest the possible combinatorial interaction between members of the ETS and myogenic BHLH families may mutually contribute to the stringency of binding site selection by these two families resulting in the differential modulation of various muscle specific genes.

Project Description:

Objectives:

1) To identify the transcription regulatory sequences in the slow and fast troponin I (TnI) genes that confine gene expression to slow- or fast-twitch muscle in response to specific frequencies of nerve-derived electrical activity. Analysis was performed in muscles injected with DNA constructs and transgenic mice, since cultured muscle cells do not manifest fiber-type specific properties.

2) To delineate sequences in the myogenin promoter that confer innervation-dependent regulation in skeletal muscle, using transgenic mice and mature muscles injected with stably transfected myoblasts.

3) To analyze if the myogenic factor MRF-4 is also regulated by denervation.

4) To characterize the function of PEF, an ets-related factor that we isolated from muscle. The interaction of PEF with the skeletal muscle basic helix-loop-helix factors was analyzed.

5) To identify the trans-acting factors that mediated regulation by electrical activity using the delineated cis-acting sequences as probes to screen expression libraries.

Methods:

Characterization of TnI regulatory sites in transgenic mice: The cloning and initial characterization of the rat TnIs gene was described in our previous Annual Report. TnIs sequences extending from 2.7 kb upstream of the transcription initiation site (position +1) down to position +50 located in the first non-coding exon were sequenced. Nested deletions in sequences residing in the upstream region were generated from both directions using ExoIII and S1 nuclease. DNA sequencing was performed using standard di-deoxynucleotide termination reactions. GeneWorks software (IntelliGenetics, Inc.) was used for the sequence and alignment analysis.

Transgenic mice were generated essentially as described by Hogan et al. [Hogan, Beddington, Constantini and Lacy (1994) Manipulating the Mouse Embryo, Cold Spring Harbor Press]. Transgenic mice were generated in (C57Bl/6 X SJL) F1 or FVB/N embryos. A series of TnI slow constructs containing different amounts of the upstream sequences were used to generate transgenic lines. Putative founders and their generations were screened by slot blot analysis of tail DNA using a CAT probe. Adult transgenic mice were used to analyze tissue- and muscle-type-specific expression of CAT activity. Tissue-specificity was determined by measuring levels of CAT activity in extracts made from tissues that either express or do not express the endogenous TnI slow gene. In order to further determine if fiber-type-specific expression was conferred by the constructs, muscle groups known to contain different proportions of fast and slow myofibers were analyzed for CAT activity.

Characterization of myogenin gene transcription regulatory elements in muscle: The generation of transgenic mice harboring chloramphenicol acetyltransferase (CAT) expression vectors driven by the mouse myogenin gene upstream regulatory sequences was described on the previous Annual Report. We analyzed mice harboring 3.7, 1.5 and 1.0kb of upstream sequence. The tissue,

developmental and innervation specificity conferred by the regulatory sequences was determined by assaying for either CAT transcripts or enzyme activity. We analyzed for innervation responsive elements in the construct by comparing CAT mRNA levels in denervated and innervated hind limb muscles; these were denervated by cutting the sciatic nerve at the upper thigh. The relative CAT mRNA levels were quantitated by RNase protection and Northern blots using the Molecular Dynamics phosphorimager.

Implantation of transfected myoblasts into mouse muscle: Myoblasts containing the stably transfected myogenin-CAT constructs were injected directly into both gastrocnemius muscles of adult mice, one leg was then denervated by transection of the sciatic nerve, and two weeks post-injection both muscles were collected and assayed for CAT activity. As controls, we injected other mice with myoblast stably transfected with CAT expression vectors driven by either the nAChR α subunit or myosin light chain enhancers. The ß-galactosidase activity was used to normalize for viable injected cells. The CAT assays were performed with muscle extracts containing equal amounts of protein or expressing the same ß-gal activity (these numbers were similar).

Cloning and analysis of muscle ets transcription factors: A cDNA library was constructed using RNA isolated from rat extensor digitorium longus (EDL) muscle, which in rat is composed predominantly of Type II fibers (>95%). An oligonucleotide, corresponding to the conserved ets domain, was used to screen the soleus muscle cDNA library at moderate stringency. The screen yielded approximately 30 positive clones, originating from 8 genes, that coded for ets-related factors. One of the cDNAs coded for a unique and novel protein which we called PEF. In order to study PEF function in transfected cells, the cDNA was cloned into the expression vector pSVT7, which contains the SV40 promoter, enhancer, and poly-adenylation sites. Rat myogenin and MyoD were expressed from the plasmid EMSV. The plasmid MLC1CAT920 consists of about 1.5 kb of the myosin light chain 1 promoter region driving a CAT reporter flanked 3' by a 920 bp enhancer region (a gift from N. Rosenthal) and the plasmid -110MCK consists of the 110 bp enhancer region of the muscle creatine kinase gene fused to -81 nt upstream of the MCK start site (a gift from H. Weintraub). The reporter plasmid 4X PEA3 and 4X mB contain 4 copies of a wild type ETS binding site or a double-point mutation that blocks ETS binding, respectively, adjacent to a TATA box (a gift from J. Hassell). HeLa and COS7 cells were transfected with these vectors using the standard calcium phosphate coprecipitation method. After 16-20 hours the media was removed and the plates were washed two times each with 3 ml PBS and were then refed with 5 ml of growth media. The cultures were allowed to grow an additional 48 hours before harvesting for CAT assays.

Major Findings:

Plasticity of the skeletal muscle and neuronal phenotype results from the selective repression and activation of gene expression in response to innervation patterns. In embryonic myotubes, the distribution of receptors and the types of contractile protein isoforms expressed change during development with motoneuron innervation. A selective distribution of nicotinic acetylcholine receptors (nAChRs) is achieved after innervation by the repression of receptor expression in the extra-junctional regions of the fiber, and the accumulation of "adult-type" receptors at the neuromuscular junction. Recently, we and others have shown that a family of muscle-specific transcription factors, which include MyoD and myogenin, regulate the expression of nAChRs. Motoneurons, eliciting different patterns of depolarization, regulate the contractile properties of the

myofibers they innervate by selectively activating expression of specific fiber-type (fast vs slow) contractile proteins. The plasticity of the muscle properties are attributed to innervation, because removal of the nerve results in the re-expression of nAChR in extrajunctional regions of the fiber and the replacement of "adult-type" myofibril proteins by their fetal counterparts; muscle re-attains its adult properties when re-innervated. Furthermore, it was elegantly demonstrated by Eccles in the 1960's that a fast-twitch muscle adopts slow-twitch properties when it is re-innervated ectopically by a nerve that normally innervates a slow muscle, and vice-versa. Conceivably, the nerve could regulate muscle properties by either the release of chemical factors and/or the patterns of depolarization it elicits. Recent experiments have shown that the electrical stimulation of denervated adult muscle, with frequency and patterns that mimic the natural depolarization of slow and fast muscle, are sufficient to down-regulate the expression of nAChR extrajunctional receptors and modify contractile properties of muscle. These experiments suggest that there should be at least two innervation-dependent regulatory cascades that differentially modulate muscle fiber-type. The molecular mechanisms underlying the regional and fiber-type-specific expression of genes during innervation are due, in large part, to the selective repression and stimulation of transcription. The long-term goals of our laboratory are to elucidate the molecular mechanisms that respond to innervation either by restricting gene expression to confined regions of the myofibril through transcriptional repression, or by stimulating transcription differentially in response to selective frequencies of electrical stimuli. Towards these goals, we have chosen as models to study the fiber-type-specific activation of TnI genes in either slow- or fast-twitch muscle, and the repression of myogenin and MRF-4 transcription by activity.

Common core elements are shared by the TnI enhancers conferring specific expression in either slow and fast muscles: We have used the regulation of the TnI genes as a model to elucidate the mechanisms that generate fiber diversification in response to epigenetic factors. Three TnI genes code for the isoforms that in the adult are specifically expressed in slow (TnI_s), fast (TnI_f) and cardiac (TnI_c) muscles. The troponins expressed in skeletal muscle form part of a complex, in combination with troponins C and T, that is involved in the regulation of acto-myosin calcium-mediated interactions during contraction. Transcription of TnI genes is activated during the myoblast terminal differentiation. During muscle maturation, the expression of the two TnI isoforms is confined to either slow or fast muscles in response to the type of moto-innervation the myofibers receive. As we previously demonstrated, specific patterns of electrical activity that mimic the endogenous frequencies of fast (100 Hz) and slow (10 Hz) nerve regulate TnI gene expression selectively. Because fiber-specification is not observed in cultured mammalian muscle, Dr. Manabu Nakayama, Dr. Jim Stauffer and Ms. Jun Cheng have used transgenic mice to identify regulatory sequences that direct the fiber-specific transcription of the slow and fast TnI genes. Multiple lines of transgenic mice were generated that harbored reporter constructs driven by systematic deletions of the upstream regions of the rat TnIs gene. Mice harboring chloramphenicol acetyltransferase (CAT) reporter constructs, driven by approximately 1900 and 950 bp of the rat TnI slow gene upstream sequence, are transcribed specifically in slow muscle, but not in fast muscles or other tissues such as heart, liver or brain. In contrast, mice harboring constructs containing the upstream TnI slow 250 or 500 bp failed to be expressed in embryonic or adult skeletal muscle. Fiber-type-specific transcription in slow muscle was restored when a 128 bp enhancer, that is located between the 950 and 500 bp upstream region of the gene, was fused to the 500 bp fragment. In order to test if the transcription specificity is conferred by the 128 bp enhancer and not the 500 bp region, we generated transgenic mice with a construct containing the 500 bp promoter fused to an intronic regulatory region derived from the quail Tn I fast gene. This 144 bp sequence was previously

shown to function as an enhancer in differentiated cultured myotubes. Mice harboring the chimeric TnI fast/TnI slow CAT construct specifically expressed the reporter gene in fast but not in slow muscles, indicating that these 2 enhancers selectively direct fiber-type-specific transcription when fused to a common TnI slow 500 bp promoter region. Alignment of the rat TnIs and quail TnIf sequences showed that the 2 enhancers share similar core elements, namely a MEF-2 site, a CCAC box, an E box, and a previously uncharacterized motif. These results suggest that related trans-acting factors, or that higher order complexes, are necessary to generate muscle diversity. (A manuscript describing this work has been submitted for publication).

Characterization of the myogenin gene regulatory sequences: Our previous work has strongly suggested a causal link of nAChR gene regulation by the MyoD-related factors, specially the innervation-dependent transcriptional regulation of receptor genes by myogenin at extra-junctional nuclei (see Previous Reports). To elucidate the molecular pathways linking the effects of muscle depolarization with the repression of gene transcription we have begun to characterize the regulatory elements in the myogenin gene, since its regulation by innervation is more proximal. To this end, transgenic mice harboring 3.7, 1.5 and 1.0kb of myogenin upstream sequence driving the expression of the CAT reporter gene were generated and analyzed by Dr. Soledad Calvo, Dr. Andres Buonanno and Ms. Jun Cheng. These myogenin upstream sequences were found to confer muscle-specific and to down-regulate reporter levels during innervation. The down-regulation of the gene was shown to result from the repressive effects of the nerve, and not simply occur as part of a developmental program, because denervation of transgenic mice increased reporter mRNA levels by at least 10-fold. We have implanted myoblasts stably transfected with myogenin\CAT reporter constructs into adult mouse muscle to further delineate the myogenin gene denervation-responsive region. The constructs present in these "chimeric muscles" are then tested for their ability to respond to innervation, by comparing the levels of reporter in the denervated vs the innervated muscles. Using this method, Dr. Gibney previously uncovered a region conferring denervation-responsiveness that resided separately from that imparting muscle-specificity. In collaboration with Dr. Pat Roseboom, band-shift assay s were used to analyze regulatory elements residing in the 0.5kb region that imparts the denervation response. Extracts from denervated muscle, but not from innervated muscle, contain factors that interact with a 30bp element residing in the 0.5kb fragment.

MRF-4 is expressed by myotubes and not satellite cells after denervation. In collaboration with Dr. Joaquim Weis, we have analyzed the expression of MRF-4 protein the innervated and denervated muscles of adult rats. In contrast to the other myogenic factors, transcripts coding for MRF-4 accumulate in skeletal muscle during maturation. Paradoxically, denervation causes a further accumulation of MRF-4 mRNAs. In order to determine if the accumulation in innervated muscles occurs selectively in different muscle types and if denervation causes expression of MRF-4 in myofibrils or satellite cells, we developed a specific antibody to the factor and analyzed its expression by immunohistochemistry. Western blotting and fluorescence immunohistochemistry on fibroblasts transfected separately with mammalian expression vectors for the 4 myogenic factors showed that the MRF-4

antisera was specific. No selective accumulation of this transcription factor was observed in specific fiber-types. Electron microscopy of sections of adult hemi-denervated rat diaphragm demonstrated that MRF-4 accumulates in the nuclei of denervated fibers and not in satellite cells; results that are consistent with our prior Northern blot time-course analysis of denervated muscles. Thus, we have shown that MRF-4 transcripts and protein accumulate in the myofibrils of denervated muscle and would suggest that this factor, like MyoD and myogenin, play a role in the re-activation of selective genes normally expressed in embryonic muscle. (A manuscript describing this work has been submitted for publication).

Identification of trans-acting factors that interact with MyoD-related factors to synergistically increase transcription of skeletal muscle-specific enhancers: As discussed above, experiments performed in transgenic mice have demonstrated that transcriptional regulation plays an essential role in confining myofibril expression to specific fiber types and conveying responsiveness to innervation. The ets family of transcription factors, many of which are involved in signal transduction, are recruited to the DNA template by interactions with other transcription factors to form higher order complexes. Dr. Jimmy Stauffer has isolated and characterized a novel ets-related factor that is similar to PEA-3 and ER81; it was named PEF for PEA-3\ER81-like factor. The full-length sequence of the PEF cDNA was obtained by the rapid amplification of cDNA ends (RACE). Analyses of PEF expression on Northern blots containing mRNA isolated from a repertoire of tissues, including slow and fast muscles, showed that these genes are transcribed in most tissues. However, PEF mRNA splicing, or the expression of closely related genes, differs among tissues. (This work has been submitted for publication).

Significance to Biomedical Research and the Program of the Institute:

The work carried out in our Unit is of general interest to the program of NICHD, since it seeks to understand the cellular and molecular mechanisms underlying muscle development. The identification of regulatory elements that determine muscle specification during development, is specially important for its use in targeted gene therapy. Our myoblast implantation experiments demonstrate that these regulatory regions function in adult muscle, and show that gene products are delivered to myofibers. In addition, the fact that we have identified elements that confine expression to either fast- or slow-twitch fibers is extremely pertinent to gene therapy, since autosomal mutations underlie diseases like Duchene's Muscular Dystrophy which predominantly affect fast muscle.

The projects directed at understanding how nAChR expression is restricted to adult synapses and regulated by innervation, are helpful for studies on Myasthenia gravis (MG). This syndrome is caused by an autoimmune response of antibodies directed against skeletal muscle nAChRs. Approximately 80% of myasthenic patients have circulating antibodies to the receptor. Although experimental autoimmune MG has been induced in laboratory animals by injection of nAChRs, the causes for the natural onset of disease are not known. It is extremely important, therefore, to understand the mechanisms that regulate the levels and

localization of nAChRs during development. The erroneous regulation of receptor levels, or its expression outside of the neuromuscular junction in the adult, could possibly result in the onset of autoimmune responses.

Proposed Course:

The major focus in the laboratory is to identify the cis- and trans-acting factors that confer specific tissue, developmental and innervation-dependent regulation of muscle genes. Although the myoblast implantation technique permits an initial analysis of innervation responsive elements, our results suggest that this technique using C2C12 myoblasts will not be useful to analyze fiber-type specific transcription. For this reason, the continued use of transgenic mice will be imperative for our studies.

Dr. Nakayama has begun a mutational analysis of sites in the TnI enhancers to identify which factors are involved in mediating fiber-type-specific transcription of these genes. With the assistance of Ms. Jun Cheng, transgenic mice are being generated for these studies. The information provided by the analysis of the cis-acting sequences will be used by Drs. Stauffer and Nakayama to identify the trans-acting factors which dictate fiber diversity.

Protocols

Animals

93-030	Buonanno	Production of transgenic mice

Publications:

Banerjee-Basu S, Buonanno A. Isolation and structure of the rat gene coding troponin I slow. Gene 1994,145:241-244.

Gibney G, Buonanno A. Analysis of neural-responsive myogenin upstream sequences by myoblast implantation. Developmental Biology, 1995 (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00712-04 LDN

PERIOD COVERED

October 1, 1994 through September 30, 1995

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

Regulation of Phenotypic Differentiation in the Developing Mammalian CNS

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

PI:	D.v. Agoston	Head	LDN,NICHD
Others:	A. Dobi	Visiting Fellow	LDN,NICHD
	E. Santha	Visiting Associate	LDN,NICHD
	C. Palkovits	Guest Worker	LDN,NICHD
	Ravi Lala	Summer Student	LDN,NICHD
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COOPERATING UNITS *(if any)*

LCB,NIMH (M. Palkovits); Dept. Neurobiol., Duke Univ. (M. Riggott, W.D. Matthew); LNS, NINDS (A. Donevan, M. O'Donovan); LN, NINDS (B. Andrews), LMB, NINDS (R. McKay), NIDDK (B. Sauer), NIMH (U. Hochgeschwender) Dept. Chemistry, Cornell Univ. (S. Chandra)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Unit on Molecular Control of Neurodifferentiation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.2	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.)*

The enkephalin (ENK) gene was used as a model to study the molecular mechanism of phenotypic differentiation with special emphasis on the identification of novel DNA binding proteins and their cis-acting elements and on their role in far-distant DNA-DNA interactions and nuclear organization during neurodevelopment. Three nuclear proteins were identified that specifically bind to the novel octamer-like motif (TTTGCAT=sept) of the ENK gene; stem-sept was expressed in multipotent neuroglial stem cells; neuro-sept and glia-sept were found to be exclusive to proliferating neuronal and glial precursors respectively and needed to be phosphorylated to bind the DNA. Specific extent of DNA bending was observed following these protein-DNA interactions. Nuclear protein(s) that binds to both a far upstream (-2450 bp) AT-rich [$(ATT)_{10}$] sequence and the proximal promoter / TATA region of the ENK gene was identified by a magnetic bead based "looping assay". The formation of the protein-DNA complex was found to be restricted to basal ganglia and cerebral cortex at the ages of P2 and P8 respectively and was found to be negatively correlated to ENKmRNA levels. The protein(s) was found to be highly charged and showed an unusually high affinity (~10^{-12} M) to this AT rich region and also to (membrane)lipids. DNA binding was found Ca2+-dependent and required at least partially phosphorylated protein(s). Four proteins of MW ~200 kd, ~ 90 kd, ~ 40 kd and ~14 kd were identified by using a combination of UV-crosslinking, mobility shift assays and DNA affinity purification. The 14 kd protein which alone forms a high mobility protein-DNA complex with the AT rich motif was selectively enriched in protein extracts prepared from the nuclear matrix. Treatment of primary developing neuronal cortical cultures with distamycin resulted in an ~15-fold increase in ENKmRNA levels as quantified by quantitative (mimic) RT PCR. The mouse enkephalin gene was further characterized and several constructs with B-gal as reporter gene were made for transgenic studies using the *Cre-lox* based targeted insertion system. Efficient DNA transfer into various primary developing neuronal cultures were developed in combination with immunomagnetic sorting of transfected cells and DNA molecular decoy for functional studies.

GPO 914-918

Project Description:

Objectives:

1) To understand the molecular basis of phenotypic differentiation in the developing mammalian central nervous system using the ENK gene as a model.

2) To identify embryonic-specific *cis*-elements of the ENK gene and define their roles during phenotypic differentiation.

3) To identify, isolate and characterize their DNA binding proteins.

4) To investigate the role of far distant DNA-DNA interactions, structural changes of the DNA and the nuclear structure during neuronal differentiation.

Methods Employed:

Mobility shift assays , competition and supershift assays were used to identify and characterize embryonic- and brain-region-specific DNA-protein complexes and DNA bending using various wild type and mutant DNA probes. Various techniques were used to isolate nuclear proteins from our frozen "developmental brain bank" and nuclear matrix associated proteins from freshly dissected embryonic brain regions. DNA footprinting techniques, including methylation interference, DNAse I, and osmium tetroxide footprinting were used to identify the specific *cis*-elements and to assess structural changes in the DNA. A biotinylated magnetic bead based assay system was used to study protein based far distant DNA-DNA interactions and for DNA affinity purification of nuclear proteins. Different protein purification methods including preparative isoelectrofocussing were used to purify DNA binding proteins. Routine recombinant DNA techniques were employed to generate various transgenic constructs. Primary developing neuronal cultures derived from fetuses at different developmental stages of cerebral cortex and striatum were established. Proliferating multipotent neuroglial stem cell cultures, which can be differentiated into glial and neuronal lineages, were used. Various physical DNA transfer methods for transient transfection and DNA molecular decoy were developed in combination with immunomagnetic isolation of transfected cells. Quantitative (mimic) RT PCR was established to quantify ENKmRNA and other transcripts.

Major Findings:

1. Three novel nuclear proteins were identified that bind the truncated octamer [(A/G_{enk})TTTGCAT=*sept*] motif located at -542 relative to the transcriptional start site of the rat ENK gene. *Stem-sept* is expressed in all brain regions between E10 and E14, but the temporal expression of this protein strictly follows the maturation gradient of the various brain regions (spinal cord first, cerebellum last). *Stem-sept* is down regulated at E16 which is paralleled with the expression of two distinct binding proteins, *neuro-sept* and *glia-sept* whose appearance is paralleled with the emergence of neuronal and glial lineages which also follows the maturation gradient of the different brain regions.

This observation was further supported by experiments done in collaboration with Dr. McKay. We established that multipotent neuroglial stem cells grown in culture under proliferating conditions express both the *neuro-* as well as the *glia-sept*. However, following changes in culture conditions that induce differentiation into the glial lineage, as indicated by the expression of the glia specific marker GFAP, *neuro-sept* expression is down-regulated and only *glia-sept* expression remained detectable. Also, only *glia-sept* but not *neuro-sept* is expressed in primary cultures of cortical astroglia.

Stem-sept does not require to be phosphorylated as suggested by different dephosphorylation experiments and it can be competed only with the septamer motif but not with the octamer one. By contrast, both *neuro-sept* and *glia-sept* require to be phosphorylated to bind the DNA and they can be competed equally with both septamer and octamer motifs. None of the proteins are recognized by any of the existing antibodies raised against various octamer-binding proteins suggesting that these proteins are novel.

The binding of the various *sept*-proteins to the DNA results in a characteristic DNA bending of seemingly different degrees.

The temporo-spatial expression pattern of proteins suggests that they may contribute to early neurodifferentiation events of neuronal and glial lineages which precede phenotypic differentiation, and the observed DNA bending may play an important role in this process by contributing to the assembly of 3D nuclear/DNA structure of developing neurons.

2. A protein complex that binds in a strict spatio-temporal manner to both to the far-upstream (-2453 bp) AT-rich region [(ATT)19 repeat] and to the proximal regulatory /TATA region of the ENK gene has been identified. The formed DNA-protein complex is resistant against 2.5 M urea, 2 % NP-40, 14 mM B-mercapto ethanol, 10 mM BAPTA and 1.2 M KCl and to enter PAGE gels. Treatment with 0.001% SDS eliminates DNA binding, and the complex also binds with high affinity to cationic lipids. The DNA binding was found Ca^{2+}-dependent and required at least partially phosphorylated protein(s). Competitive mobility shift assays with various mutants of the ATT motif indicated that the protein complex requires a specific DNA structure characterized by AT rich sequences characteristic to matrix attachment regions (MARs) rather than specific nucleotide sequences characteristic to conventional DNA binding proteins/transcriptional factors. The protein complex seems to bind to the DNA in an all-or-nothing fashion as evidenced by protein titrations, suggesting cooperative binding between proteins or between the protein subunits. The length of the repeat was another factor in the strength of the bond. As the length of the oligonucleotide became shorter, the protein became less tightly bound to the DNA, implying a possible binding multimer that is cooperatively bound, but separated by a non-bound DNA section. This is a common pattern for nuclear matrix proteins.

Dialysis analysis indicated that the size of the protein complex is approximately 350 kd. UV crosslinking and DNA affinity purification showed that the complex is formed by four proteins of MW ~200 kd, ~ 90 kd, ~ 40 kd and ~14 kd. The 14 kd protein is selectively enhanced in nuclear matrix preparations and forms a high mobility protein-DNA complex with the AT rich motif. However, most abundant protein of the complex is the ~40 kd protein as indicated by high enrichment following DNA affinity purification.

The very characteristic the spatio-temporal expression pattern of this complex in the developing brain suggested a neuronal cell type- and developmental-specific repressor role. This hypothesis was supported by *in vivo* experiments using the drug distamycin that specifically binds to the minor groove and thereby perturbs minor groove-protein interactions typical of nuclear matrix proteins. At the age of E20 when the complex is being expressed distamycin treatment of primary cortical neuronal cultures that normally express low levels of ENK mRNA resulted in a ~15-fold increase in ENKmRNA. This finding further supports the proposed repressor role for the complex and the first time indicates the involvement of the nuclear matrix in phenotypic differentiation of the CNS.

3. In collaboration with Drs. Hochgeschwender and Sauer we have made the first three transgenic constructs using the *Cre-lox* based targeted insertion system containing B-gal as reporter gene.

4. We further studied the di-nucleotide repeat (TG/AC)n, located between -674 -618 of the rENK gene. Analyzing the migration patterns of the (AC) and (TG) strands following chemical degradation we have established that single stranded (TG) and (AC) cannot form stable double stranded DNA because the overall conformation of the two strands are different. This structural feature of the repeat may be responsible for the Ca^{2+}-induced DNA sliding with consequence on the 3D structure of the DNA.

Significance to Biomedical Research and the Program of the Institute:

The unparalleled and precisely balanced diversity of neuronal phenotypes in the mammalian central nervous system is the cellular prerequisite of normal neuronal functions. This diversity is the result of a tightly controlled developmental process. Much is known about early developmental events but late molecular events in neurodevelopment such as phenotypic differentiation is almost completely unknown at the molecular level. Also, the complexity of the mammalian CNS requires many levels of gene regulation and likely a coordinated and combined regulatory mechanism is required to achieve precise regulation. Understanding the regulation of phenotypic differentiation has many implications for future studies on the general mechanism that regulate neurodevelopment and differentiation. Equally if not more importantly it will yield critical information to understand the molecular basis of neurological and psychiatric disorders, many of them are believed to be the resulted from abnormal development. The involvement of the enkephalin phenotype has been suggested to contribute to symptoms of various disorders including autism, Huntington's disease, epilepsy and suspected in drug abuse. Very likely, more and more neuropsychiatric diseases and disturbances will be proven to be at least partly caused by faulty neurodevelopmental process. As "timing" seems to be one of the "master controller" during neurodifferentiation, markers that indicate developmental stages can be of great use. Our approach ("sample and probe") has already been identified significant number of novel, development-specific DNA binding proteins and these can be used as markers that precisely indicate the stage of differentiation in the developing mammalian central nervous system.

Proposed Course:

Our highest priority is to understand the *in vivo* role for the identified DNA motifs during the various stages of phenotypic differentiation as well as to isolate and characterize their specific DNA binding proteins.

1) Our mobility shift assays and DNA footprinting have led to the identification of three development-specific novel elements on the ENK gene that bind proteins in spatio-temporal manner.

We will study the *in vivo* role of these motifs in both transgenic models and in primary developing neuronal cultures using transient transfection and DNA molecular decoy.

a) Transgenic studies

We will determine *cis*-requirements for correct development-, cell- and brain region-specific expression of the ENK gene using the first construct (pMENK/B-gal/lox5.0) that has been made in Brian Sauer's CRE-lox vector (pBS392). The basal and induced expressions of the reporter gene under control of the 5 kb 5′ regulatory region of the ENK gene were successfully tested in a cell culture model. In the likely case of correct expression pattern in PMENK/B-gal/lox5.0 animals, in the next generation of transgenes we will attempt to perturb the developmental- and cell-specific expression pattern of the ENK gene by mutating all three elements [$(TG/AC)_{28}$; septamer and$(ATT)_{19}$] simultaneously. In the likely case of perturbed expression pattern of the reporter gene the following generation of transgenic animals will bear the constructs that are mutated in only one motif. This way both the possible combinatorial effect of the three motifs as well as the role of individual motifs can be studied.

b) Primary neuronal culture project: Parallel with the transgenic effort we will perform both transient transfection assays as well as DNA molecular decoy using various primary neuronal culture systems and constructs identical to those used for transgenes as well as double-stranded decoy DNA molecules. IL2R co-transfected cells will be immunomagnetically sorted (MACS) and ENKmRNA levels will be quantified by mimic RT PCR. In collaboration with Dr. MacKay's laboratory, we plan to use his neuroglial stem cell culture system as a cell culture model for both for transient transfection as well as for DNA decoy to elucidate the function of the septamer motif.

2) We will purify the DNA binding proteins of the (ATT)19 repeat by the newly developed single step DNA affinity chromatography, microsequencing the proteins, and using degenerate oligonucleotides we will clone the proteins. Because both the septamer and (TG)28 binding proteins bind as monomers to their motifs, their isolation can be attempted by expression cloning using the DNA motif for screening. To identify trans-acting factors which do not bind directly to DNA but act on transcriptional regulation through protein-protein interaction, we will use a two-hybrid system which has been developed to identify cDNA clones encoding proteins which specifically interact with the protein of interest or subdomain of the protein. This two-hybrid system is commercially available in the form of existing and custom-made systems by Clontech (Matchmaker).

Protocols:

Animals:

95-008 Molecular and cell biological studies of neuronal differentiation

Publications:

Agoston Dv, Komoly S, Palkovits, M. blockade of neuronal activity is a cofactor in axotomy: selective up-regulation of galanin expression in septo-hippocampal cholinergic neurons. Exp Neurol 1994;126:247-55.

Agoston Dv, Palkovits CG, Fitzgerald SC, Brenneman DE. Regulation of *c-fos* and its responsiveness in developing mouse spinal cord cultures. Dev Brain Res, in press.

Dobi AL, Matsumoto K, Santha E, Agoston Dv. Guanine specific chemical sequencing of DNA by osmium tetroxide. Nucl Acid Res 1994;22:4846-7.

Dobi AL, Palkovits M, Palkovits CG, Santha E, Agoston Dv. Protein-DNA interactions during phenotypic differentiation. Mol Neurobiol 1995;10:185-203.

Senna M, Bravo DT, Agoston Dv, Waschek JA. High conservation of upstream regulatory sequences on the human and mouse vasoactive intestinal peptide (VIP) genes. J DNA Sequences 1994;5:25-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 HD 00713-01 LDN

PERIOD COVERED: October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Regulation of Gene Transcription and Neurite Outgrowth by Neural Impulse Activity

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

PI:	R.D. Fields	Head	LDN,NICHD
Others:	K. Itoh	Visiting Fellow	LDN,NICHD
	B. Stevens	Biologist	LDN,NICHD

COOPERATING UNITS (if any)
Dr. Melitta Schachner, Depart. Neurobiology, Swiss Federal Institute of Technology, Zurich, Switzerland (M. Schachner); Brain Research Institute, University of Zurich, Zurich Switzerland (C. Bandtlow)

LAB/BRANCH
Laboratory of Developmental Neurobiology

SECTION
Unit on Neurocytology and Physiology

INSTITUTE AND LOCATION
NICHD, 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.)

Our studies show that expression of the neural cell adhesion molecule L1 can be regulated by action potential stimulation, by controlling levels of L1 mRNA. Regulation is selective for specific frequencies of stimulation, and for specific types of neural cell adhesion molecules (NCAM is not affected). Adhesion of neuroblastoma cells to DRG axons is reduced after 5 days electrical stimulation at the frequency that is effective in lowering L1 expression, but no changes in cell-cell adhesion are produced by stimulus patterns that do not alter L1 levels. Association of Schwann cells with axons is reduced for up to 4 days after stimulation at 0.1 Hz, but not after 1 Hz stimulation. Fasciculation of neurites can be regulated by impulse activity at an appropriate frequency.

Intracellular signaling from neural impulses can be sensitive to temporal features of action potential stimulation, rather than to the concentration of second messengers or products in signaling reactions activated by the stimulus. Transcription of the *c-fos* gene can be more strongly activated by action potential stimuli producing low amplitude intracellular calcium transients repeated at short intervals, compared to action potential bursts that produce much larger intracellular calcium transients, repeated at less frequent intervals. Phosphorylation of the transcription factor CREB at Ser 133, does not correlate with *c-fos* transcription for some patterns of stimulation, particularly those consisting of intense bursts repeated at longer intervals.

Accommodation of growth cones to electrically-induced collapse is associated with a decrease in trans-membrane calcium currents, which results from removal of calcium channels from the cell membrane after 24 hours of action potential stimulation at appropriate frequencies. Growth cone responses to action potentials involve different intracellular calcium signaling pathways than those activated by the receptor-mediated growth cone collapsing factor NI-35.

Project Description

Objectives

The Unit on Neurocytology and Physiology, directed by Douglas Fields, is investigating intracellular signaling from the membrane to the nucleus in response to patterned neural impulse activity. The current goal of the Unit is understanding how expression of genes controlling the structural and functional properties of neurons is regulated by specific patterns of impulse activity in developing neural circuits. This work has focused on activity-dependent regulation of neural cell adhesion molecules, second messenger systems, phosphorylation of transcription factors controlling immediate early gene transcription, and the influence of action potentials on growth cone motility.

Methods Employed

Dorsal root ganglion neurons dissociated from fetal mouse spinal cord are maintained in a multi-compartment cell culture preparation equipped with platinum electrodes for chronic electrical stimulation. Other cell culture preparations including Schwann cells dissociated from the sciatic nerve of mice and neuroblastoma cells.

Electrophysiological recording, advanced imaging techniques, and molecular methods are employed to study neuronal responses to action potential stimulation. Growth cone responses to guidance factors and action potentials are studied by low-light level time-lapse video microscopy. Ratiometric imaging of calcium-sensitive dyes is used to measure the temporal and spatial dynamics of electrically-evoked calcium transients in neurons. Immunocytochemistry, electron microscopy and confocal microscopy are used for morphological studies.

Specific mRNA transcripts in cultured neurons are measured by polymerase chain reaction, competitive PCR, and Northern blots. Western blot, immunoprecipitation and kinase assays are used to monitor expression of neural cell adhesion molecules, immediate early genes, neurotrophin receptors, CaM Kinase and MAP kinase activity, and phosphorylation of the transcription factors SRF and CREB.

Surgical implantation of miniperfusion pumps to infuse protease inhibitors into the brain are being used in combination with trans-synaptic neuronal labeling to investigate the formation of ocular dominance columns in visual cortex of kittens.

Major Findings

Regulation of Neural Cell Adhesion Molecules by Specific Patterns of Neural Impulses. Nervous system structure is modified by electrical activity in developing neural circuits, but the molecular mechanisms are not well understood. Neural cell adhesion molecules, such as L1 and NCAM, influence development of the nervous system by regulating cell adhesion, transmembrane signaling, neurite outgrowth, fasciculation and myelination. Our recent research on mouse DRG neurons in an *in vitro* preparation shows that expression of specific neural cell adhesion molecules is regulated by neural impulse activity. Moreover, this regulation is sensitive to specific temporal patterns of impulses.

Kouichi Itoh has performed measurements, using competitive RT-PCR, which show that stimulation of surprisingly low frequency (1 action potential every 10 seconds) decreases the level of L1 mRNA more than 10 fold in DRG neurons. Western blots confirm that this decrease in message results in significantly lower expression of L1 at the protein level. Interestingly, other patterns of stimulation (1 action potential per second, for example) do not alter L1 mRNA or protein, and another neural cell adhesion molecule, NCAM, is not affected by the stimulus. Regulation of neural cell adhesion molecules by specific patterns of neural impulses could be an important mechanism regulating cellular interactions that coordinate structure and function of the nervous system during development. The signaling mechanism responsible for sensitivity to specific patterns of impulses in neurons is currently under study.

Effects of Impulse Activity on Cell Adhesion. Morphogenesis of the nervous system requires recognition and adhesion of appropriate cells to form proper structural/functional complexes. We have developed two assays of neural cell adhesion in multicompartment cell cultures to test whether cellular adhesion is influenced by impulse activity of DRG neurons. Adhesion of test cells (neuroblastoma N2a cells) plated on stimulated DRG neurites is reduced after stimulation at the same frequency of impulses that is effective in lowering expression of L1 (0.1 Hz). Higher frequency stimulation or blockade of activity are without effect on adhesion or L1 expression. Pre-incubation of N2a cells with antibodies against L1 inhibits their adhesion to DRG axons, indicating the involvement of L1 in this adhesion assay.

Schwann cells provide critical functions in development of peripheral nerves, and during regeneration after injury, by promoting neurite outgrowth, providing neurotrophic support, consolidating unmyelinated axons into bundles, and myelinating large diameter axons to enable rapid long-distance conduction. **Beth Stevens** has conducted experiments in which Schwann cells, purified from the sciatic nerve of mice, are plated onto DRG axons that have received different frequencies of electrical stimulation for 5 days. This work shows that association of Schwann cells to DRG axons is highly sensitive to the pattern of impulse activity in the axons. The effects on Schwann cell association with axons are observed only in response to specific frequencies of impulse activity (0.1 Hz, but not 1 Hz), and the effects persist for up to 4 days after the stimulus treatment. The persistent reduction in number of Schwann cells on DRG axons 4 days after 0.1 Hz stimulation may result from a combination of processes, including adhesion, survival, or proliferation of the Schwann cells.

Effects of Impulse Activity on Axon Fasciculation. During normal development, DRG neurons pass through a progression of distinct developmental phases that include neurite outgrowth, defasciculation of axon terminals to innervate peripheral targets, fasciculation and consolidation of axon terminals innervating functional sensory structures, and synaptogenesis with motor neurons in the spinal cord. Each of these developmental phases is accompanied by distinct patterns of spontaneous neural impulse activity, suggesting that functional activity within these developing neural circuits may provide an important regulatory influence on these developmental processes. We have tested whether the association between distinct patterns of impulse activity and defasciculation and fasciculation of DRG axons are functionally related. Using quantitative digital morphometry, this work shows that axons stimulated at the specific frequency that is effective in lowering L1 expression produces significant defasciculation of DRG neurites in the multicompartmental culture preparation. Several molecules may participate in

activity-dependent regulation of axon fasciculation, but as with L1 expression, 0.1 Hz stimulation for 5 days leads to significant defasciculation, whereas 1 Hz stimulation or the absence of impulse activity produces no changes in fasciculation or L1 expression. Stimulus patterns that are effective in lowering L1 expression, and the associated functional effects we observe, both parallel endogenous changes in impulse patterns that fire spontaneously in mouse DRG neurons *in utero*. These changes in firing patterns have long been correlated with distinct developmental phases, but prior to these observations concerning L1 expression, a possible molecular mechanism for a causative relationship between these firing patterns and axon fasciculation has not been available.

Intracellular Signaling from Patterned Neural Impulses. A major area of current research concerns is how extracellular signals regulate expression of genes that are necessary for adaptive responses of cells to changing environmental conditions. Considerable activity is being directed toward identifying the sequence of reactions linking extracellular stimulation to the processes regulating gene expression, but how these signaling reactions might extract and transmit information from temporally varying stimulus patterns is not known. This question is particularly important in the nervous system, where information is conveyed in the temporal pattern of neural impulses.

We are addressing this question by using an *in vitro* preparation of neurons dissociated form the DRG of mice, stimulated via electrical impulses of different patterns. Using this method of stimulation in combination with RT-PCR, ratio fluorescence calcium imaging, immunocytochemistry, and protein phosphorylation assays, we are pursuing our previous findings that expression of immediate early (IE) genes can be activated by specific patterns of impulse activity. Several stimulus patterns have been identified in which expression of the IE gene cannot be understood as a simple response to increasing intensity of stimulation (i.e., increasing frequencies of action potentials), but appears to discriminate differences in temporal features of the stimulus. By monitoring the temporal dynamics of the activation and inactivation of known reactions in the signaling cascades, we hope to begin to understand whether signaling cascades have resonant properties, such that trains of impulses at specific frequencies or patterns can activate signaling pathways in a selective manner.

Calcium Signaling and Regulation of Gene Transcription in Neurons. Calcium is critical in mediating intracellular signaling and in regulating gene expression in neurons. Imaging methods that allow calcium transients to be studied in living neurons in response to electrical stimulation are being used to investigate whether discrimination of different impulse patterns may result from differences in calcium influx. Changes in resting calcium concentration, amplitude, and dynamics of calcium increase and recovery after stimulation have been quantified in neuronal cell bodies, and subcellular domains, including axons, dendrites, the nucleus, and growth cones. These measurements are correlated with levels of transcription of the immediate early gene *c-fos*, using RT-PCR in DRG neurons in culture. We find that transcription of *c-fos* is largely independent of the number of action potentials delivered in the stimulus, and that for a number of stimulus patterns the amount of intracellular calcium generated by impulse activity fails to correlate with levels of *c-fos* transcription. In the case of repeated bursts of impulses, a better correlation is found between *c-fos* transcription and the interval between successive bursts of action potentials, rather than the amplitude of the intracellular calcium or duration of each stimulus burst. For example, pulse trains of

comparatively long duration (10 Hz, for 9 sec), which produces greater increase in intracellular calcium $[Ca^{++}]_i$ (735 nM), fails to activate *c-fos* expression when repeated at 5 min intervals, but brief pulse trains (10 Hz, 1.8 sec), which induce less increase in $[Ca^{++}]_i$ (485 nM), significantly increases expression of *c-fos* when delivered at more frequent intervals (every 1 minutes). This evidence of the importance of temporal aspects of second messenger generation is consistent with previous work showing that even a single action potential, which produces the smallest possible increase in $[Ca^{++}]_i$ (about 20 nM), can stimulate gene transcription if delivered at appropriate intervals (every 10 sec for the *c-fos*).

Dynamics of CREB Phosphorylation in Response to Action Potentials. Nuclear transcription factors are key substrates for calcium dependent protein kinases which help convey action potential stimulation to the nucleus to control expression of genes regulating adaptive responses of neurons. Phosphorylation of the nuclear transcription factor CREB by cAMP or calcium has been shown to be critical in regulating transcription of the *c-fos* gene in neurons, and in consolidating long term memory in mice and invertebrates. Using an antibody specific for CREB phosphorylated at Ser 133, we have studied the kinetics of phosphorylation of this transcription factor in response to action potential stimulation of specific frequencies, and correlated this with *c-fos* expression and the changes in intracellular calcium produced by the stimulus in DRG neurons. Our work shows that phosphorylation in response to action potentials at a frequency of 10 Hz is rapid, reaching maximal values in less than 10 sec, but dephosphorylation of CREB at the critical site of Ser 133 proceeds with a much slower time course, declining by 1/2 within about 5 minutes after a brief stimulus burst. Phosphorylation of CREB at Ser 133 parallels the rapid rate of increase in $[Ca^{++}]_i$, but we find that $[Ca^{++}]_i$ recovers to normal levels within several seconds; much faster than CREB dephosphorylation. This work has revealed a number of stimulus patterns in which *c-fos* expression is not consistent with levels of CREB phosphorylation at Ser 133. For example, high levels of $[Ca^{++}]_i$, if repeated infrequently, e.g. 9 sec 10 Hz stimulation every 5 min, produces large increases in phosphorylation of CREB at Ser 133, yet this stimulus induces minimal *c-fos* transcription. This discrepancy between CREB phosphorylation and transcription of *c-fos*, indicates that CREB phosphorylation is not necessarily sufficient for transcription of this gene in response to action potentials, and that the dynamic responses of parallel signaling reactions regulating gene transcription need to be considered to better understand how transcription is regulated by patterns of impulses in the nervous system.

Growth Cone Responses to Action Potentials. Our previous research has shown that action potentials induce growth cone collapse, similar to responses mediated by receptors on the growth cone for specific guidance molecules in the mammalian brain. This research showed that growth cone collapse was associated with the large increase in intracellular calcium that accompanies action potential depolarization. However, after 24 hours exposure to this electrical stimulus, growth cones no longer collapse in response to action potentials. Ratio fluorescence imaging showed that although high levels of intracellular calcium were reached in accommodated growth cones, the kinetics of calcium increase were slowed significantly. This suggests that a slower rate of increase in intracellular calcium, will fail to activate mechanisms inducing the collapse response. Either changes in calcium buffering and removal mechanisms or decreased calcium influx through voltage-sensitive calcium channels could slow electrically-induced calcium transients in these neurons. Using calcium imaging, we have found that chronic impulse activity also leads to slower electrically-induced calcium accumulation in the cell body of DRG neurons after chronic stimulation. This allowed the use of electrophysiological

methods to measure differences in calcium influx through voltage-sensitive calcium channels in DRG neurons after chronic electrical stimulation. Experiments performed in collaboration with Dr. Nelson and colleagues, using whole-cell patch clamp recording of calcium currents, reveal that voltage-sensitive calcium currents are reduced after chronic stimulation of DRG neurons. Receptor binding studies indicate that this reduction is accompanied by loss of L-type calcium channels from the plasma membrane. This adaptive regulation appears to contribute to the resistance of growth cones to electrically-induced collapse after a period of chronic stimulation. In addition, activity-dependent changes in calcium signaling could regulate a wide variety of other calcium-dependent processes in neurons, possibly including gene expression or synaptic plasticity.

Signal Transduction Pathways in Growth Cone Responses to Action Potentials and Substrate-Bound Factors. Recent work in collaboration with Dr. Christine Bandtlow of the University of Zurich, is exploring differences in signaling reactions mediating growth cone collapse in response to a protein isolated from CNS myelin (NI-35), and collapse induced by action potentials. Both these stimuli are associated with an increase in intracellular calcium in the growth cone, and each is dependent on influx of calcium through voltage-sensitive calcium channels. Calcium imaging and voltage clamp methods showed that calcium fluxes become attenuated in accommodated growth cones. We therefore tested whether the membrane protein isolated from central nervous system myelin (NI-35) would also induce attenuated calcium responses in growth cones accommodated to electrically-induced collapse. This research indicates that NI-35 stimulates large increases in intracellular calcium in cell bodies of DRG neurons that are not significantly different before or after chronic electrical stimulation. This suggests that divergent signal transduction mechanisms, involving partially independent calcium-signaling cascades, are activated by the two growth cone collapsing stimuli.

Significance to Biomedical Research and the Program of the Institute

Nervous system development and recovery from nervous system injury are critically dependent upon selective cell-cell adhesion, fasciculation, and growth cone guidance. The regulation of these and other developmental processes by extracellular signals is essential for coordinating the complex structural/functional interrelations necessary for development and regeneration of the nervous system. Elucidating the responses of signaling reactions that link action potential stimulation to gene transcription, and that in turn activates specific adaptive responses in neurons, will ultimately enable better interventions to prevent or correct developmental deficits and promote recovery from nervous system injury.

Proposed Course

We will continue to study the responses of signaling reactions to temporally varying stimulation in DRG neurons, and seek to understand the cellular basis for the temporal specificity to different frequencies of stimulation. Future work will emphasize spatial heterogeneity in calcium transients in the nucleus and cytoplasm of neurons, the temporal dynamics of activation of kinases that preferentially phosphorylate transcription factors CREB and SRF, and calmodulin activation by action potentials in growth cones and DRG neurons.

Protocols:

Animals:

94-013 Nelson Developmental neurobiology in culture systems

94-016 Fields Effects of proteases on ocular dominance columns of visual cortex

Publications

Fields RD. Regulation of neurite outgrowth and immediate early gene expression by patterned electrical stimulation. Prog in Brain Res 1944;102:125-36.

Fields, R.D. and P.G. Nelson (1994) Role of electrical activity in synapse formation. In: Stenger DA, McKenna TM, eds. Enabling Technologies for Cultured Neural Networks. San Diego: Academic Press Inc, 1994, pp. 237-60.

Nelson PG, Fields RD. Developmental plasticity of the brain: Problems and solutions in dynamic imaging of the synaptic bouton. In: T. Hiruma, ed. Biomolecular Mechanisms and Photonics: Cell-Cell Communication (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01202-7 LDN

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Regulation of expression and function of neuropeptides during development

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

PI:	Y. Peng Loh	Section Chief	LDN, NICHD
Others:	William P. Hayes	IRTA	LDN, NICHD

COOPERATING UNITS *(if any)*

Lab. Biol. Chem., NCI (J. Battey), Lab. Neurochem., NINDS (S. Wray, J. Mill and H. Chin); Univ of Montreal (J. Chan); Lab. Cell. Mol. Neurophys., NICHD (V. Gallo)

LAB/BRANCH

Laboratory of Developmental Neurobiology

SECTION

Section on Cellular Neurobiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	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.)*

Project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01004-12 LMG

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Regulation of Amino Acid and Nucleotide Biosynthesis in Saccharomyces cerevisiae

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

PI: A.G. Hinnebusch, Research Microbiologist, (All listed personnel LMG:NICHD)

M. Garcia	Vis. Fellow	M. Marton	IRTA Fellow	G. Pavitt	Visiting Fellow
C. Vasquez	Vis. Fellow	P. Romano	IRTA Fellow	J. Anderson	IRTA Fellow
W. Yang	Vis. Assoc.	R. Rolfes	IRTA Fellow	E. Duenas	Guest Researcher
B. Jackson	Biologist	K. Natarajan	Vis. Fellow		
C. Drysdale	IRTA Fellow	F. Zhang	(Ebon)		
L. Phan	Guest Researcher				

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Section on Molecular Genetics of Lower Eukaryotes

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
11.06	10.06	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.)*

We are studying transcriptional and translational control mechanisms that regulate amino acid and purine biosynthetic genes in yeast in response to nutrient availability. GCN4 is a transcriptional activator of amino acid biosynthetic genes that is regulated at the translational level by the eIF-2α kinase GCN2. We have proposed that phosphorylation of eIF-2 by GCN2 under starvation conditions stimulates *GCN4* translation by inhibiting guanine nucleotide exchange on eIF-2 catalyzed by eIF-2B, thereby reducing the concentration of the ternary complex eIF-2/GTP/Met-tRNA$^{Met}_i$. A large number of point mutations were isolated in the GCN3, GCD7, and GCD2 subunits of eIF-2B that uncouple *GCN4* translation from eIF-2α phosphorylation without affecting eIF-2B catalytic activity. These mutations cluster in two regions of sequence similarity shared among the three proteins which may define an interaction surface between eIF-2B and eIF-2(αP). Many of the mutations affect residues conserved between yeast and mammalian eIF-2B, and the rat homologue of GCN3 can partially substitute for GCN3 in yeast cells. GCD2, GCD7, and GCN3 can form an eIF-2B subcomplex in vivo that reverses the effects of eIF-2α phosphorylation, probably by sequestering eIF-2(αP). We have shown that GCD10 is a component of the 8-subunit eIF-3 complex, and that mutations in *GCD10* are suppressed by overexpressing tRNA$^{Met}_i$. These results suggest that *gcd10* mutations reduce binding of ternary complexes to 40S ribosomal subunits. GCN1 and GCN20 are components of a protein complex required in vivo for phosphorylation of eIF-2α by GCN2. An N-terminal segment of GCN20 and a domain in GCN1 related to translation elongation factor 3 (EF-3) mediate complex formation between the two proteins. GCN20 contains ATP-binding cassettes found in membrane transporters; however, these sequences are partially dispensable for GCN20 function, and GCN1 and GCN20 are distributed uniformly throughout the cytoplasm. Based on their homology to EF-3, we propose that GCN1/GCN20 interact with ribosomes and facilitate binding of uncharged tRNA to GCN2. GCN2 contains a pseudo-kinase domain in its N-terminus that is conserved in the Neurospora homologue of GCN2 that is also required for regulating kinase function in vivo. The transcriptional activation domain of GCN4 contains 7-8 subdomains, each consisting of 2-3 bulky hydrophobic amino acids surrounded by acidic residues. These subdomains can cooperate in different combinations to activate transcription. An *ADE5,7* promoter fragment sufficient for adenine-repressible transcription contains three 6-10nt elements that probably function as binding sites for the BAS1, BAS2, and ABF1 transcription factors. Overexpressing BAS2 increases *ADE5,7* expression under repressing conditions, suggesting that adenine repression involves reducing the ability of *ADE* gene promoters to compete with other yeast genes for binding limiting amounts of BAS2.

Project Description:

Objectives:

To understand at the molecular level how the yeast *S. cerevisiae* regulates its capacity for synthesizing amino acids and nucleotides according to the availability of these nutrients in the environment. One facet of this regulation, known as general amino acid control, involves increased expression of a large number of amino acid biosynthetic genes in response to starvation for any amino acid. Synthesis of GCN4 protein, the transcriptional activator in this system, is stimulated under starvation conditions by a translational control mechanism involving short open reading frames (uORFs) in the *GCN4* mRNA leader, several general translation initiation factors, and a protein kinase known as GCN2. Genetic and biochemical experiments are being conducted to understand how the uORFs regulate the flow of scanning ribosomes to the *GCN4* start codon according to the availability of amino acids. A combination of genetic, molecular and biochemical approaches are being used to identify and characterize the trans-acting factors, both positive and negative, that mediate the regulatory functions of the uORFs. Some of these factors are subunits of the general translation initiation factors eIF-2 or its guanine nucleotide exchange factor eIF-2B; others appear to be components of previously unidentified general initiation factors; still others, including the protein kinase GCN2 and its positive effectors GCN1 and GCN20, are dispensable regulatory factors dedicated to *GCN4* control. We wish to identify the biochemical functions of each factor and determine its position in the signal-transduction pathway that detects uncharged tRNA in amino acid-starved cells and modifies the translational machinery in a way that stimulates *GCN4* expression. This regulatory mechanism involves phosphorylation of the α subunit of eIF-2 by the protein kinase GCN2 and consequent reduction in the ability of eIF-2B to recycle eIF-2. This same mechanism operates in mammalian cells to inhibit total protein synthesis in response to various stress conditions, including amino acid starvation. By combining the powerful genetics and molecular biology of yeast with the biochemical analysis of translation initiation, we hope to provide a detailed molecular description of this highly conserved mechanism for regulating protein synthesis. The human eIF-2α kinase DAI (double-stranded RNA-activated inhibitor of translation) is an important regulator of cell growth and differentiation and a critical component of the interferon response to viral infection in humans. We have shown that DAI can functionally substitute for GCN2 in yeast cells and we are using the *GCN4* genetic system to probe the mechanism of DAI activation by dsRNA.

In addition to studying the regulation of *GCN4* translation, we are conducting an in-depth mutational analysis of the transcriptional activation domain of the GCN4 protein, with the goal of obtaining a better understanding of the detailed structure of this domain and the identification of specific transcription factors with which GCN4 interacts in stimulating the expression of its target genes. We have also undertaken a study of the transcriptional control of adenine biosynthetic genes in response to exogenous purines. We are defining the minimal cis-acting sequences at the *ADE5* gene necessary for adenine repression of this gene and studying the regulatory proteins that bind to these sites, including BAS1, BAS2 and GCN4. We hope to learn how the expression and activity of these regulatory proteins is modulated by purine availability in the cell. Parallel studies of general amino acid control and the regulation of nucleotide biosynthesis in yeast should provide an integrated view of how eukaryotic cells control the levels of substrates (amino acids and nucleotides) and the machinery for protein and nucleic acid synthesis in the face of a changing nutritional environment.

Major Findings

I. Mechanism of translational control of *GCN4* expression.

A. Evidence that the efficiency of *GCN4* translation is inversely coupled to the concentration of eIF-2•GTP•Met-tRNA$^{Met}_i$ ternary complexes in the cell.

Phosphorylation of the α subunit of translation initiation factor-2 (eIF-2α) on residue Serine-51 is a prominent mechanism for regulating protein synthesis in mammalian cells. The phosphorylated form of eIF-2 inhibits translation initiation by impairing the conversion of eIF-2·GDP to eIF-2·GTP by the guanine-nucleotide exchange factor eIF-2B. In yeast, phosphorylation of eIF-2 by the protein kinase GCN2 stimulates translation of *GCN4* mRNA under conditions of amino acid starvation. According to our model, essentially all ribosomes that bind to the 5' end of *GCN4* mRNA translate uORF1, under both starvation and nonstarvation conditions, and ca. 50% remain attached to the mRNA and resume scanning downstream as 40S subunits. Under nonstarvation conditions, when the active form of eIF-2 is abundant, these 40S subunits rapidly rebind the eIF-2•GTP•Met-tRNA$^{Met}_i$ ternary complex and regain the ability to recognize an AUG codon as a translational start site. Consequently, most reinitiate at uORFs 2, 3, or 4, and then dissociate from the mRNA, failing to reach the *GCN4* start codon. Under starvation conditions, eIF-2α is phosphorylated by the protein kinase GCN2 and, by analogy with mammalian systems, this reduces the level of eIF-2•GTP•Met-tRNA$^{Met}_i$ ternary complexes by inhibiting the recycling factor eIF-2B. Following translation of uORF1, many ribosomes now scan the entire distance between uORF1 and uORF4 without rebinding the ternary complex. Lacking the initiator tRNA^{Met}, they cannot recognize the AUG start codons at uORFs 2, 3, and 4 and continue scanning downstream. While traversing the leader segment between uORF4 and *GCN4*, most of these ribosomes re-bind the ternary complex and reinitiate translation at *GCN4*.

One of the key tenets of our model is that increased translation of *GCN4* is triggered by a reduction in the concentration of eIF-2•GTP•Met-tRNA$^{Met}_i$ ternary complexes. In support of this idea, we showed that reducing the number of chromosomal genes encoding initiator tRNA^{Met} mimics the effect of eIF-2 phosphorylation in causing derepression of *GCN4* translation in the absence of amino acid starvation or GCN2 function. In addition, overexpression of all three subunits of eIF-2, which leads to a ~10-fold increase in the heterotrimeric eIF-2 complex in vivo, prevents derepression of *GCN4* in response to eIF-2 phosphorylation by GCN2. It also partially suppresses the slow-growth phenotype associated with hyperphosphorylation of eIF-2 by GCN2^c enzymes, and this suppression is augmented when tRNA$^{Met}_i$ is co-overexpressed with the eIF-2 complex. These results provide strong support for the idea that phosphorylation of eIF-2 inhibits general translation and specifically stimulates *GCN4* expression by reducing the levels of the ternary complex in the cell.

B. Saturation mutagenesis of the regulatory domains in the GCD2, GCD7 and GCN3 subunits of eIF-2B.

Another important piece of evidence supporting our model is that mutations can be isolated in subunits of eIF-2B that suppress the phenotypes associated with eIF-2α hyperphosphorylation by hyperactivated GCN2^c protein kinases. The suppressor mutations include point mutations and deletions in *GCN3* a nonessential subunit, and point mutations in *GCD7* and *GCD2* two of the four essential subunits of the yeast eIF-2B complex. These mutations could decrease the affinity of eIF-2B for phosphorylated eIF-2 and thus prevent sequestering of eIF-2B in an inactive state; alternatively, they could allow eIF-2B to catalyze nucleotide exchange on eIF-2. The C-terminal half of GCD2, GCD7 and GCN3, are very similar in amino acid sequence, suggesting that the interaction of each protein with eIF-2 involves a structural feature that is common to all three. To test this idea, we set out to identify all of the amino

acids in GCD2, GCD7 and GCN3 that are critically required for negative regulation of eIF-2B by phosphorylated eIF-2. Towards this end, we have isolated and characterized a large number of dominant or semi-dominant point mutations in *GCD2 GCD7*, and *GCN3*with the suppressor phenotype described above. For both *GCD2* and *GCD7*, we have obtained point mutations that render phosphorylated eIF-2 completely ineffective in down-regulating general protein synthesis and in stimulating *GCN4* translation (Gcn$^-$ phenotype). These mutations are actually more effective than a deletion of *GCN3* in suppressing the toxic effect of eIF-2 hyperphosphorylation by PKR. Thus, it appears that all three proteins make important contributions to the inhibitory effects of eIF-2(αP) on eIF-2B activity.

The results obtained thus far identify two clusters of Gcn$^-$ mutations which affect residues located in regions of homology between GCD2, GCD7 and GCN3. One of the two clusters in each protein is located at the extreme C-terminus, the domain of greatest sequence similarity among the three. A second cluster occurs within a region of high similarity near the N-termini of GCN3 and GCD7 and in the center of GCD2 (which contains a large N-terminal domain not shared with GCD7 and GCN3). It is noteworthy that all of the *GCD2* mutations fall within the region of the protein that is related in sequence to GCD7 and GCN3. In a few cases, amino acids occupying identical positions in the sequence alignments have been mutated in two of the three proteins. Together, these results suggest that homologous segments in GCD2, GCD7 and GCN3 are devoted to the regulatory interactions between eIF-2B and phosphorylated eIF-2. These homologous segments might be juxtaposed on the surface of eIF-2B and make direct contact with different residues in the phosphorylated N-terminal domain of eIF-2α, as suggested previously. Alternatively, one of the two regulatory segments in each protein might interact with eIF-2α while the other could mediate a conformational change in eIF-2B that distorts the active site when phosphorylated eIF-2 is bound. The segments located between the two clusters of Gcn$^-$ mutations in each protein are also very similar in sequence. Perhaps these segments perform a structural role in mediating subunit interactions between GCD2, GCD7, and GCN3.

C. Suppression of eIF-2(αP) toxicity by overexpression of eIF-2B subcomplexes.

A second approach we have taken in probing the structure and function of eIF-2B is to overexpress different combinations of the eIF-2B subunits. We showed previously that overexpressing all 5 subunits, or all 4 subunits except GCN3, suppresses the toxicity of eIF-2α phosphorylation in *GCN2^c* strains. More recently, we observed the same phenotype when only GCD2, GCD7 and GCN3 were overexpressed from high copy-number plasmids. In fact, overexpressing just GCD7 and GCD2 conferred nearly the same suppression as did the combination of all three proteins. In contrast, overexpressing each of the individual subunits and many other combinations of two or three subunits of eIF-2B conferred no suppression of *GCN2^c* mutations. One interpretation of these results is that GCD7, GCD2 and GCN3, or just GCD7 and GCD2 alone, can form stable subcomplexes that sequester phosphorylated eIF-2 and neutralize its inhibitory effect on the native eIF-2B complex. In support of this interpretation, we succeeded in co-immunoprecipitating nearly all of the excess GCD7 and much of the excess GCN3 with anti-GCD2 antibodies from cells overexpressing GCD2, GCD7 and GCN3. Thus, we now have biochemical evidence for subcomplex formation by these proteins in vivo. An alternative explanation for our results is that subcomplexes containing GCD2, GCD7 and GCN3 have nucleotide exchange activity and thus can functionally replace native eIF-2B. This explanation seems unlikely because overexpressing the subcomplex does not complement the growth defects of mutations in the GCD1 subunit that are suppressed when eIF-2 is overexpressed.

D. In vivo analysis of the mechanism for inhibiting eIF-2B by phosphorylated eIF-2.
Overexpression of eIF-2 in strains containing an activated GCN2^c kinase leads to the production of much higher levels of the phosphorylated form of the protein than are present in the same strain containing wild-type amounts of eIF-2; yet, we found that the toxicity of GCN2^c proteins is suppressed by overexpressing eIF-2. This apparent paradox can be explained by noting that the ratio of phosphorylated to nonphosphorylated eIF-2 was reduced in the strain overexpressing eIF-2 versus the parental strain. If we propose that the mechanism of inhibition of eIF-2B by eIF-2(αP) is competitive inhibition, rather than irreversible binding of a non-competitive inhibitor, we can explain why the degree of inhibition depends more on the ratio of eIF-2(αP):eIF-2 rather than the absolute amount of phosphorylated eIF-2. We can eliminate the trivial possibility that overexpression of eIF-2 simply decreases the requirement for eIF-2B, perhaps as a result of spontaneous nucleotide exchange on eIF-2, because several mutations in subunits of eIF-2B are not complemented by overexpression of eIF-2. Therefore, our results indicate that eIF-2(αP) acts as a competitive inhibitor of eIF-2B rather than forming an extremely stable inactive complex with it.

E. GCD10 is the RNA binding subunit of eIF-3.
Mammalian eIF-3 is an 8-subunit complex that stimulates several steps in the initiation pathway in cell-free translation systems. Yeast contains a structurally similar complex that can functionally replace mammalian eIF-3 in an in vitro translation system containing all the initiation factors from Hela cells except for eIF-3. In collaboration with Mercedes Tamame's and John Hershey's laboratories, we have now obtained strong biochemical evidence that *GCD10* encodes the 54.6kDa RNA-binding subunit of yeast eIF-3. Mutations in *GCD10* lead to constitutive derepression of *GCN4* translation in the absence of *GCN2* and *GCN3,* and to temperature-sensitive growth on rich medium, the same phenotypes associated with *gcd* mutations affecting subunits of eIF-2 or eIF-2B. We cloned *GCD10* and found that a deletion of the gene is lethal. In addition, we observed dissociation of polysomes and accumulation of 80S subunits after shifting *gcd10* temperature-sensitive mutants to the non-permissive temperature, consistent with a defect in an essential translation initiation factor. After producing antibodies against GCD10, we showed that the protein is present in a high molecular weight complex, and that a fraction of GCD10 is physically associated with polysomes and ribosomal subunits. GCD10 co-purifies with eIF-3 biochemical activity and other subunits of the eIF-3 complex through Superose-6 gel filtration chromatography and MonoS ion-exchange chromatography, and is identical in electrophoretic mobility to the 62 kDa subunit of eIF-3 described previously. This 62 kDa subunit binds RNA in vitro and we showed that GCD10 has RNA binding activity by a Northwestern assay using radiolabeled globin mRNA as the probe. We also discovered that our GCD10 antibodies cross-react with the RNA-binding 66kDa subunit of human eIF-3. Finally, we showed that GCD10 is specifically co-immunoprecipitated with the ~90 kDa subunit of eIF-3 encoded by *PRT1*

Biochemical studies on mammalian eIF-3 have implicated this factor in several steps of initiation, including dissociation of 80S ribosomes into free 60S subunits and 46S preinitiation complexes containing eIF-3 and 40S subunits, binding of eIF-2/GTP/Met-tRNA$^{Met}_i$ ternary complexes to form 43S complexes, and binding of mRNA to 43S complexes containing both eIF-3 and the ternary complex. We have proposed that *gcd10* mutations reduce the ability of eIF-3 to rebind to 40S subunits and form 46S complexes following termination at uORF1. This would result in "naked" 40S subunits scanning downstream from uORF1 which have a reduced ability to rebind ternary complexes (Fig. 8). Alternatively, *gcd10* mutations could decrease the ability of eIF-3 to stimulate binding of eIF-2/GTP/Met-tRNA$^{Met}_i$ ternary complexes to 46S complexes rather than delaying the formation of these complexes. Either of these last two defects would decrease the rate at which ternary complexes can

rebind to 40S subunits scanning downstream from uORF1, explaining why *gcd10* mutations cause ribosomes to ignore uORFs 2-4 and reinitiate at *GCN4* in the absence of eIF-2 phosphorylation by GCN2.

It is thought that eIF-3 functions at multiple steps of the initiation pathway and interacts with most of the other known initiation factors, including eIF-1A, eIF-2A, eIF-3A, eIF-4, eIF-4A, eIF-4B, and eIF-5. It could thus be imagined that eIF-3 provides a platform on the 40S subunit which facilitates binding of these factors in the correct orientation with respect to one another and with the decoding sites on the ribosome. One way of testing this idea is to employ genetic suppressor analysis to identify specific interactions between particular subunits of eIF-3 and the components of other initiation factors or ribosomal proteins. Towards this end, we have isolated high copy plasmids from wild-type yeast genomic libraries that complement temperature-sensitive *gcd10* mutations. Five of the six plasmids partially suppress the derepression of *GCN4* (Gcd$^-$ phenotype) seen in *gcd1, gcd2* and *gcd13* mutants in addition to *gcd10* mutants; however, none suppresses the temperature-sensitive phenotypes of these other *gcd* mutations, or of *prt1* mutations, suggesting a specific interaction with GCD10. Hybridization of the cloned fragments to the Olson-Riles ordered genomic library shows that the suppressor genes derive from seven genomic loci. Five of these loci contain one of the structural genes for initiator $tRNA^{Met}$ and in one case, we have shown that disruption of this gene abolishes suppressor activity. Thus, it appears that overexpression of initiator $tRNA^{Met}$ can compensate for the general initiation defect in *gcd10*mutants. The simplest explanation for this finding is that binding of the ternary complex to small ribosomal subunits is defective in the *gcd10* mutants. This provides in vivo evidence that eIF-3 stimulates binding of ternary complexes to 40S subunits and supports the idea that *gcd10* mutations are specifically defective for this aspect of eIF-3 function. The fact that multi-copy $tRNA^{Met}_i$ genes do not suppress mutations in *PRT1* encoding another subunit of eIF-3, seems to indicate that *PRT1* mutations alter this or some other function of eIF-3 in a way that cannot be overcome by simply increasing the concentration of ternary complexes.

F. Analysis of novel GCD factors

Mutations in the *GCD13* and *GCD14* genes have the same phenotypes as mutations in any of the four GCD genes encoding subunits of eIF-2B. Thus, GCD13 and GCD14 could be additional subunits of eIF-3 or components of another factor that influences the production or utilization of the ternary complex in translation initiation. In accordance with this idea, we found that *GCD13*mutations lead to polysome dissociation in vivo when mutants are incubated at the nonpermissive temperature. Efforts to clone *GCD13* by conventional approaches have failed; therefore, we mapped *GCD13* to the telomeric region of the left arm of chromosome XV. Using this information we are attempting to isolate the gene from ordered lambda clones or cosmids containing this portion of XV.

We confirmed that *GCD14* mutations lead to constitutive derepression of *GCN4* expression in the absence of GCN2 and GCN3. The *GCD14*gene has been isolated and found to encode a protein of ca. 40 kDa that contains a degenerate RNA recognition motif (RRM), and thus could be an RNA binding protein. Based on peptide sequencing done in John Hershey's lab, GCD14 does not appear to be the 39kDa subunit of eIF-3.

G. GCN1 and GCN20 are components of a heteromeric protein complex that stimulates the phosphorylation of eIF-2α by GCN2.

GCN1 and GCN20 are positive regulators of *GCN4* translation that function by stimulating the ability of GCN2 to phosphorylate eIF-2 in vivo under amino acid starvation conditions. We have shown that GCN1 and GCN20 interact in vitro by co-immunoprecipitation experiments and by using the yeast two-

hybrid system; thus, they are components of a protein complex that is required in vivo to couple GCN2 kinase activity to amino acid availability. Interestingly, ca. 800 amino acids of GCN1 shows sequence similarity to the fungal-specific translation elongation factor EF-3. It is thought that EF-3 functions in stimulating release of uncharged tRNA from the E (exit) site of the ribosome and thereby stimulates binding of charged tRNA to the A site. Remarkably, GCN20 also shows strong sequence similarity to a portion of EF-3 containing two ATP-binding cassettes characteristic of the "ABC" family of transporter proteins. GCN1, in contrast, does not contain the signature sequences of ABC domains and is most similar to EF-3 in a region N-terminal to its ABC domains. Thus, GCN20 and GCN1 are similar to different portions of EF-3.

The great majority of ABC proteins are membrane transporters that use the energy of ATP hydrolysis to pump substrates against a concentration gradient. The typical ABC transporter, typified by the multiple-drug resistance protein MDR, consists of four membrane-associated domains. Two of these domains contain six hydrophobic α-helical membrane-spanning segments that form the pore through which substrates cross the membrane. The other two domains are peripherally located on the cytoplasmic face of the membrane, bind ATP and couple ATP hydrolysis to the transport process. GCN20 seems to lack regions sufficiently hydrophobic to function as transmembrane domains; however, in prokaryotes at least, the individual domains of the transporter can be expressed as separate polypeptides. It is thus conceivable that GCN20 contains the two ATP-binding domains and interacts with one or more transmembrane proteins in carrying out a transport function. One possibility would be that GCN20 interacts with GCN1, which does contain numerous hydrophobic regions, to comprise one of the transporters identified in yeast that delivers amino acids from the cytoplasm to the vacuole.

A few members of the large family of ABC proteins, such as EF-3, are not associated with membrane transport events. Therefore, GCN20 and GCN1 could be components of an ABC complex with no role in membrane transport that acts more directly to stimulate GCN2 kinase activity by uncharged tRNA. One possibility, motivated by their sequence similarity to segments of EF-3, would be that GCN1 and GCN20 interact with the ribosome and facilitate an interaction between uncharged tRNA bound at the ribosomal A site and the HisRS-related domain of GCN2. This activity could be akin to the proposed function of EF-3 in stimulating release of uncharged tRNA from the ribosomal E site. We have identified proteins from diverse eukaryotic organisms, including *C. elegans*, rice, Arabadopsis, and humans, that are more closely related to GCN20 than to any other known ABC proteins, and contain sequence similarity in regions flanking the ABC domains that are not conserved among diverse members of the ABC family. These sequence similarities could indicate that GCN20 belongs to a new subfamily of ABC proteins with a biological function conserved from yeast to mammals. If so, higher eukaryotes may contain a mechanism for coupling the rate of translation initiation to the level of uncharged tRNA similar to that uncovered in *S. cerevisiae* involving GCN2 and its positive effectors GCN1 and GCN20.

We have used our antibodies against GCN1 to localize the protein in yeast cells by indirect immunoflourescence. Our results to date indicate that the protein is uniformly distributed throughout the cytoplasm. Its distribution appears distinctly different from that of a known vacuolar membrane protein, VAT2, that was analyzed in parallel. These results do not support the model that the GCN1/GCN20 complex are components of a membrane transporter. We have also been investigating the importance of the ABC domains in GCN20 for its regulatory function by making deletions and site-directed substitutions of conserved residues in the Walker A and B motifs of the ATP-binding cassettes. Surprisingly, mutations in these motifs reduce but don't eliminate GCN20 function. In fact, a deletion allele lacking all 634 residues C-terminal to amino acid 118, which includes both

ABC domains, retains substantial complementing function. In contrast, a deletion of residues 4-118 that leaves both ABC domains intact completely abolishes GCN20 function. These mutations do not significantly lower GCN20 protein levels. The N-terminal 118 residues is sufficient for interaction with the C-terminal two-thirds of GCN1 in the yeast two-hybrid system. In addition, a GCN20-lacZ fusion protein containing only the N-terminal 118 residues of GCN20 can be co-immunoprecipitated with GCN1, whereas a nearly full-length GCN20-lacZ fusion lacking only residues 4-118 fails to interact with GCN1. Thus, the N-terminal domain of GCN20 is both necessary and sufficient for its function as a positive regulator of GCN2 and for its physical interaction with GCN1. The fact that the ABC domains of GCN20 are partly dispensable for its positive regulatory function also seems to be at odds with the idea that GCN1 and GCN20 form an amino acid transporter because ATP hydrolysis is essential for the function of ABC transporter proteins. However, we found a predicted ABC protein highly similar to GCN20 (Yer036), in the yeast genome database that could conceivably substitute for the ABC domains of GCN20 in a *gcn20* mutant that retains only the N-terminal 118 residues.

Using the yeast two-hybrid system, we showed that the N-terminal 118 amino acids of GCN20 could interact with the C-terminal two-thirds of GCN1. We have been extending this analysis by testing different overlapping segments of GCN1 for interaction with the N-terminus of GCN20. Our results thus far indicate that all GCN1 fragments that interact with GCN20 contain the EF-3-related segment of GCN1. The smallest of these segments contains 345 amino acids and is coincident with the region most highly related to EF-3.

H. GCN2-independent derepression of *GCN4* translation.

The regulatory mechanism described above applies to the derepression of *GCN4* translation that occurs under conditions of prolonged amino acid-limited growth. There is evidence that a different pathway is responsible for the transient derepression of *GCN4* that accompanies a "shiftdown" from amino acid-rich to minimal medium. We uncovered another instance of *GCN2*-independent induction of *GCN4* translation in strains overexpressing tRNAs under conditions in which the excess tRNA cannot be aminoacylated. The most complete derepression occurred in strains overexpressing a mutant $tRNA^{Val}$(AAC) with a mutation in the acceptor stem that is expected to abolish aminoacylation. Overexpression of the mutant $tRNA^{Val}$ derepresses *GCN4* translation under nonstarvation conditions in a manner dependent on the uORFs and independent of *GCN2* and Ser-51 on eIF-2α. Overexpression of the mutant $tRNA^{Val}$ did not affect cellular growth under nonstarvation conditions in an otherwise wild-type strain; however, it exacerbated the slow-growth phenotype of a $GCN2^c$ mutant. These findings suggest that the presence of excess unchargable $tRNA^{Val}$ lowers the level of eIF-2 activity by a mechanism that does not involve eIF-2α phosphorylation. A weaker *GCN2*-independent derepression was observed in *gcn2*Δ strains overexpressing wild-type $tRNA^{His}$ under histidine starvation conditions, indicating that the *GCN2*-independent mechanism can be activated by an excess of uncharged wild-type tRNA.

II. Regulation of the mammalian eIF-2α kinase DAI in yeast.

In mammalian cells, stimulation of the latent protein kinase activity of the double-stranded-RNA-activated inhibitor of translation, DAI, occurs in response to viral infection. The N-terminus of DAI contains two copies of a sequence motif found in several dsRNA-binding proteins and numerous deletion and point mutations have been introduced into these sequences that affect dsRNA binding in vitro. We showed that DAI expressed at low levels in yeast mimics the function of GCN2 in stimulating translation of *GCN4*, and inhibits total protein synthesis when expressed at high levels. Exploiting these findings, we went on to demonstrate that the dsRNA-binding domain is required for

DAI kinase function in yeast cells and that the dsRNA-binding motifs are involved in the activation mechanism. Secondly, we find that the N-terminal copy of the dsRNA-binding domain plays a greater role than the C-terminal copy in activating kinase function in yeast cells. Thus, the requirements for dsRNA binding in vitro and for kinase activation in vivo closely coincide, providing direct evidence that dsRNA binding to the repeated motifs stimulates DAI kinase activity in vivo.

We have been attempting to identify sites of autophosphorylation in PKR that are important for its activation by dsRNA in vivo. Mike Mathew's lab have identified several tryptic peptides from the N-terminal regulatory domain of PKR that are autophosphorylated in vitro by PKR purified from cultured human cells. In collaboration with their group, we have analyzed the effects of making alanine substitutions in the various Ser and Thr residues in these peptides on PKR function in yeast. Our results thus far indicate that substitutions at a combination of these potential phosphorylation sites partially impairs PKR activation. This result implies that, although these sites may be important for activation, there are probably additional sites of autophosphorylation in the catalytic domain of PKR. Accordingly, we have also been examining the importance of two Thr residues located in the insert between kinase subdomains VII and VIII as potential autophosphorylation sites. The activity of several protein kinases, including PKA, MAPK, MAPKK and CDK requires phosphorylation or autophosphorylation of one or two closely spaced Ser or Thr residues in this segment. In the case of CDK, this phosphorylation is thought to alter the conformation of a loop that protrudes into the active site and prevents proper positioning of the protein substrate. It can also promote proper interactions between the N-terminal and C-terminal lobes of the kinase domain, as in the case of PKA and MAPK. We have preliminary evidence that Ala substitutions at Thr-445 and Thr-450 each decrease PKR kinase function in vivo, and completely inactivate it when combined in the same protein. These mutant proteins are expressed at high levels and migrate in SDS-PAGE with the same mobility as the subdomain II K296R mutant protein; thus, the mutations at Thr-445 and Thr-450 appear to prevent autophosphorylation of PKR on most, if not all of its phosphorylation sites.

III. Mechanism of transcriptional activation by GCN4.

A. Mutational analysis of the GCN4 activation domain.

Previous work by Struhl and his colleagues established that GCN4 contains a potent transcriptional activation domain located in the center of the protein between residues 107-144. There were indications that additional activation determinants were located N-terminal to this central acidic activation domain (CAAD); however, they appeared to be insufficient for high level activation in the absence of a portion of the CAAD. We set out to locate more precisely the activation determinants in the N-terminal portion of GCN4 and to compare the relative efficiencies of transcriptional activation by the CAAD versus the N-terminal activation domain (NTAD) when GCN4 is expressed at physiological levels. Towards this end, numerous deletion and point mutations were constructed in a single-copy *GCN4* gene containing the native promoter and translational control elements, and analyzed for their effects on gene expression after inducing the mutant proteins by histidine starvation. The levels of all mutant proteins were quantified by immunoblotting using antibodies raised against the DNA-binding and dimerization domain of GCN4. Because activation domains in several well characterized mammalian activators, most notably VP16 and yeast GAL4, contain hydrophobic residues critically required for the activation functions of these proteins, we also wished to determine whether this structural feature extended to the CAAD or NTAD of GCN4 protein.

Our analysis showed that GCN4 contains a potent activation domain in its N-terminal 100 amino acids that confers nearly wild-type transcriptional activation of *HIS3* or *HIS4* in the complete absence of the CAAD. Similarly, the CAAD can promote high-level transcription when the NTAD is missing. The

CAAD and NTAD are both strongly dependent on the ADA2 protein for activating transcription at *HIS3* and *HIS4*. The CAAD was found to be critically dependent on two clusters of aromatic and bulky hydrophobic residues: Met-107, Tyr-110, Leu-113 and Trp-120, Leu-123 and Phe-124. The latter corresponds to a short stretch of amino acids (Trp-X-Ser-Leu-Phe), that is conserved between the activation domains of GCN4 and its homologues in Neurospora (cpc-1) and Aspergillus (G. Braus, personal communication). The NTAD was found to be dependent on a pair of Phe residues at positions 97-98. Consistent with the deletion analysis, point mutations in the hydrophobic and aromatic residues in the CAAD impair GCN4 function only when combined with a deletion of the NTAD or with substitutions at Phe-97, Phe-98. Likewise, deletions in the NTAD or point mutations at Phe-97 and Phe-98 impair GCN4 function only when the CAAD is missing or bears mutations at the aforementioned hydrophobic and aromatic residues located between Met-107 and Phe-124. Combining together substitutions at all eight hydrophobic residues in full-length GCN4 essentially eliminated activation of *HIS3* and reduced activation of *HIS4* to 20% of wild-type. These findings indicate that the activation domain of GCN4 is more complex than was previously imagined and shares significant structural similarities with activation domains found in other acidic activators.

In accordance with the results just described, a deletion that removes the entire CAAD and Phe-97 and Phe-98 does not completely abolish activation of *HIS4*. Surprisingly, overexpression of this mutant protein achieved by removing the uORFs is lethal under nonstarvation conditions. This lethality is dependent on the GCN4 DNA binding domain, suggesting that it results from sequestration of one or more general transcription factors in non-productive complexes by the excess mutant GCN4 protein. Based on these observations, we set out to identify activation determinants in the NTAD located in the region N-terminal to position 97. To accomplish this goal, PCR was employed in a random mutagenesis of the NTAD between residues 17-100 in a *GCN4* allele lacking the CAAD, and alleles with greatly reduced GCN4 function were isolated and sequenced. Thirty out of 42 alleles analyzed contained a mutation at one of 6 Phe residues present in the NTAD segment that we mutagenized (Phe-45, Phe-48, Phe-67, Phe-69, Phe-97, and Phe-98). Using site-directed mutagenesis, we found that single alanine substitutions of these Phe residues could account for the Gcn$^-$ phenotype of all 30 alleles.

In one of the 12 mutant alleles in which a Phe residue was not altered, the mutant phenotype resulted from a lysine substitution at Glu-88. This was the only mutation at an acidic residue that had a strong effect on GCN4 function. Moreover, we found that an alanine substitution at Glu-88 has little effect on activation, indicating that an acidic residue is not required even at this position. Interestingly, Glu-88 maps within the only short stretch of amino acids in the NTAD that is conserved between GCN4 and its relatives in Neurospora and Aspergillus: Thr^{82}-X-Leu-X-X-Pro-X-Leu^{89}, located 8 residues N-terminal to the critical Phe-97, Phe-98 pair. Using site-directed mutagenesis, we found that Leu-89 is the sole conserved residue in this segment that is required for activation by GCN4. Thus, the Lys substitution at Glu-88 probably reduces activation by interfering with the function of the adjacent residue, Leu-89. It is noteworthy that leucine has a bulky hydrophobic side-chain and can partially substitute for certain critical Phe residues in the VP16 activation domains.

Two additional Phe residues are present in the N-terminal domain of GCN4 (Phe-9 and Phe-16) just outside the region that we mutagenized by PCR. Using site-directed mutagenesis, we found that Phe-9 is also critical, whereas Phe-16 makes only a minor contribution to NTAD function. A methionine at position 14 is also dispensable for activation by the NTAD. The latter results show that not all bulky hydrophobic or aromatic residues in the NTAD are functionally important. This conclusion was underscored by the fact that alanine substitutions at Leu-65 and Leu-71(located very close to the critical Phe-67 Phe-69 pair) and at Leu-84, Val-93 and Val-94 (in the vicinity of Phe-97 Phe-98), have

no effect on NTAD function. To determine whether Phe is uniquely required at residue 45, we randomized this codon and isolated alleles covering a wide range of GCN4 function. Sequencing these alleles indicated the following functional hierarchy for different amino acids at position 45:

Trp, Phe > Leu > Tyr > Ile > Val,Ser > Thr, Pro, Glu, Gln, Cys, Ala, Lys, Gly,

with the first group conferring wild-type activation and the last showing the null phenotype. Thus, either an aromatic or bulky hydrobphobic residue seems to be required at position 45. Finally, we verified that alanine substitutions at the three new Phe clusters in the NTAD at positions 9/16, 45/48 and 67/ 69 reduce activation by full-length GCN4 only when combined with point mutations in the CAAD at Met-107, Tyr-110, Leu-113 and Trp-120, Leu-123 and Phe-124. In addition, we used immunoblot analysis to verify that these mutations do not reduce the level of GCN4 protein in the cell.

Taken together, our results indicate that GCN4 contains a minimum of 7 clusters containing 2-3 aromatic or bulky hydrophobic amino acids distributed throughout the N-terminal 150 amino acids that comprise its two activation domains. We cannot determine from our data whether these residues are required for the overall structure of the activation domains or for specific contacts they make with co-activators or basic transcription factors. It is worth noting, however, that certain transcription factors contain critical hydrophobic residues, including TBP and $TAF_{II}230$, and it has been suggested that hydrophobic interactions are important in stabilizing contacts between activators and their targets in the transcriptional machinery.

IV. Genetic analysis of the regulation purine biosynthetic genes.

A. Identification of the minimal cis-acting elements in the *ADE5,7* promoter required for adenine-repressible transcription.

The transcription of genes encoding enzymes of de novo purine nucleotide biosynthesis, is repressed by addition of adenine to the medium. The BAS1 and BAS2 proteins are required for high-level expression of *ADE5,7, ADE8, ADE1* and *ADE2* under derepressing conditions (minimal medium lacking adenine), and it is believed that adenine repression involves down-regulating the ability of BAS1 or BAS2 to stimulate transcription. One way this could occur would be if a repressor protein binds to the *ADE* genes when excess adenine is present in the medium. According to this model, a negative regulatory site would be present in the promoters and its removal would lead to constitutively derepressed expression. To test this possibility, we set out to identify the minimal sequence element from *ADE5,7* sufficient for adenine-regulated transcription. We found that a 74 bp fragment containing two TGACTC elements separated by 33 bp confers BAS1/2-dependent, adenine-repressible transcription on a *CYC1-lacZ* construct that contains a TATA box but lacks the native *CYC1* UAS elements. The gene-proximal TGACTC sequence in this fragment is required for promoter function and purine regulation, whereas the distal element augments expression that is fully dependent upon the proximal site. Interestingly, the proximal TGACTC sequence is more critical than the distal sequence at *ADE2* as well. Unexpectedly, deletion of the region between the two TGACTC sequences decreased expression dramatically from the *ADE5,7-CYC1* hybrid construct.

To map in greater detail the sequence requirements for this regulatory element, we are making clustered substitutions in consecutive 3-bp intervals extending across a 67 bp fragment that contains only the proximal TGACTC sequence and ca. 30 5'- and 3'-flanking nucleotides. From the results obtained thus far, we find that substitutions in the TGACTC core element and in the 6 nt immediately 3' to this sequence (GTGTCC) eliminate both expression and adenine regulation of the hybrid promoter, presumably due to inactivation of the BAS1 binding site. Changes in the sequence TAATAA located 10 nt 3' to the TGACTC site also greatly reduce expression and adenine regulation. These latter

nucleotides might also be required for BAS1 binding in vivo; alternatively, they may comprise a BAS2 binding site. Consistent with a requirement for sequences located between the two TGACTC elements, substitutions in the sequence CCGTCGGTAGTGACA located 24 nt 5' of the proximal TGACTC sequence either reduce or eliminate both expression and adenine regulation. Inspection of this sequence and consideration of the relative effects of mutations at different positions in the sequence strongly suggests that it constitutes a binding site for ABF1 protein. None of the ca. 50 clustered substitution mutants we analyzed thus far have the derepressed phenotype expected from inactivation of a negative control site where a repressor protein would bind. Therefore, our results are most consistent with the idea that adenine repression of transcription involves antagonizing the function of the positive factors BAS1, BAS2 or ABF1, through a protein modification like phosphorylation or by a protein-protein interaction with a repressor protein. BAS1 is the most likely target for regulation because both ABF1 and BAS2 interact with promoters that are presumably unresponsive to purine levels.

B. Genetic selections for trans-acting mutations that abolish adenine-mediated repression of transcription.

We wish to to identify additional factors that might be involved in regulating BAS1 or BAS2 by adenine and to delineate the regulatory domains in BAS1 or BAS2. Towards these ends, we have constructed yeast strains in which defects in adenine repression lead to an easily detectable growth phenotype. These strains contain a fusion of the *ADE5,7* promoter to the *HIS3* coding region replacing the wild-type *HIS3* gene. These strains are His^+ on minimal medium but His^- when grown on of adenine, due to repression of the *ADE5,7-HIS3* construct. By selecting for His^+ revertants on adenine-containing medium, we hope to isolate mutations that abolish adenine repression of *ADE5,7*. These strains also contain an integrated *ADE5,7-lacZ* construct to identify mutations that derepress *ADE5,7* in *trans*, an *ade2* allele to eliminate adenine uptake mutants that are derepressed because of low intracellular adenine pools, and a *gcn4*Δ allele to avoid isolation of *gcd* mutations. We have begun using these strains to select three kinds of regulatory mutations.

First, we mutagenized a plasmid-borne copy of *BAS1* by hydroxylamine and selected two dominant alleles that allow growth of the test strain on +Ade -His medium. The products of these *BAS1* alleles may be insensitive to adenine repression or may have increased affinity for DNA or other proteins with which BAS1 interacts to activate transcription. It is unlikely that they simply increase BAS1 protein levels because wild-type *BAS1* on a high copy plasmid does not confer a His^+ phenotype in the test strain. These two alleles have a relatively weak phenotype; therefore, we are screening a second pool of *BAS1* plasmids mutagenized with an *E. coli* mutator strain to obtain alleles with a stronger His^+ phenotype. In addition, we will sequence the two existing mutations and construct a double mutation. We will also screen a pool of *BAS2* plasmids mutagenized with the mutator strain for the same phenotype.

In a second approach, we screened high copy plasmid yeast genomic libraries for dosage suppressors. Among the His^+ transformants analyzed thus far, the only suppressor plasmid we obtained (that does not contain *HIS3*) encodes *BAS2*. As just mentioned, high copy-number *BAS1* does not suppress the His^- phenotype of the tester strain (although we have not confirmed that BAS1 is overexpressed from this plasmid). These findings could indicate that BAS2 is rate-limiting for activation of *ADE5,7* transcription, in accordance with the fact that BAS2 is involved in regulating other genes besides *ADE* genes. It also suggests a model in which BAS1 must recruit BAS2 to an *ADE* promoter to activate transcription, and this function of BAS1 is diminished by excess adenine. We are now determining whether overexpression of BAS2 leads to constitutively derepressed *ADE5,7-lacZ* expression, as

predicted by the model just described, or whether *ADE5,7* expression is elevated under derepressing conditions but remains adenine-repressible even when BAS2 is overexpressed. The latter would indicate that recruitment of BAS2 from other promoters is not the adenine-repressible parameter.

In a third approach, we have isolated chromosomal mutations that suppress the His^- phenotype of the test strain on +Ade -His medium. Thus far, we obtained 10 His^+ revertants in which expression of the *ADE5,7-lacZ* fusion is derepressed 2-3 fold on medium containing adenine, indicating the presence of a trans-acting mutation that impairs adenine repression. We are investigating whether this phenotype is conferred by a single mutation in each revertant and determining the number of complementation groups involved. Once this analysis is completed, we will clone the wild-type alleles of the suppressor genes by complementing the His^+ phenotype of the mutants. It will also be worthwhile to carry out insertional mutagenesis with the Snyder library using the tester strains described above and selecting for His^+ mutants on +Ade -His medium. If the key regulatory factors are nonessential, this approach should allow rapid identification and isolation of the genes involved.

Significance to Biomedical Research and the Program of the Institute:

Yeast is a eukaryotic organism that carries out many of the fundamental processes of eukaryotic cells and offers a level of molecular genetic analysis that cannot be achieved with multicellular organisms. General amino acid control and the regulation of purine biosynthetic genes involve a variey of strategies for regulating gene expression. Investigating the molecular details of these two systems is providing useful paradigms for coordinate transcriptional activation of unlinked genes, transcriptional repression, gene-specific translational control, functional modifications of protein complexes involved in general translation initiation, and the involvement of protein kinases and protein phosphatases in signal-transduction pathways. The results of these inquiries can be expected to provide valuable insights into how higher eukaryotic cells control the availability of amino acid and nucleotide substrates for protein and nucleic acid synthesis in response to changes in the environment. In fact, *GCN4* translational control appears to be a gene-specific instance of a highly conserved mechanism that regulates protein synthesis in mammalian cells under conditions of stress such as amino acid starvation. The regulatory mechanisms under study in yeast are critical elements of the network of interrelated controls that couple growth rate and division to environmental stimuli in all eukaryotic cells.

Proposed Course of the Project.

A. Investigation of the role of poly-A binding protein (PAB) in reinitiation on *GCN4* mRNA.
Previous studies have suggested that inactivation of PAB impairs general translation initiation and that this defect can be partially overcome by a variety of mutations that reduce 60S subunit biogenesis. This has led to the idea that PAB is involved in some aspect of 40S-60S subunit joining; however, its precise role in translation initiation is unknown. We propose to isolate mutations in PAB that perturb *GCN4* translational control as a means of increasing our understanding of PAB function in translation. There are three principal ways that perturbing PAB function could affect *GCN4* expression. First, it could decrease the ability of ribosomes to remain attached to the mRNA, or to resume scanning and re-form an initiation complex, following termination at uORF1. This would produce a Gcn^- phenotype. Second, it could decrease the dissociation of ribosomes from the mRNA following termination at uORF4, causing a Gcd^- phenotype. A third possibility is that PAB could be required for efficient subunit joining during reinitiation. If PAB must rebind to ribosomes as they scan downstream from uORF1, as postulated for the eIF-2·GTP·Met-$tRNA_i^{Met}$ ternary complex, then reduced-function mutations in PAB should have a greater effect on reinitiation at uORF4 than at *GCN4* and produce a Gcd^- phenotype. They would lead to increased *GCN4* expression only when uORF1 and uORF4 are

both present in the leader, whereas a Gcd$^-$ phenotype arising from reduced ribosome dissociation following uORF4 translation (the second mechanism above) would be seen with uORF4 alone.

To isolate mutations in PAB affecting *GCN4* translation, we will mutagenize the cloned gene on a plasmid, subclone various segments into a wild-type copy of *PAB* and analyze the resulting plasmids in a *pabl*Δ strain as the only copy of *PAB* by plasmid shuffling. Mutant plasmids will be screened for altered sensitivity to 3-AT or 5-FT to identify Gcn$^-$ or Gcd$^-$ phenotypes, respectively. It will be particularly interesting if mutations having different effects on *GCN4* are found to map in different domains of PAB. This mutational analysis should also be helpful in identifying portions of the protein that are specifically involved in translation initiation as opposed to other potential functions of PAB in poly(A) tail metabolism and mRNA turnover. In addition, mutations with a 3-AT^s phenotype could be used in suppressor analysis to identify proteins that interact with PAB in the translation initiation pathway.

B. Molecular genetic analysis of eIF-2B.

1. In vitro analysis of protein-protein interactions in the GCD2/GCD7/GCN3 subcomplex.

From immunoprecipitation experiments, we obtained biochemical evidence that GCD2, GCD7 and GCN3 can form a stable subcomplex in vivo. To test our prediction that the GCD2/GCD7/GCN3 subcomplex can interact with eIF-2, we intend to purify the subcomplex from yeast, or reconstitute it in vitro from proteins overexpressed in *E. coli,* and probe for interactions with eIF-2 purified from yeast. With the higher protein concentrations that can be achieved with purified preparations, we hope to detect complexes that are not stable enough to be detected by immunoprecipitations from cell extracts. We have constructed plasmids for overexpression of GCD2, GCD7 and GCN3 in *E. coli* and established conditions for purification of these proteins from bacteria. We can now purify 1-2 mg of glutathione-S-transferase (GST)-tagged versions of GCD7 and GCN3, from which the GST moiety can be cleaved in vitro by thrombin, and a His-tagged version of GCD2. We will attempt to form a trimeric complex with purified GCD2, GCN3 and GST-GCD7 fusion proteins and isolate the complex on a glutathione column. If we find that GCD2 must be present in the reactions in order to retain GCN3 on a glutathione column with GST-GCD7, this will be a good indication of specific complex formation between all three proteins. One important application for this assay will be to identify residues in GCD2 that are required for its interaction with GCD7 and GCN3. Initially, we will analyze the effects of deleting different segments of GCD2 on its ability to be specifically retained by the GST-GCD7 protein on a glutathione column. It should not be necessary to purify the various mutant GCD2 proteins from the bacterial extracts since their binding to the column can be assayed by immunoblot analysis. We should be able to study interactions between GCD2 and GCD7 by this approach even if the trimeric complex with GCN3 cannot be formed, since we have in vivo evidence for a GCD2-GCD7 complex. Using the His-tagged version of GCD2, we can also identify residues in GCD7 required for its interaction with GCD2, and it will be interesting to learn whether regions of homology between the two proteins mediate their physical interactions with one another.

If we succeed in reconstituting a GCD2-GCD7-GCN3 subcomplex, we will attempt to demonstrate binding of purified eIF-2 to this complex and analyze the effects of phosphorylating eIF-2 on the interaction. Wild-type eIF-2 will be purified according to our previously published procedures or as described below, and phosphorylated in vitro using recombinant PKR as already described. The eIF-2 will be applied to a nickel or glutathione column containing bound GCD2-GCD7-GCN3 trimeric complex and its retention on the column will be analyzed or by immunoblotting using antibodies against subunits of eIF-2. The K_D for the mammalian eIF-2/eIF-2B complex has been estimated between 0.1-1.0 nM (S. Kimball, personal communication). Thus, even if the K_D for interaction between eIF-2 and the GCD2-GCD7-GCN3 trimeric subcomplex is only 1 μM, we should be able to detect it using this technique if we bind ca. 25 μg of the reconstituted trimeric complex to ca. 50 μL of resin. We will establish the specificity of an interaction between eIF-2 and the eIF-2B

subcomplex by showing that it depends on both GCD2 and GCD7 and that the affinity is higher when GCN3 is also present in the complex. We anticipate that phosphorylation of eIF-2 will increase its affinity for the trimeric complex and that introduction of one or more Gcn⁻ mutations into the GCD2, GCD7 or GCN3 proteins will either diminish the affinity for phosphorylated eIF-2 or increase the affinity for nonphosphorylated eIF-2. An increase in affinity will be detected by retention of the same fraction of applied eIF-2 in the presence of less trimeric complex bound to the column.

2. Studies on the regulation of mammalian eIF-2B by phosphorylated eIF-2.
There is a high degree of sequence similarity between GCD2, GCD7 and GCN3 and the corresponding subunits of mammalian eIF-2B. Interestingly, a number of the Gcn⁻ alleles we isolated in these subunits alter residues that are conserved between the yeast and mammalian proteins. We wish to determine whether these amino acids are involved in regulating mammalian eIF-2B by eIF-2(αP) in the same way that they function in yeast. To test this idea, we used site-directed mutagenesis to introduce the amino acids present in five Gcn⁻ alleles of *GCD2* at the corresponding positions of the rat eIF-2Bδ cDNA. These mutant cDNAs are being inserted into different vectors designed for expression in mammalian cells. We will examine the effects of expressing these mutant subunits of eIF-2B on the response to eIF-2 phosphorylation in mammalian cells by the following two assays.

It has been established that certain expression vectors activate PKR in COS cells and this leads to greatly reduced expression of the DHFR gene contained on the same plasmid. This inhibition has been attributed to localized activation of PKR by double-stranded RNAs formed between DHFR mRNA and antisense RNAs made from the same plasmid. Overexpression of the non-phosphorylatable eIF-2α-S51A protein from a different plasmid can rescue expression of DHFR from the PKR-sensitive vector. The overexpressed of eIF-2α-S51A protein replaces wild-type eIF-2α in the trimeric eIF-2 complex and thereby reduces the amount of eIF-2 in the cell containing the phosphoryated form of the α subunit. We will determine whether overexpression of the δ subunit of rat eIF-2B bearing one or more Gcn⁻ mutations has the same effect on DHFR expression as that described for eIF-2α(S51A). Each of the constructs containing wild-type or mutant rat eIF-2Bδ cDNAs inserted in the vector pMTVA- will be co-transfected into COS-1 cells with plasmid pD61 encoding the DHFR reporter gene and expression of DHFR will be analyzed as described. As controls, the DHFR reporter plasmid will be co-transfected with pMTVA- derivatives encoding wild-type eIF-2α or eIF-2α-S51A, provided by R. Kaufman. If we find that expression of one or more mutant forms of eIF-2Bδ leads to increased DHFR expression relative to that given by the wild-type δ subunit, this will imply that the mutation renders mammalian eIF-2B less sensitive to the inhibitory effects of eIF-2(αP), just as occurs in in yeast. These experiments should also reveal the extent to which eIF-2B mediates the regulatory effects of eIF-2(αP)in mammalian cells.

In collaboration with Scot Kimball's lab the same mutant and wild-type rat eIF-2Bδ cDNAs will be expressed from the pBK-RSV vector in rat cells and analyzed for their effects on eIF-2B nucleotide exchange activity in cell extracts. They have established procedures for increasing eIF-2α phosphorylation by treating rat GH3 pituitary tumor cells with Brefeldin A and shown that eIF-2B in the whole cell extracts exhibits reduced nucleotide exchange activity for eIF-2·[^{3}H]GDP binary complexes added as substrate. We will determine whether overexpression of any of the mutant forms of eIF-2Bδ in the rat cells can rescue eIF-2B exchange activity. It is hoped that the various Gcn⁻ mutations will have similar relative effects on eIF-2B activity in COS-1 cells, as measured indirectly by rescue of DHFR expression, and in the rat cells as measured directly by assaying eIF-2B exchange activity.

C. Studies on GCD10 and the eIF-3 complex in GCN4 translational control and general translation initiation.

1. Domain structure of GCD10 and identification of its RNA binding site.

To begin our analysis of the role of RNA binding by GCD10 in eIF-3 function we will identify the minimal segment of GCD10 required for RNA binding in vitro and evaluate its importance for GCD10 function in vivo. We will construct nested deletions progressing from the N- and C-termini of GCD10 in a His-tagged allele tailored for expression in *E. coli* and for in vitro transcription/translation in rabbit reticulocytes. The mutant proteins will be tested for RNA binding by Northwestern and by binding to RNA immobilized on Separose. After identifying the minimal fragment of GCD10 required for RNA binding in vitro, we will analyze the in vivo effects of deletions in this domain. Assuming that the RRM-like sequence will be required for RNA binding in vitro, we will also examine effects of point mutations in the conserved RNP-1 and RNP-2 elements on RNA-binding in vitro and GCD10 function in vivo. For all these experiments, a myc epitope-tagged *GCD10* allele will be mutagenized and introduced into yeast in place of wild-type *GCD10* by plasmid shuffling. We will determine whether the *gcd10* mutations reduce growth rate or lead to derepression of *GCN4* (Gcd$^-$ phenotype). If the mutations are lethal, they will be tested for a dominant slow-growth or Gcd$^-$ phenotype. If a dominant-negative phenotype is observed, we will determine whether the mutant protein competes with wild-type GCD10 for incorporation into eIF-3 by immunoprecipitating with antibodies against PRT1 and analyzing the immune complexes with anti-myc antibodies. If so, mutagenesis of the dominant-negative allele will be carried out to isolate mutations that abolish this phenotype without reducing the level of the mutant protein. These mutations should identify residues in GCD10 required for stable complex formation with the other eIF-3 subunits. This interpretation will be tested by coimmunoprecipitation experiments. We will also rescue the existing *gcd10* chromosomal mutations by plasmid gap-repair and sequence the rescued alleles. These mutations may identify a functional domain of GCD10 that is distinct from those involved in RNA binding and eIF-3 subunit interactions but which is critical for repression of *GCN4* translation; eg. a domain that interacts with eIF-2. If so, we will begin in vitro mutagenesis of *GCD10* to isolate additional mutations affecting this domain by mutagenizing a high-copy plasmid bearing *GCD10* and selecting for growth on 5-FT plates (Gcd$^-$ phenotype) in a strain containing wild-type chromosomal *GCD10*. By selecting for dominant Gcd$^-$ mutations, we should isolate mutations that alter GCD10 function rather than simply reducing its expression or ability to interact with other components of eIF-3.

2. Genetic identification of proteins that interact with GCD10.

We are in the process of localizing and sequencing the remaining high copy suppressor of *gcd10* mutations to determine if it encodes an additional subunit of eIF-3. We also will begin to screen high copy plasmid genomic libraries for suppressors of a *prt1* temperature-sensitive mutation, and a P_{GAL1}-cDNA expression library for suppressors of both *gcd10* and *prt1* mutations. This suppressor analysis may identify proteins that specifically interact with these subunits of eIF-3 in addition to isolating the remaining subunits of the eIF-3 complex. If we do not obtain any new suppressor genes in these approaches, we will turn to the isolation of synthetic lethal mutations that render *gcd10* or *prt1* mutants inviable at a permissive or semi-permissive temperature.

John Hershey's lab at U.C.-Davis is obtaining peptide sequences from the 135, 33, 29 and 21 kDa subunits of yeast eIF-3 and has agreed to share this information with us. By this approach, they have already identified the 39 and 135 kDa subunits as sequenced ORFs of previously unknown function, encoded on Chromosome XIII, whereas none of our suppressors maps to XIII. Presumably, the structural genes for the remaining subunits of eIF-3 will be cloned either as one of our high copy suppressors or from the peptide sequences obtained in Hershey's lab. It is important to verify that all of the proteins that co-purify with eIF-3 in Hershey's purification scheme can be co-immunoprecipitated with the GCD10 and PRT1 proteins. Thus, we will construct epitope tagged alleles of the 39 and 135kDa subunits (and any others that are identified in the yeast database

from the peptide sequences) and replace the wild-type chromosomal alleles with the tagged versions. These strains will be used to determine whether the epitope-tagged proteins can be quantitatively immunoprecipitated with GCD10 and PRT1. These cloned genes can be used in the future to study interactions between GCD10 and other subunits of eIF-3 with the same techniques described above for studying GCD7-GCD2 interactions.

3. Analysis of *GCD13* and *GCD14*.

To learn more about the function of GCD14, we will test it for RNA binding activity using the assays described above for GCD10. We will also analyze existing temperature-sensitive *gcd14* mutations for polysome profiles indicative of general defects in translation initiation. We will construct an epitope-tagged functional allele of *GCD14*, or raise antibodies against a trpE fusion protein expressed in bacteria. Using antibodies against GCD14, we will investigate whether the protein is associated with polysomes, 40S or 60S subunits fractionated by velocity sedimentation on sucrose gradients, and also test for interactions with eIF-2·eIF-2B or eIF-3 by co-imunoprecipitation experiments. The isolation of dosage suppressors of *gcd14* mutations will also be initiated. We will screen the P_{GAL1}-cDNA expression library for a *GCD13* cDNA clone.

D. Regulation of protein kinase GCN2

1. Analysis of the N-terminal regulatory domain and potential autophosphorylation sites.

Ilse Barthelmess' group isolated a gene from *N. crassa* (*cpc-3*) encoding a GCN2 homologue (NcGCN2), with 40% and 32% sequence identity to the protein kinase and histidyl-tRNA synthetase-related domains of GCN2, respectively. Deletion of *cpc-3* impairs derepression of the *GCN4* homologue in Neurospora, called *cpc-1*. In collaboration with their lab, we are completing the sequence of an ca. 500-residue N-terminal domain of NcGCN2 and find that it shows significant similarity to the corresponding region in GCN2 (25% identity). In both proteins, this region contains a degenerate kinase catalytic domain of ca. 300 residues. In GCN2, there are consensus copies of kinase subdomains VII-XII in the correct order, whereas the NcGCN2 pseudo kinase domain (ΦPK) contains subdomains II, and VIII-XI. N-terminal to the ΦPK, both GCN2 and NcGCN2 contain an ca. 80-amino acid segment rich in positively and negatively charged residues. It seems likely that this charged segment is located on the surface of the protein and, could perhaps be a binding site for an effector protein.

Two instances of ΦPK domains have been reported in mammalian proteins. The JAK family of tyrosine protein kinases (that interact with cytokine receptors) contain a ΦPK domain of unknown function in addition to a conventional PK domain, whereas atrial natriuretic peptide (ANP) receptor contains a ΦPK domain N-terminal to a guanylate cyclase catalytic domain. For the latter, the ΦPK segment appears to be a negative regulatory domain that mediates stimulation of the cyclase activity by ATP and also provides a binding domain for a novel protein phosphatase that may be involved in desensitizing the the ANP receptor by dephosphorylation. By analogy with the ANP receptor, the ΦPK domain of GCN2 may play an important role in regulating kinase activity in response to amino acid or purine limitation, or under unknown conditions of starvation or stress, by binding another kinase or phophatase that modifies the authentic kinase domain, as there is evidence that GCN2 is phosphorylated by another kinase in vivo. The ΦPK domain contains all the conserved residues in the C-terminal lobe located near the peptide substrate binding groove in PKA therefore, it might compete with the authentic kinase domain for binding eIF-2α. Alternatively, it might physically interact with the authentic kinase domain, or mediate dimer formation between two molecules of GCN2.

We will take several genetic approaches to address the importance of the ΦPK domain. In one approach, we will use site-directed mutagenesis to substitute conserved amino acids in ΦPK subdomains VII-XII with alanines. In addition, we will construct chimeras that replace the yeast ΦPK domain with that from the Neurospora homologue. If this chimera is functional, it will imply that the exact set of invariant residues left intact is not critically important for ΦPK function. We will also generate small in-frame deletions throughout

the region (using two-codon SacI site insertions made previously, and mutagenize the ΦPK domain at random sites in the *E. coli* mutator strain XL1-Red (Stratagene), and screen the mutant plasmids for reduced GCN2 function (Gcn$^-$ phenotype) or constitutive activation of GCN2 (Gcd$^-$ phenotype) using amino acid analogues as indicators of *GCN4* expression. This random mutagenesis should identify residues in the ΦPK that are not conserved among protein kinases, but which are involved in the regulatory function of the domain, either as binding sites for other regulatory proteins or as points of contact between the pseudo and authentic kinase domains or between protomers in a GCN2 homodimer. We will also make deletions in the 80-residue N-terminal highly charged segment and, depending on their phenotypes, introduce clustered alanine substitutions throughout this segment of GCN2. In addition, we will replace it with the corresponding segment from Neurospora NcGCN2 which, although highly charged, shows little primary sequence similarity to the yeast segment.

Another very interesting target for mutagenesis that could be involved in the function of the ΦPK domain is a segment between subdomains VII and VIII of the authentic PK domain of GCN2. The activity of several protein kinases, including PKA, MAPK, MAPKK and CDK requires phosphorylation or autophosphorylation of one or two closely spaced Ser or Thr residues in this segment . In the case of CDK, this phosphorylation is thought to alter the conformation of a loop that protrudes into the active site and prevents proper positioning of the protein substrate. It can also promote proper interactions between the N-terminal and C-terminal lobes of the kinase domain, as in the case of PKA and MAPK. GCN2 contains a larger than usual segment separating subdomains VII and VIII, containing a total of 7 serine and threonine residues. One or more of these residues could be critical sites of autophosphorylation or phosphorylation by another kinase, or sites of dephosphorylation by a protein phosphatase. We have mutated one of these residues, Thr-813, to Gly and observed a leaky Gcn$^-$ phenotype. We will mutate additional Thr and Ser residues in this segment to Ala, singly and in combination with an Ala substitution at Thr-813, and analyze the effects on *GCN4* expression in vivo. If one or more of these residues is found to be critical for GCN2 function in vivo, we will determine whether function can be restored by substitutions with Asp or Glu, which may functionally substitute for phosphoserine or phosphothreonine. In addition, we will determine whether the mutations reduce autophosphorylation of GCN2 in our standard immune complex assays, or its level of phosphorylation in vivo by labeling cells with radioactive phosphate, immunoprecipitating GCN2 from whole cell extracts, and analyzing the labeled protein by SDS-PAGE. If there are multiple phosphorylation sites, it will be necessary to analyze the tryptic phosphopeptides produced in vitro and in vivo by mutant and wild-type proteins. If the critical site is for autophosphorylation, it will be necessary to show that substitution of the site with Ser (if the wild-type residue is Thr) or Thr (if the wild-type residue is Ser) leads to the production of a novel phosphopeptide. If autophosphorylation occurs in trans following dimerization, we may also suceed in showing that wild-type GCN2 can phosphorylate the critical residue using a K559V defective kinase as substrate.

2. Analysis of GCN1/GCN20 interactions.

The importance of the EF-3-related domain of GCN1 for protein-protein interactions with GCN20 suggests that the positive regulatory function of GCN1/GCN20 is related to the role of EF-3 in translation elongation. To pursue this possibility further, we would like to determine whether residues in GCN1 that are conserved with EF-3 are of critical importance for *GCN1* function. We also wish to identify specific amino acids that are required for protein-protein interactions with GCN20, as these may illuminate the role of GCN20 in the putative EF-3-like function of GCN1. GCN1 encodes a very large protein of 2,672 amino acids, and in addition to mutational analysis of the EF-3-related domain (residues 1329-2140), we would like to determine whether other segments of the protein are important for its regulatory function. We will begin a mutational analysis of GCN1 by testing internal deletions of ca. 300-amino acid segments, constructed using naturally occurring and engineered restriction sites for: (1) loss of *GCN1* function, revealed by sensitivity to 3-AT; and (2) constitutively activated *GCN1* function, leading to 5-FT resistance. This approach may allow us to eliminate relatively large

segments of GCN1 that may not be required for its regulatory function. In a second approach, we have used the *E. coli* mutator strain XL-1 Red (Stratagene) to carry out random mutagenesis of *GCN1*, and we are subcloning segments of 1-2 kb from this plasmid pool into an unmutagenized *GCN1* plasmid pool. Screening of these pools will be restricted to those segments found to be important for GCN1 function by the internal deletion analysis, beginning with the segment corresponding to the EF-3-related region of GCN1. Mutant alleles will be subjected to Western analysis and those which produce full-length GCN1 at wild-type levels will be sequenced to identify the mutation. The mutant proteins will be tested for protein-protein interactions with GCN20 using the co-immunoprecipitation assays described above. Any *gcn1* mutations that reduce co-immunoprecipitation of the two proteins will be examined for the ability to be suppressed by overexpression of GCN20 from a high copy plasmid. If an in vitro assay for GCN2 kinase activity showing GCN1-dependence is available at this time, the *GCN1* mutations will be analyzed for effects on GCN2 kinase function in vitro. Any *GCN1*^{c} alleles we obtain will be tested to determine whether they are dependent on *GCN20* for their derepressed phenotype.

3. Genetic screening for novel proteins that interact with GCN2.

At present, we have not detected a physical interaction between the GCN1/GCN20 complex and GCN2 and little is known about how these proteins regulate GCN2 kinase activity by uncharged tRNA. The location of GCN2 on the ribosome and possible roles of specific ribosomal proteins in this regulation are also unknown, as is the mechanism for coupling GCN2 kinase activity to purine levels. It is unlikely that the *gcn* class of mutations has been saturated, and there may be additional proteins besides GCN1 and GCN20 involved in controlling GCN2 activity. For these reasons, we will pursue several genetic approaches aimed at the identification of proteins that interact with GCN2. In one approach, we will use yeast two-hybrid constructs containing different segments of GCN2 to screen the Elledge cDNA library and the Clontech genomic library. We will begin with a bait construct containing the ΦPK domain of GCN2 and proceed as necessary to one containing the HisRS-like domain, following the same plan outlined above for analyzing two-hybrid interactions with GCN1 bait plamids.

In a second approach, we will attempt to saturate the *gcn* class of mutations by random insertional mutagenesis of the yeast genome using the insertion library constructed in Mike Snyder's lab. This is a plasmid library of genomic fragments containing Tn3::*lacZ*::*LEU2* transposons inserted at random sites. The mutagenized yeast fragments are removed from the plasmid vector by digesting with NotI and then used to make chromosomal insertions by transforming a yeast strain to Leu^{+}. Using this library, we will mutagenize a haploid strain that is wild-type for all known *GCN* and *GCD* genes (except for *GCN4*) and contains an integrated *HIS3-gus* fusion under the control of GCN4 and an integrated *GCN4-lacZ* fusion. The strain will carry one of our mutant alleles of *GCN4* which contains only the N-terminal activation domain. This *GCN4* allele confers high-level activation of GCN4-regulated genes under our standard starvation conditions but is defective relative to wild-type *GCN4* under more severe starvation conditions. By incorporating this attenuated allele in place of wild-type *GCN4,* we hope to increase the sensitivity of our screen for *gcn* mutations. (In addition, we may identify mutations in nonessential transcriptional co-activator proteins, such as *GCN5*, that impair transcriptional activation mediated by the N-terminal activation domain; see below). Transformants containing insertions in different loci will be screened for increased sensitivity to 3-AT, sulfometuron (inhibits Ile and Val synthesis), and azaadenine (azA) (inhibits purine biosynthesis), indicating a Gcn^{-} phenotype. Transformants showing sensitivity to 3-AT and SM or to azA will be subjected to quantitative assays of the two integrated fusions after growth in the presence of 3-AT or aZA and ranked according to the magnitude of their derepression defects. (Note that 1/6 of the chromosomal insertions will fuse *lacZ* in-frame to the disrupted gene. For this class of insertion mutants, we will conduct Western analysis of *GCN4* expression using antibodies against GCN4.) Any mutants showing reduced *GCN4* expression under starvation coditions will be analyzed genetically to determine

whether this phenotype results from a single insertion. Mutants showing reduced *HIS3-gus* expression but normal *GCN4* expression will be set aside for studies on GCN4 transcriptional co-activators (see below). For all others, we will isolate genomic sequences adjacent to the insertion site using the plasmid integration-excision scheme of Burns et al. and determine the sequence of 100-200 nt. If the sequence does not correspond to a known *GCN* gene or match an entry in the yeast database, we will determine the map location by hybridization to the Riles-Olson lambda library and clone the wild-type gene from the appropriate lambda or cosmid clone purchased from ATCC. The gene will be localized by complementation testing, completely sequenced, and a chromosomal deletion will be constructed to determine the null phenotype with respect to *GCN4* expression and eIF-2α phosphorylation.

E. Molecular mechanism of transcriptional activation by GCN4

1. Biochemical analysis of potential interactions between GCN4 and transcriptional mediators.

Our mutational analysis of GCN4 has identified two functionally distinguishable activation domains that each contain multiple clusters of 2-3 aromatic or bulky hydrophobic amino acids that are critically required for the function of that domain. Five such clusters have been identified in the NTAD and 2 are located in the CAAD. Some or all of these residues could mediate important interactions between GCN4 and its target proteins in the transcriptional machinery. Genetic analysis has implicated the ADA2, ADA3 and GCN5 proteins as mediators of transcriptional activation by GCN4 and several other acidic activators; however, at present, there is no evidence that GCN4 physically interacts with these proteins or with any of the general transcription factors. We plan to use the point mutations we generated in the activation domains of GCN4 to identify target proteins in the transcription machinery and to identify the domains in these proteins that contact GCN4.

We are testing GCN4-GST fusion proteins for interactions with candidate proteins synthesized by in vitro transcription/translation, focusing initially on the TATA-binding protein TBP. A panel of GCN4-GST fusion proteins are being overexpressed in *E. coli*, including full-length GCN4, deletion derivatives lacking the NTAD, CAAD, or both, and several full-length constructs containing mulitple point mutations in both the NTAD and CAAD. Bacterial extracts containing these proteins are mixed with aliquots of in vitro translation extracts containing ^{35}S-methionine-labeled yeast TBP, after which we determine the amount of radiolabeled TBP that is retained with the GST-GCN4 proteins on glutathione-agarose beads (GST pull-down assay) . We have also carried out reciprocal reactions in which ^{35}S-methionine-labeled GCN4 proteins synthesized in vitro are tested for interactions with a GST-TBP fusion protein. In control experiments, we have reproduced the interaction between VP16 and TBP that depends on the C-terminal activation domain of VP16. In addition, we observed GCN4-GCN4 homodimer formation. Although we detected an interaction between TBP and GCN4, it appeared to be relatively weak compared to the TBP-VP16 interaction. In addition, the DNA binding-dimerization domain at the C-terminus of GCN4 seemed to be both necessary and sufficient for the in vitro interaction with TBP. Consequently, we will not pursue this interaction for the time being. We also used the GST pull-down assay to explore interactions between yeast TFIIB and GCN4, but thus far have not observed any interaction between them. In future experiments we will to examine possible interactions between GCN4 and the individual ADA2, ADA3 and GCN5 proteins synthesized by in vitro transcription/translation, as described above.

It is possible that GCN4 can interact with the ADA2, ADA3 and GCN5 proteins only when the latter are present in a complex. To explore this possibility, we will add GST-tagged mutant and wild-type GCN4 proteins expressed in *E. coli* to whole cell yeast extracts prepared from strains expressing either untagged ADA2 or a functional HA-tagged form of ADA2 (provided by L. Guarente). We will then determine what fraction of the GCN4 proteins can be co-immunoprecipitated with HA-ADA2 and what fraction of HA-ADA2 can be co-purifed with GCN4 on a glutathione-agarose column. Similar experiments have been used to demonstrate that GAL4-VP16 and native GAL4 can interact with ADA2 or with the mediator complex.

We will also examine whether components of the holoenzyme mediator complex, purified from yeast as described by Kim et al, can be recovered with GCN4-GST proteins on glutathionine agarose. This last experiment will be done in collaboration with Christoph Hengartner in Rick Young's who has provided us with partially purified holoenzyme and antibodies against several of the SRB protein components of the mediator complex. In addition, Tony Weil has supplied us with a partially purified preparation of a TFIID-like complex from yeast, consisting of TBP and associated factors (A. Weil, personal communication). We will probe for interactions between these proteins and GST-GCN4 proteins using glutathione agarose chromatography and antibodies against some of the TFIID protein components provided by Weil's group. If we detect an interaction between GCN4 and the mediator or TFIID complex that is abolished by one or more mutations in the GCN4 activation domain, we will follow up with GST-GCN4 pull-down experiments using individual subunits of the appropriate complex. Young's and Weil's groups are in the process of purifying these proteins after over expressing them in insect cells from bacculovirus vectors, and they have indicated their willingness to collaborate with us in testing the purified proteins for specific binding to GST-GCN4 proteins in vitro.

2. Genetic screens for transcriptional factors that interact with GCN4.

a. Suppressors of a mutant GCN4 protein that is lethal when overexpressed.

We have set up several genetic screens aimed at identifying functionally important interactions between GCN4 and components of the transcriptional machinery. One approach already underway is to isolate chromosomal mutations that overcome the dominant lethal effect of overexpressing a mutant GCN4 protein which lacks the CAAD but retains the NTAD, and is produced at ca. 4-fold higher levels than wild-type GCN4. Because the toxic effect of this construct is dependent on both a functional DNA binding domain and activation determinants in the NTAD, we presume that the mutant GCN4 protein sequesters one or more essential transcription factors. We reasoned that it might be possible to isolate mutations that eliminate a nonessential mediator that is required for sequestering the essential transcription factor by the mutant GCN4 protein, as this approach was used previously to isolate mutations in *ADA2* that overcome the toxicity of an overexpressed GAL4-VP16 protein in yeast. Since we found that the toxicity of overexpressing the mutant GCN4 protein is not suppressed by deletion of *ADA2*, we hope to identify a distinct mediator that interacts with the NTAD of GCN4. It might also be possible to isolate mutations in a general transcription factor that reduces its affinity for GCN4 without eliminating essential interactions with other activators.

We have isolated spontaneous revertants of the lethal phenotype conferred by overexpressing the mutant GCN4 protein from an uORF-less construct carried on a single-copy *URA3* plasmid. After removing the toxic *GCN4* allele from the revertants by 5-FOA counter-selection, we identified strains which exhibited 3-AT-sensitivity, indicating a possible defect in the ability of the resident wild-type GCN4 to activate transcription. Meiotic analysis was used to identify those revertants in which 3-AT sensitivity (in the presence of wild-type *GCN4*) and suppression of the lethal *GCN4* mutant allele were conferred by a single mutation. Several of these mutants also displayed a slow-growth phenotype on rich medium (Slg$^-$) that co-segregated with the other phenotypes. For most of these Slg$^-$ revertants, this phenotype could be partially complemented by introducing a single-copy plasmid bearing *SPT13*, encoding yeast TBP. Accordingly, the endogenous *SPT13* gene was isolated from these revertants by gap repair and was found to be indistinguishable from wild-type *SPT13* in complementing the Slg$^-$ phenotype of our revertants. Hence, none of these revertants appear to contain suppressor mutations in *SPT13*. The fact that an extra copy of *SPT13* partially complemented their Slg$^-$ phenotype does suggest, however, that they contain mutations affecting a general transcription factor. We have verified that the suppressor mutations do not reduce the steady-state level of the GCN4 protein, and we are attempting to clone the wild-type alleles for three of the suppressor mutations with particularly strong Slg$^-$ phenotypes.

In a related approach, we will screen a high copy-number plasmid library for genes that when overexpressed can overcome the lethality of overexpressing the mutant GCN4 protein described above. If this *GCN4* construct is lethal because a co-activator or general factor is being sequestered by the mutant GCN4 protein, then overexpressing the sequestered factor should relieve the toxicity. Even if the sequestered factor is a multimeric complex, this approach could succeed because overexpressing only one subunit of the complex might be enough to sequester the toxic GCN4 protein and prevent it from interacting with the native co-activator complex. A high copy-number *URA3* plasmid library will be introduced into a wild-type *GCN4* yeast strain and a pool of ca. 10^6 transformants will be isolated and transformed with the toxic *GCN4* allele on a single copy-number *LEU2* plasmid. Transformants that arise will be tested for the ability to lose the library plasmid and grow on 5-FOA. Failure to grow on 5-FOA except after loss of the *LEU2* plasmid will indicate the presence of a dosage suppressor of the lethal *GCN4* construct. Transformants of this type will be analyzed to determine whether the suppressor plasmid lowers the expression of the mutant GCN4 protein, and then tested for exacerbation or suppression of the Gcn$^-$ phenotype of a panel of leaky *gcn4* alleles bearing different mutations in the activation domain. Exacerbation of the Gcn$^-$ phenotype could occur if the encoded protein is a component of a heteromeric co-activator complex that can compete with native co-activator for interaction with defective GCN4 proteins. Suppression of the Gcn$^-$ phenotype could occur if the overexpressed protein is a monomeric co-activator whose interaction with GCN4 is impaired by one the *gcn4* mutations being tested. We will also determine the null phenotype of the suppressor gene. If a deletion strain is viable, we will examine it for Gcn$^-$ phenotypes (3-AT-sensitivity and defective derepression of *HIS-lacZ* fusions) and for defects in activation by GAL4 and HAP4 using the appropriate lacZ reporter constructs dependent on these activator proteins. We will epitope-tag the gene with the *myc* epitope and use anti-myc antibodies to probe for co-immunoprecipitation of the suppressor gene product with components of the ADA2/ADA3/GCN5 complex, or with the mediator or TFIID complexes described above. The suppressor protein will also be tested for direct interactions with GCN4 protein in vitro using the GST pull-down assay described above.

b. Suppressors of a leaky *GCN4* allele with point mutations in the activation domains.
In an independent genetic screen, we will isolate chromosomal suppressors of the 3-AT-sensitive phenotype of the *gcn4-1843* allele, which contains alanine substitutions in two of the activation modules in the CAAD (at Met-107, Tyr-110, Leu-113 and Trp120, Leu-123, and Phe-124) and in one activation module of the NTAD (Phe-97 and Phe-98). Because it contains mutations in both the CAAD and NTAD, this allele confers very weak activation in vivo despite an ca. 4-fold higher than wild-type level of protein. Assuming that these mutations weaken interactions between GCN4 and one or more co-activator proteins or basic transcription factors, we anticipate that chromosomal mutations can be isolated in these factors that will restore their interaction with mutant GCN4. By testing these mutations for the ability to suppress other *gcn4* alleles that contain a different set of point mutations, we may identify suppressors that specifically restore interactions with particular activation modules of GCN4. We may also isolate mutations in other components of the transcription machinery which bypass the requirements for those activation modules which are lacking in the *gcn4* allele. It is conceivable that certain transcription factors that are normally recruited or activated by GCN4 could be mutated to mimic the effect on them that wild-type GCN4 normally exerts. Even if they map in known proteins, such bypass mutations would highlight the importance of these proteins in transcriptional activation by GCN4.

We have constructed a pair of isogenic strains containing *gcn4-1843* integrated on a *URA3* plasmid at *ura3-52*, plus a *GCN4-lacZ* fusion at *TRP1* and a *HIS3-GUS* construct at *LEU2*. We are collecting 3-AT-resistant revertants in one of these strains and cross-screening them for resistance to sulfometuron-methyl (SM) and 5-methyltryptophan (5-MT) (that inhibit isoleucine-valine and tryptophan biosynthesis, respectively), and for resistance to cyclohexamide (to screen out multiple drug resistance). Revertants that are resistant to 3-AT, SM

and 5-MT, but sensitive to cyclohexamide, will be assayed for expression of the *HIS3-GUS* and *GCN4-lacZ* reporters. Those showing increased *HIS3-GUS* expression but unchanged *GCN4-lacZ* expression will be analyzed to determine whether they contain a back-mutation at *GCN4* or an unlinked suppressor. This will be done by evicting the *gcn4-1843* allele on the integrating *URA3* plasmid on 5-FOA medium, followed by transformation with a single copy plasmid bearing *gcn4-1843*. Revertants containing trans-acting suppressors will revert to a 3-AT-sensitive phenotype upon eviction of the *gcn4-1843* allele and re-acquire 3-AT-resistance upon reintroduction of *gcn4-1843* on a plasmid. Revertants that retain the 3-AT^r phenotype after eviction of the *gcn4-1843* allele will be set aside for the present, because they contain GCN4-independent suppressors. All revertants that contain unlinked GCN4-dependent suppressors will be crossed to the isogenic strain containing the *gcn4-1843* allele and subjected to meiotic analysis. Revertants found to contain single suppressor mutations responsible for their 3-AT-resistant phenotypes will be tested for allele-specific suppression by transforming the versions of the revertant strains which lack the integrated *gcn4-1843* with plasmid-borne *gcn4* alleles containing different point mutations in the activation domain. Suppressors that fail to suppress, or which even exacerbate, the 3-AT-sensitive phenotype of other leaky *gcn4* alleles will be particularly interesting. These may contain a mutation in a co-activator protein that restores its interaction with an altered GCN4 activation module but that weakens its interaction with the wild-type version of that module. Genetic analysis will be used to assign suppressors to complementation and linkage groups and the wild-type genes will be cloned by complementing either the suppressor phenotype, or a suitable secondary phenotype associated with the suppressor mutations.

The same strain just described containing *gcn4-1843* is also being used to screen high copy plasmid yeast libraries for dosage suppressors. A mediator protein or basic transcription factor that functions as an individual polypeptide might suppress a GCN4 activation defect when overproduced. In addition, if GCN4 is phosphorylated as a means of stimulating its activation function, we might identify a protein kinase, an activator of a kinase, or an inhibitor of a phosphatase as a dosage suppressor of a defective *gcn4* allele. The virtue of this approach is that a plasmid library can be screened rapidly by direct selection for 3-AT-resistant transformants, and plasmid-associated suppressors can be readily distinguished from chromosomal suppressors by determining whether the suppressor phenotype disappears when the plasmid is lost from the strain. The same genetic criteria listed above for chromosomal suppressors will be applied to evaluate high copy-number plasmid suppressors.

F. Regulation of purine nucleotide biosynthesis

Our interpretations of the mutational analysis of the *ADE5,7* promoter will be evaluated by conducting gel retardation assays on various mutant and wild-type DNA fragments using BAS1, BAS2 and ABF1 proteins expressed in *E. coli,* or produced by in vitro transcription/translation, following published procedures. From these experiments we hope to learn whether the requirement for particular nucleotide positions for transcription and adenine repression in vivo can be accounted for by their effects on binding BAS1, BAS2, or ABF1 in vitro. They should also reveal the identify of the bindingsite(s) for BAS2 at *ADE5,7* and indicate whether the binding of one protein is dependent on the other two. We will continue with our analysis of protein fusions to identify the adenine-repressible activity of BAS1 or BAS2. In addition, we will proceed with our genetic selections for trans-acting mutations that abolish adenine-mediated repression of transcription to identify additional factors that might be involved in regulating BAS1 or BAS2 by adenine and to delineate the regulatory domains in BAS1 or BAS2, all as described above in the progress report.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

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

Our research program focuses on the mechanisms of retroelement action. Our approach to understanding the complex interactions between the retroelement and its host is to study retrotransposons, a family of elements that are closely related to retroviruses. A significant advantage to studying retrotransposons is they exist in hosts such as yeast that can readily be studied using sophisticated molecular genetic techniques. In the process of characterizing yeast transposition, we have collected strong evidence that Tf1 reverse transcriptase uses a novel self-priming mechanism to initiate cDNA synthesis. This is in complete contrast to the tRNA mechanisms thought to be used by all other LTR-containing elements. In this report we describe the characterization of the minus-strand strong-stop DNA that provides additional support for the self-priming mechanism. Genetic and biochemical analysis of Tf1 RT mutations in the active site of the polymerase allowed us to observe priming intermediates consisting of transcripts that had the first 11 bases removed. This data suggested a molecular model for priming that includes a cleavage of the first 11 bases of the transcript and the priming of reverse transcription from the 3'OH of the 11th base.

The analysis of a large family of point mutations near the primer binding site (PBS) confirmed the presence of a new 39 base pair RNA structure that is essential for transposition. As this large structure includes the 11 base pairs shown to be important for priming, we speculate that the newly detected structure may also participate in the self-priming mechanism. The assembly of functional Tf1 particles has been a paradox since other retroelement particles assemble with a molar excess of capsid protein that accumulates because the levels of the Pol proteins are restricted by reading frameshifts or stop codons. Tf1 however, expresses all its protein from within a single open reading frame as a primary translation product and we found that Tf1 particles contain a 26-fold excess of Gag compared to IN protein. By looking at cultures in different stages of growth, we have been able to observe an IN degradation process that leads to this excess of Gag. In addition, we found that most of the IN degradation occurred before Tf1 cDNA is synthesized indicating that the particles with a 26-fold excess of Gag are intermediates in transposition. We have used immunoblot analysis to reveal that IN degradation occured in cells starved for glucose and not in those cell starved for nitrogen, suggesting that the loss of IN is not a time-dependent process but occurred only after certain growth conditions are met. To determine the factors required for transposition, we have created a large set of mutant strains that are defective for transposition. Thus far, we have identified six host genes that are required for either protein accumulation, particle stability, or integration.

Project Description

Objectives:

Our efforts are designed to elucidate the molecular basis of each step in the process of retroelement proliferation. The most medically important class of these elements are the retroviruses, members of which are responsible for AIDS as well as several types of cancer caused by the proviral induction of oncogene expression. However, many aspects of particle assembly, reverse transcription, nuclear entry and chromosome integration are difficult to study because retrovirus hosts possess a high level of genetic complexity. Our approach to understanding these events is to study retrotransposons, a family of elements that are closely related to retroviruses. A significant advantage of studying retrotransposons is they exist in yeast, a host that can be studied readily by using sophisticated molecular genetic techniques. The similarities of retrotransposons to retroviruses includes the presence of two long terminal repeats (LTRs) and open reading frames (ORFs) with coding sequences homologous to retroviral protease (PR), reverse transcriptase (RT), and integrase (IN). The first step in the transposition pathway is synthesis of a full-length mRNA with sequence that begins in the 5' LTR and terminates in the 3' LTR. This is directly analogous to the initial step in retrovirus particle formation. Retroviral and retrotransposon mRNAs are translated into proteins that assemble along with the mRNA into large particle structures. Both retroviral and retrotransposon particles undergo a maturation process that includes the proteolytic processing of precursor proteins and the reverse transcription of the mRNA. Retroviral particles are able to escape the host cell and integrate their DNA into the genome of neighboring cells, while retrotransposon particles complete transposition by simply inserting their DNA into the genome of the original host cell. Because each step in retrotransposition is directly related to a retrovirus process, results from the investigation of yeast retrotransposition will be relevant to aspects of retrovirus behavior.

The retrotransposon we study is the Tf1 element of the fission yeast, *Schizosaccharomyces pombe*. The transposon is 5 kb and contains a single ORF of 1340 amino acids with coding sequences homologous to PR, RT, and IN. We have previously demonstrated, using an *in vivo* assay, that at least one of our cloned copies of Tf1 is active and can transpose at a significant frequency. The system we have developed allows us to use a battery of powerful molecular and genetic techniques to identify and characterize factors that contribute to the transposition process. We are initially focused on the interactions between the host cell proteins and the element because it is these that are the least understood in the retrovirus systems. Genetic experiments on retroviruses have defined the principal role of each of the major virus proteins by altering the virus encoded factors and assaying for an effect on virus functions. Studying retroelements in yeast allows us to explore the contributions of the host proteins to the entire process of transposition.

The genetic approach we are using to identify proteins that function in transposition is to use a new form of the transposition assay to screen individual colonies for mutations that alter the frequency. We developed a genetic screen at NIH for transposition that depends on the overexpression of Tf1 transcripts containing a selectable marker that is reverse transcribed during transposition and inserted into the host genome. We experimented with various combinations of growth media and expression systems with the result that we increased our previously reported transposition frequency of 0.5% per 10 cell generations to 18%. This improvement allowed us to develop this new transposition assay that is now being used to screen large numbers of cells for mutations that alter the transposition frequency. Mutations in genes that contribute to transposition will be used to clone the genes required for transposition from an *S. pombe* genomic library.

Another goal associated with the mechanisms of transposition is to learn which parts of the Tf1 proteins are required for mobility and what are their roles in the process. Towards this goal, we have started to assemble a large set of random mutations in Tf1 itself that block transposition. By characterizing the positions and properities of these mutations, we hope to associate each protein with a part of the transposition pathway and perhaps attribute new functions to some of these domains.
Further characterization of Tf1 protein expression is also a goal because of its unique aspects among LTR-containing retroelements. Retroviruses and LTR-containing retrotransposons encode their Gag (capsid) and Pol (PR, RT, and IN) proteins in separate ORFs that are interrupted by a frameshift or a stop codon. This organization causes a molar excess of Gag to Pol protein to be expressed which is important for the assembly of particles that normally contain as much as 20-200 times more Gag than Pol. Tf1 coding sequence for Gag and Pol is contained within a single ORF and we have used three separate experiments to show that all Tf1 protein is expressed within a single primary translation product. Last year we determined that the Tf1 proteins do form virus-like particles similar to those made by the other retrotransposons studied to date. One of our present objectives is to determine the ratio of Gag to Pol proteins within these particles and develop a model for how these ratios are achieved as expressed from a single ORF.

A unique aspect of Tf1 is that its sequence contains no homologies to tRNA molecules at the site where reverse transcription is initiated, the primer binding site (PBS). This is in direct contrast to all other LTR-retroelements which have 15-20 bases of perfect homology to specific tRNA molecules that serve as primers for reverse transcriptase during the first step of cDNA syntheis. Although we have detected the presence of an RNA primer at the Tf1 PBS, it appears not to be a tRNA. Our goal is to determine the identity of the reverse transcriptase primer as well as the mechanism used. Recent results indicate that Tf1 uses a novel selfpriming mechanism of reverse transcription that defines a new family of retroelements.

Methods Employed

We have used a wide variety of molecular, genetic, and biochemical techniques in experiments described in this report. Our genetic approaches include several standard yeast techniques such as transformation, gene replacement, mating, tetrad dissection, diploid formation and mutagenesis. We are also using the two-hybrid system as a general method for identifying any *S. pombe* proteins that interact with Tf1 proteins.

Our standard transposition assays take advantage of two drug marker selections. We use 5-fluoroorotic acid to select for cells that lose plasmid copies of the URA3 gene. Our transposons are marked with the bacterial *neo* gene that provides *S. pombe* with resistance to high concentrations of G418. We have also started to use an artificial *S. pombe* intron placed within marker genes in the transposon to assay for reverse transcription events.

Biochemical methods include the use of various yeast expression systems to improve production of the Tf1 proteins. We now use the *nmt1* promoter of *S. pombe* to regulate high levels of protein synthesis. The Tf1 ORF is fused to the *nmt1* promoter in a multicopy plasmid. The presence of 10 μM vitamin B1 reduces expression 50-fold. *nmt1* is the strongest *pombe* promoter thus far characterized. Extracts from these expression strains are then subjected to sucrose gradient sedimentation as well as other purification protocols. Fractions from these gradients are immunoprecipitated by using antibodies raised against Tf1 proteins.

A full range of molecular biology techniques are utilized to make reagents and characterize them. Constructs are made by using restriction enzymes, phosphatases, DNA polymerases, PCR and ligase. We routinely sequence the DNA of complex constructs. Immunoblots, DNA blots and RNA blots are all used to characterize various strains.

Major Findings:

A. Priming of Tf1 reverse transcription.

1. Evidence that Tf1 uses an unusual mechanism of self-primed reverse transcription.

The role of tRNA in the priming of reverse transcription is thought to be common to all LTR-containing elements since the retroviruses and LTR-retrotransposons that have been sequenced have a PBS that is complementary to known tRNA species (Figure 1A). In light of the conservation of reverse transcription priming, it is surprising that no tRNA primer has been identified for Tf1. In addition to the absence of tRNA homology, Tf1 also lacks the highly conserved UGG in the PBS of retroelements that hybridizes to the last 3 bases of the tRNA molecule. Evidence for the presence of an RNA primer that functions precisely at the conventional PBS location of Tf1 came from previous analyses of DNA isolated from virus-like particles. This DNA was used as template in primer extension analyses and treatment with RNase demonstrated that there are at least 9 bases of RNA at the 5' end of the minus strand strong-stop DNA. The position of this RNA is immediately downstream of the 5' LTR and defines the PBS for Tf1. Instead of postulating that an uncharacterized tRNA serves as the primer, we proposed in our last annual report that Tf1 mRNA undergoes a novel self-priming mechanism of reverse transcription. The first 11 bases of the Tf1 transcript are exactly complementary to the PBS location identified by the primer extension experiments. This observation raised the possibility that the first 11 bases of the transcript folds back and anneals to the PBS. An RNA endonuclease would be able to cut the first 11 bases from the transcript so that the 3' OH required to prime DNA synthesis would become available (Figure 1B). Alternatively, RT might prime DNA synthesis from a 2'OH of an internal nucleootide as has been reported for the bacterial retron reverse transcriptase [Hsu, 1992 #1424; Lampson, 1989 #836].

Experiments decribed in our last annual report showed that the mutations in Figure 1C at the PBS or the 5' end of the transcript reduced transposition more than 30-fold. When two mutations were combined to reestablish complementarity, transposition frequencies were restored to 80% of the wild type level [Levin, 1995 #1575]. Consistent with the hypothesis that the mutations block priming, we fould that the individual mutations resulted in a drop in the levels of minus-strand strong-stop DNA as isolated from Tf1 particles. Strains with the compensating double mutations produced wild type levels of strong-stop DNA.

2. Analysis of minus-strand strong-stop DNA.

In all cases where the strong-stop species was observed on DNA blots, the signal was a doublet composed of two closely migrating bands. In hopes of learning more about the priming mechanism, the doublet nature of the minus-strand strong-stop species was investigated. We used DNA blot analysis of sequence gels in combination with radiolabeled probes consisting of several oligonucleotides to map the 5' and 3' termini of the two strong-stop species. The sequence ladders on these gels indicated that the size of the two strong-stop species differed by about 11 bases (+/-1 base). The results indicated that the larger band is the full-sized strong stop species with a 3' end that corresponded with the first

base of the Tf1 transcript and a 5' end that has the RNA primer with the PBS sequence. The smaller species has the same RNA primer at the 5' end but the 3' end is missing the 11 bases that are templated by the beginning of the Tf1 transcript. The shortened strong-stop molecule is likely due to a premature termination of reverse transcription resulting from either a cleavage of the mRNA or an attachment of a 2'-5' linkage at the 11th base of the Tf1 transcript. Regardless of which of the two events caused the termination of reverse transcription, the presence of the shortened strong-stop product provides support for the self-priming model.

3. *In vivo* experiments to distinguish between two types of self-priming mechanisms Despite the genetic and biochemical evidence that the folded RNA structure is important for priming, several questions about the mechanism are left unanswered. One very significant question is how does the RNA structure allow for priming given that no 3' OH is made available by the formation of the fold-back loop. A likely possibility is that an endonuclease is responsible for cleaving off the 1st 11 bases of the Tf1 transcript so that the 3'OH of the 11th base can serve as the site of priming. Another explanation for the lack of a 3' OH is that the Tf1 reverse transcriptase may actually prime DNA synthesis from a 2' OH forming a branched intermediate similar to that produced by the bacterial retron reverse transcriptases [Hsu, 1992 #1424; Hsu, 1990 #998]. Our observation that minus strand strong-stop DNA synthesis is blocked near the 11th base of the Tf1 transcript supports the idea that either a cleavage event or a 2' OH linkage occurs at the 11th base of the transcript.

To explore the nature of the priming mechanism, we mapped the 5' ends of Tf1 transcripts extracted from particles to test for the presence of Tf1 mRNA missing its first 11 bases. The result was that large amounts of Tf1 transcript were detected, but all of it had 5' ends at the previously mapped 5' start site [Levin, 1992 #1196]. One complication of this result is that the putative cleavage event is likely coupled to reverse transcription so that if transcripts are cleaved, they would immediately be degraded by RNaseH. In addition, our inability to detect evidence for Tf1 transcripts connected to strong stop DNA by a branch point might have been due to RNaseH degradation of the mRNA.

To preserve priming intermediates from degradation, we used site-directed mutagenesis to generate five Tf1 plasmids each with a mutation in an active site residue of RNaseH. If these mutations greatly reduce RNaseH activity, any cleaved transcripts would be protected from degradation and thus could be observed by S1 nuclease mapping or primer extension. In addition, mutations that leave RT polymerase activity intact would also allow us to observe Tf1 transcripts attached by a branch point to strong-stop DNA if a 2' OH group is used for priming. Several groups have identified 3 amino acid positions within RNaseH of HIV and moloney leukemia virus that can be mutated to produce an RT with normal polymerase activity and no RNaseH function [Repaske, 1989 #1479; Mizrahi, 1994 #1478]. Using these studies as a guide, as well as the identification of active site residues from X-ray crystal structures of other RNaseH molecules [Davies, 1991 #1598; Katayanagi, 1990 #1599; Yang, 1990 #1600; Kohlstaedt, 1992 #1601], we individually changed two essential aspartic acids (443 and 498, HIV numbers) to asparagines and alanines while a glutamic acid (478) was changed to a glutamine. These mutations were incorporated into the Tf1 transposition plasmid and their proteins expressed in our *S. pombe* strains. Transposition assays of the mutant Tf1 elements were used to verify a lack of mobility, and *in vitro* RT assays of the Tf1 particles were used to identify the mutations that preserve DNA polymerase activity. All 5 mutations caused a severe reduction in transposition frequency. Replacement of either of the aspartic acids with glutamine resulted in particles with wild type levels of RT activity as measured by *in vitro* assays that include synthetic primer and template. We found that the alanine replacements of the aspartic acids retained approximently half of the RT activity and the glutamine replacement of the glutamic acid had no detectable activity. Extracts from partially purified

particles were used to isolate Tf1 mRNA to be examined for cleavage at the 5' end as well as for covalent linkage to strong-stop DNA. None of the RNA isolated from any of the mutant or wild type strains exhibited evidence of a 5' end cleavage as indicated by primer extension studies. These results ostensibly suggested that cleavage of the first 11 bases of the transcript was not required for self-priming. However, examination of the particles for DNA indicated that none of the mutant RT polymerases were able to produce minus-strand strong-stop material. Therefore, the lack of any observable cleaved mRNA could have been due to a complete lack of priming events caused by the RNaseH mutations. In addition, the absence of minus-strand strong-stop DNA in the mutants suggested that the priming event may actually require RNaseH activity.

An additional *in vivo* experiment designed to detect the generation of a primer via a cleavage event of the Tf1 transcript avoided mutations in RNaseH in case it itself possesses the putative cleavage avtivity. We used versions of Tf1 RT that have defective polymerase activity with the objective of trapping priming intermediates that have undergone the putative cleavage event but not extension. We expected that mutations in the polymerase domain of RT would allow the accumulation of cleaved Tf1 transcripts because RNaseH would be unable to degrade the cleaved mRNA due to the lack of DNA:RNA duplex. 3 Tf1 assay plasmids with mutations in the conserved YXDD box of the RT polymerase were created. RNA from mutant and wild type particles was examined by primer extension and the results indicated that 20%-50% of the RNA from the mutant particles had suffered a cleavage event that removed just the first 11 bases. Wild type particles showed no cleavages indicating that RNaseH was degrading the RNA as cleavage events occurred. S1 nuclease mapping experiments are in progress to independently examine the 5' end of the mutant RNA for cleavage sites. Should these confirm the presence of cleaved RNA in particles, we will combine the RT polymerase mutations with the RNaseH mutations to test the role of RNaseH activity in the cleavage event. A role of RNaseH in the cleavage of the Tf1 self-priming RNA would be particularly interesting given recent reports that RNaseH from retroviruses possess an activity designated RNaseH* that results in cleavage of RNA in an RNA:RNA duplex [Hostomsky, 1994 #1385].

4. The 11 base pair RNA duplex required for self-priming comprises one third of a much larger structure of continuous RNA base pairs.

An important question left unanswered by our current self-priming model resulted from the close inspection of the RNA sequence in this leader region. Figure 2 shows a large potential RNA structure containing the 11 base pairs of the PBS as well as 28 other continuous base pairs. We are interested in defining the function of this large potential structure in priming as well as other processes such as packaging of Tf1 mRNA into the virus like particles. To this end, we have randomly mutagenized the entire region of secondary structure by PCR and isolated a total of 78 mutations, 40 of which represent single base mutations that reduce transposition significantly. These strains have been subjected to our quantitative transposition assay and the results indicated that approximently half exhibited at least 50-fold less transposition activity while the others showed 5 to 50-fold lower transpositon. Mutations of both classes were distributed throughout the PBS region, the adjacent 11 base pair region, and the 5 base pair section just upstream of the PBS. To address the effect of the mutations on expression, each of these Tf1 elements was subjected to immunoblot analysis and found to synthesize wild type levels of Gag and IN protein.

We have recently developed a DNA blot method for the detection of Tf1 cDNA that enables us to measure amounts of mature double-stranded reverse transcript. *S. pombe* cell extracts are phenol extracted and ethanol precipitated. The resulting nucleic acid is restriction digested with enzymes that

produce cDNA fragments with very different sizes from the plasmid Tf1 sequences. This method was used to screen each of 40 different Tf1 elements with mutations in the RNA leader structure. The results showed that each of the mutants produced lower levels of cDNA than wild type. When the amounts of cDNA were quantitated, the reduction in reverse transcription caused by each mutation correlated well with the magnitude of their transposition defect. Although this data suggests each mutation only effects priming levels, we are also testing the mutations for defects in the packaging of RNA into particles, another activitiy that would result in lower levels of cDNA synthesis.

B. The ratio of Gag compared to IN in Tf1 particles.

1. The Tf1 Gag protein accumulates to significantly higher levels than IN.

We have previously shown that the Tf1 primary translation product is a single 140 kDa polypeptide that is cleaved by PR to form the mature Gag, PR, RT and IN proteins. Further characterization of the Tf1 proteins is important because their expression is unique among LTR containing retroelements. Retroviruses and LTR-containing retrotransposons encode their Gag (capsid) and Pol (PR, RT, and IN) proteins in different ORFs that are separated by a frameshift or a stop codon. This organization causes a molar excess of Gag with respect to Pol protein to be expressed because a small number of ribosomes are able to translate past the stop codon of Gag. Excess Gag is important for assembly of particles because Gag serves as a structural component of the particle. Since the Tf1 coding sequence for Gag and Pol is contained within a single ORF, there is no obvious mechanism for overproducing Gag protein. The high levels of transposition exhibited by Tf1 and the particle nature of its proteins compelled us to develop methods for determining the ratio of Gag to Pol proteins in whole yeast extracts as well as in partially purified virus-like particles.

As reported last year, two different methods were developed to calibrate the immunoblot signals produced by anti-Gag and anti-IN antisera so that quantitative blotting could be used to evaluate the relative levels of these two proteins. Extracts from a PR$^-$ *S. pombe* strain that contained only the 140 kD primary translation product were used to calibrate anti-Gag and anti-IN antisera since the Tf1 product contained equal amounts of Gag and IN epitopes. Our second method for antisera calibration was based on Gag and IN protein expressed in bacteria and purified using Ni columns. Both methods for comparing the strength of the antisera generated similar results that indicated the anti-IN antiserum produced 1.5 to 3 times more signal than the anti-Gag antiserum. Using the calibrated antisera, we found immunoblots of *S. pombe* proteins showed that whole cell extracts as well as partially purified particles contained 26 times more Gag than IN. This significant excess of Gag in the Tf1 particles represented a composition that resembled that found in retrovirus and retrotransposon particles [Stromberg, 1974 #1451; Panet, 1975 #1450; Farabaugh, 1993 #1389].

Despite the observation that Tf1 particles appeared to assemble with the high levels of Gag expected for typical retroelement particles, the mechanism for adjusting the ratios of Gag to IN was very different than the frameshifting mechanism. We surmised that since Tf1 proteins are all expressed at the same levels, a selective degredation mechanism must result in the reduction of IN levels. In support of this reasoning, we found that extracts made from log-phase yeast cultures contained equal amounts of Gag and IN while cultures of higher cell density contained the lower amounts of IN relative to Gag that are usually observed.

2. The analysis of Tf1 particle development in *S. pombe* cultures.

This year we initiated detailed time-course experiments to carefully monitor the changes in IN levels as cells reached stationary phase. In agreement with observations reported last year, log phase cells in these cultures possessed approximently 1:1 ratios of Gag to IN and the stationary phase cells showed much less IN than Gag. Most of the IN degradation occurred within a 6 hour period between the OD_{600} levels of 9.2 and 14.0. The final Gag to IN ratio observed in this experiment was 40:1. To determine if the Tf1 particles that contained excess Gag played a functional role in transposition, we examined the same cells from the time course for reverse transcription products. A blot of DNA extracted from whole cells, cut with *Bst*XI, and hybridized with a *neo* probe was used to measure the synthesis of Tf1-*neo* cDNA. The restriction cut allowed us to distinguish between *neo* sequences derived from reverse transcripts verses plasmids because Tf1 has a single *Bst*XI site 2 kb from the 3' end of the element. Additional evidence that the 2 kb band was derived from the Tf1 cDNA came from examination of an isogenic strain with a Tf1 plasmid that had a frameshift mutation in PR and therefore produced no RT. This mutant strain contained the plasmid band but not the 2 kb cDNA band. The 2 kb cDNA band derived from the double-stranded Tf1 cDNA was observed to increase greatly in the cells that contained reduced IN protein. The levels of the 2 kb band were quantitated and normalized to the amount of plasmid derived signal in each lane. The increase in Tf1 cDNA content observed during the entire time course was 8-fold and the amounts were linearly increasing even in cells from the last time point. The bulk of this increase occurred after the Gag to IN ratios increased to the levels observed in stationary phase. The fact that most of the cDNA is produced following degradation of the majority of IN protein was consistant with the hypothesis that particles containing a large molar excess of Gag compared to IN are functional in producing double-stranded cDNA. We cannot however determine whether the small amounts of cDNA produced in log phase cells are synthesized by particles of this type or by particles containing equal molar amounts of Gag and IN.

3. Examination of stationary phase cultures for conditions that trigger IN degradation.

The degradation of Tf1 IN in cells from stationary phase cultures is a regulated process that results in the high ratios of Gag to IN typical of retroelements. Several aspects of stationary phase conditions are candidates for initiating the degradation of IN. Nitrogen, sulfate, and glucose are all nutrients that may be limiting in stationary phase cultures and thus initiate IN degradation. The low pH of these cultures or the accumulation of ethanol are also properities of stationary phase that might result in IN degradation. To determine which if any of these conditions might effect the ratio of Gag to IN, we grew cells expressing Tf1 to midlog densities and placed them in media that contained different levels of the agents being tested. The results form this survey indicated that cells starved for nitrogen or sulfate exibited the normal degradation of IN seen in stationary phase cells. However, we found that cells starved for glucose, were unable to degrade IN and therefore resulted in stationary cells that contained equal amounts of Gag and IN long after cells in complete medium exhibited the drop in IN levels. These results suggested that the turnover of IN may be a process that only occurs in cells arrested in G_0 since nitrogen and sulfate starvation of *S. pombe* causes G_0 arrest while glucose starvation causes a nonspecific arrest [Moreno, 1991 #1429]. Never the less, we plan to use plates with reduced glucose to assay the transposition frequency of cells that possess equal levels of Gag and IN. These experiments will indicate whether IN degradation is an esential feature of transposition.

C. The contribution of Tf1 RT to transposition.

To identify essential functions of RT for transposition, we have characterized a set of point mutations created either by random or site-directed mutagenesis.

1. Mutations in RNaseH that block transposition but not reverse transcription.

We have previously reported the isolation of a large set of random point mutations in Tf1 that reduced transposition activity. These were screened using our homologous recombination assay to identify mutations that, never the less, produced normal levels of reverse transcript. We identified 7 Tf1 mutations that exhibited these properities and as expected, most of the base changes were found to be located within IN. Surprisingly, 2 of the mutations were within the C terminus of the RNaseH domain of RT. This was an unexpected result because studies of RNaseH mutants in retroviruses have indicated that the synthesis of mature double-stranded cDNA requires RNaseH activity to release single stranded DNA intermediates from their RNA templates [Champoux, 1993 #1442; Blain, 1995 #1602]. To establish if the level of reverse transcription is effected in the two mutant strains, we extracted total DNA from each and subjected it to DNA blot analysis using conditions that allowed us to visualize restriction products from both ends of the cDNA. Consistant with the homologous recombination assays, the blot results showed that one of the strains had fully normal levels of reverse transcript while the other exhibited about 2-fold less cDNA than wild type Tf1. One possible explanation of our results is that the mutants specifically affect the ability of RNaseH to cleave the residule RNA primers off the 5' ends of the cDNA without reducing the enzyme's general ability to degrade RNA annealed to DNA. The result of this defect would be to allow RT to product completed cDNA but the presence of the RNA primers would inhibit the integration reaction. Whether or not this is the cause for the low transposition frequencies of the 2 RNaseH mutations, we appear to have identified a role of RNaseH in the integration process.

2. Site-directed mutations of Tf1 RT define essential functions.

Despite the presence of highly conserved amino acid residues in the sequence of Tf1 that indicate the presence of an RT, we had no direct evidence that the domain actually encodes a functional reverse transcriptase. As discussed in section A3 (*In vivo* experiments to distinguish between two types of self-priming mechanisms), we generated 8 site-directed mutations within conserved residues of Tf1 RT. Within the RNaseH domain, we changed two conserved aspartic acids (443 and 498, HIV numbers) to asparagines and alanines while glu478 was changed to a glutamine. The result of each of these 5 RNaseH mutations was a Tf1 element that exhibited no detectable transposition activity. In addition, the mutations resulted in undectable levels of homologous recombination between the Tf1 plasmid and cDNA, indicating the levels of reverse transcripts was low. To gauge the effect of these RNaseH mutations on the RT polymerase activity, we partially purified particles from the mutant transposons and assayed them for RT activity by adding oligo dT as primer and poly rA as template. The results indicated that D443N, D498N, D443A, and D498A all showed high levels of activity that were either equilivant to wild type or within 2-fold of wild type RT. The high level of RT activity possessed by these mutant RTs indicate that the defects in transposition were not due to lower stability of RT but probably the lack of RNaseH activity. This conclusion was supported by the absence of any products of reverse transcription as measured by DNA blotting. As a result, these mutations provided the first physical data that the Tf1 RT possesses RNaseH activity essential for transposition.

To address the role of the polymerase domain of RT, three additional mutations were produced in the conserved "YXDD" box that constitutes a portion of the polymerase active site in other retroelements. The mutations created were D185N, D185L, and D186N (HIV number system). The transposition frequencies of all three strains were undectable as were their frequencies of homologous recombination. *In vitro* assays of particles produced by these mutant strains indicated the levels of RT activity was

reduced by at least 60-fold as a result of each mutation. The results from the mutations in polymerase and RNaseH strongly indicate Tf1 does encode an RT that possess functions essential for transposition.

D. The characterization of host mutations that inhibit transposition.

In order to improve our understanding of the retrotransposition process and by analogy, retrovirus infection, we initiated a large scale genetic screen for *S. pombe* strains that were defective for transposition. As reported last year, cultures of *S. pombe* that contained our Tf1 assay plasmid were mutagenized with EMS under conditions that caused 80% lethality on minimal medium. After these strains were colony purified, 5,000 were patched onto our transposition assay media and screened for reduced frequencies of transposition. Approximently 176 strains from two mating types reproducibly transposed at significantly lower levels than the parent strains. All these candidates were tested for the presence of single genetic lesions affecting transposition and for the magnatude of the deficiency caused by each mutation. As a result, we chose to focus our experiments on 6 mutant strains that had transposition frequencies 5 to 25-fold lower than that of wild type cells.

1. Recombination analysis of 6 mutant strains to identify the minimum number of genes that effect transposition.

Each of the original mutant strains were crossed with others from the same set and the resulting spores were tested for transposition to determine if any two strains carried mutations within the same gene. If two strains are mated that each possess mutations in the same gene, none of the resulting spores will transpose at wild type frequencies. If the mutations exist in different genes that segregate independently, one quarter of the spores will show normal transposition activity. The results indicated that each of the 6 mutant strains contained genetic lesions in different genes that we have termed *hop1* through *hop6*.

2. The evaluation of each transposition mutation for effects on specific steps in the transposition pathway.

1. *Hop1*

The strains with the *hop1-1* mutation grow at wild type rates and possess a mutation that reduced its transposition frequency by 25-fold when measured with our quantitative assay. The frequency of homologous recombination between Tf1 cDNA and the Tf1 plasmid was also measured quantitatively and found to be 50-fold lower in *hop1-1* cells compared to wild type levels. The low frequencies of homologous recombination suggested that the *hop1-1* mutation caused a defect early in the transposition process that resulted in lower levels of reverse transcript. Results from DNA blots of *hop1-1* strains were consistent with the recombination data in that no reverse transcripts were detected. Immunoblots of *hop1-1* strains demonstrated a dramatic lack of Gag and IN protein accumulation that very likely caused the low levels of cDNA due to a simple lack of particle formation. We have measured mRNA levels of Tf1-*neo* in *hop1-1* strains and find at most a reduction of 2-fold that may not be the main cause of the transposition defect but merely the result of transcript destabilazition due to low rates of Tf1 translation. We are currently pursuing the possibility that the lack of Tf1 protein is due to either reduced translation of the Tf1 transcript or increased degradation of the Tf1 proteins. We have made lacZ fusions to the first codon of the Tf1 ORF as well as to internal locations to identify which Tf1 sequences are effected by the *hop1-1* defect. The isogenic *S. pombe* strains with and without the *hop1-1*

mutation are now being constructed to test the lacZ plasmids. In addition, we have fused the complete Tf1 ORF to the *nmt1* promoter after the *nmt1* 5' untranslated region to determine if the *hop1-1* mutation caused a lack of Tf1 protein accumulation due strictly to protein degradation and not lack of translation. Preliminary results indicate that when the Tf1 proteins are expressed from the *nmt1* transcriptional leader, the *hop1-1* mutation has no effect on Gag or IN acumulation suggesting that the lack of Tf1 protein accumulation caused by *hop1-1* was due to a defect in translation linked to the 5' untranslated region of the Tf1 mRNA.

Because the lack of homologous recombination is so dramatic, we were able to identify plasmids from an *S. pombe* genomic library that suppressed the *hop1-1* defects. We are now characterizing these plasmid candidates to determine if they contain the *hop1* gene.

2. *hop2*

Strains with the *hop2-1* mutation have an 8-fold transposition defect and a 4-fold drop in their homologous recombination frequency. The phenotype of the *hop2* strains is similar to *hop1* cells in that the levels of Tf1 protein accumulation was low although the amounts of cDNA detected by DNA blot were not greatly reduced. We are testing these strains for defects in translation and protein stability using the same lacZ fusions developed for characterizing *hop1*.

3. *hop3*

The *hop3-1* mutation caused a 10-fold drop in both the transposition and homologous recombination assay frequencies. Unlike the *hop1* and *hop2* mutations, *hop3* cells were able to produce wild type levels of mature Tf1 proteins in log phase cultures. However, 1 day after *hop3-1* cells reached stationary phase, they exhibited a sharp drop in Gag levels suggesting a loss of protein stability. Although we observed the *hop3-1* cells contained normal levels of reverse transcript, the loss of Gag and possibly IN could result in lower IN activity or less efficient cDNA presentation to the nucleus. To reveal the cause of low Tf1 protein accumulation in stationary phase cells we are testing these strains for defects in translation or protein stability using the same lacZ fusions developed for characterizing *hop1*. Preliminary results indicate that the Gag protein in stationary phase *hop3-1* cells is absent even when expressed from a plasmid with the *nmt1* 5' untranslated region fused to the Tf1 ORF. This suggests that the lack of Gag is due to degradation, not a defect in translation.

4. *hop5*

Cells with the *hop5-1* mutation transpose with frequencies 12-fold lower than wild type strains. Results from the homologous recombination assay also indicated a 12-fold defect. As observed for *hop3-1* cells, DNA blots made from *hop5-1* strains showed normal levels of mature reverse transcript were produced. Immunoblots indicate that this mutation has no effect on the levels of Tf1 protein accumulation. We speculate that the defect caused by *hop5-1* is similar to that of *hop3-1* in that a block may occur in the presentation of the cDNA:IN complex to the nucleus.

Significance to Biomedical Research and the Program of the Institute.

The medical significance of our research stems from the close relationship between retroviruses and retrotransposons. The high level of similarity in structure between these two types of retroelements

results in the large number of mechanisms that the elements share. The mechanism of IN mediated insertion for retrotransposons and retroviruses has been shown to require the same reaction intermediatesb [Eichinger, 1988 #419]. The early steps of reverse transcription are primed from the same position of the elements and both types of retroelements use polypurine sequences for priming plus strand reverse transcription. The structure of transposon virus-like particles is analogous to retrovirus particles in their composition of capsid and Pol proteins. The medical importance of retroviruses is dominated by the role of HIV in the AIDS epidemic. HIV is a retrovirus that appears to undergo the same types of reverse transcription, proteolysis, integration and particle formation as do retrotransposons. Because many aspects of retrovirus function, including particle assembly, reverse transcription, nuclear entry and chromosome integration, are difficult to study in hosts that possess a high level of genetic complexity, the molecular genetic analysis of retrotransposons in yeast provides a promising approach to answering many of the important questions that are at the center of the AIDS crisis.

The study of transposons in general and retrotransposons in particular is relevant to the understanding of neoplastic disease mechanisms. Transposons are endogenous mutagens of cells; chemical mutagenesis is strongly correlated with carcinogenesis; therefore studies of these endogenous biological mutagens is highly pertinent to an understanding of cancer. Moreover, the growth of a wide variety of tumors is associated with activation of endogenous retroviral expression and rearrangements of the genomic DNA in tumor cells. Many studies clearly indicate that the activation of cellular oncogenes is directly responsible for mammalian neoplasms. In many cases, such gene activation is caused by retrovirus or retrotransposon transpositional insertion into a proto-oncogene locus. For this reason, the thorough understanding to be gained from studies of retrotransposons inhabiting the genomes of genetically tractable organisms, particularly yeast, is directly relevant to the understanding of both oncogene activation mechanisms and the DNA rearrangements that occur in cancer cells.

Proposed Course

A. Self-primed reverse transcription.

1. The mechanism of mRNA cleavage.

We are pursuing several approaches to identify the chemical nature of the self-priming mechanism of Tf1 reverse transcription. We have previously shown that the interaction between the PBS and the 5'end of the Tf1 transcript is required for synthesis of the minus-strand strong-stop DNA. The chemical nature of the priming event is still unknown. One possibility is that a 3'OH is provided by a site-specific nicking enzyme that creates a primer by cutting off the first 11 bases of the Tf1 transcript. Our data from the RT polymerase mutations has provided the first evidence for a cleavage of the Tf1 transcript after the 11th base. Tf1 mRNA isolated from particles with the mutant reverse transcriptases was analyzed by primer extension and found to have been cleaved between the 11th and 12th bases. We will also subject the particle RNA preparations to S1 nuclease analysis to seek independent evidence for the absence of the first 11 bases. Because the mutations in the conserved residues of RNaseH surprisingly resulted in much lower amounts of minus-strand strong-stop DNA, we will test the hypothesis that RNaseH is the source of the cleavage activity. We will compare Tf1 elements with mutations in the polymerase domain to Tf1 elements with mutations in both the polymerase and RNaseH domains to determine if the addition of the RNaseH mutation reduces the level of cleaved mRNA.

2. *In vitro* analysis of the self-priming mechanism.

As an additional means of studying the chemistry of priming, we will develop an *in vitro* priming assay to generate large quantities of priming intermediates. Initially, we will attempt to produce a priming reaction from purified RT and Tf1 transcript. RT expression vectors will be produced and tested for use in bacteria as well as baculovirus and *Pichia pastoris*. Once RT is successfully purified from an expression system, we will combine RT, *in vitro* transcribed Tf1 mRNA, and ^{32}P labeled dNTP's under a varity of conditions. These reactions will be tested for the synthesis of minus-strand strong-stop DNA. Mutations in RNaseH and/or the polymerase domain of RT will be tested in the assay to characterize the source of the cleavge activity. We will also test reactions with mRNA alone to determine if the cleavage is autocatylitic. The products of the *in vitro* reactions will also be examined for evidence of a 2'OH priming mechanism. The products of priming reactions containing radiolabeled Tf1 mRNA will be digested with the nucleases P1 and T2 to determine if the bond between the RNA primer and the synthesized DNA is a 2'-5' or a 3'-5' linkage [Ruskin, 1990 #917; Ruskin, 1985 #747].

3. The function of the 39 base pair RNA structure.

In conjunction with the study of the mRNA cleavage mechanism, we will continue to explore the role of the 39 base pair RNA structure that contains the PBS. The data from our mutagenesis of this structure indicated that sequence along its entire length are critical for transposition. We now plan to evaluate if these mutations can be rescued by the addition of compensitory mutations, suggesting that the RNA structure is important and not the individual sequences. Mutations from each of the 4 sections of the RNA structure will be further studied to identify their individual contributions to the transposition process. These mutations will be tested for their effects on translation, mRNA packaging into particles, self-priming, reverse transcription, and integration.

B. The mechanism of Tf1 VLP assembly.

1. Identification of growth conditions that induce IN degradation

We have shown that cells starved for nitrogen or sulfate undergo IN degradation while cultures starved for glucose maintain equal molar amounts of Gag and IN. This observation will allow us to test the transposition efficiency of Tf1 in cells that starve for glucose to evaluate the importance of IN degradation to transposition. If functional particle development requires IN degradation then the cells starved for glucose will be unable to transpose even several days after stationary phase is reached. If however, the degradation of IN is not required for transposition, the glucose starved cells should undergo normal levels of transposition. This question of IN degradation will be addressed below in separate experiments.

2. The role of IN degradation in transposition.

Although we propose that the reduction in mature IN protein observed in stationrary phase cells is a regulated step of Tf1 particle assembly, the possibility exists that assembly occurs before IN degradation and excess IN not incorporated into the VLP's is degraded simply due to greater exposure to cellular proteases. Our observation that much of Tf1 reverse transcription occurred after IN degradation indicated that the particles with excess Gag are functional and represent intermediates in the transposition process. The idea that IN degradation is a programmed aspect of particle development is consistent with our observation that the majority of the RT is also degraded in stationary phase cells

(J. Lin, A. Atwood, and H. Levin, unpublished). A resolution to the question of how important is the reduction of Pol protein to the assembly process requires that we develop direct genetic means of manipulating the Gag to Pol ratios in stationary phase cells.

We plan to test the importance of high Gag to IN ratios in transposition by overexpressing IN from a separate plasmid with a promoter that is active in stationary phase cells. If particle development requires IN levels be reduced much below the amount of Gag protein, the increase in IN provided by the overexpression plasmid should cause a defect in transposition. The *fbp1* promoter is active in stationary phase and will be tested for use in this experiment [Hoffman, 1989 #858].

Another approach we will take to define the importance of Gag to IN ratios is to reduce Pol protein levels throughout particle formation by inserting "leaky" frameshift mutations between Tf1 Gag and PR, PR and RT, and between RT and IN. The frameshift sequences from Ty3, Ty1 and M1 have been characterized in *S. cerevisiae* and will be tested in *S. pombe* for the purposes of this experiment [Dinman, 1992 #1361; Farabaugh, 1993 #1389; Belcourt, 1990 #824]. If the excess Pol proteins are simply an impediment to assembly, the reduction in expression caused by the frameshift mutations will have no detrimental effect on transposition and may actually cause an improvement in activity.

3. Characterization of the activity that degrades IN.

An important aspect of Tf1 particle assembly that we will address here is the source of the IN degradation activity. The protease or proteases that degrade the IN and RT in stationary phase cells could either be a transposon encoded protein such as PR, or it could be a host protein. We will study the stability of mature-sized IN expressed on a plasmid in the absence of all the other Tf1 proteins to determine if host factors alone are sufficient to regulate the IN degradation in stationary phase cells without the association of other Tf1 proteins. If no IN degradation is observed in these stationary phase cells, or if IN is unstable even in log-phase cells, the possibility will be tested that regulated degradation of IN requires the presence of Gag, RT, and IN assembled into particles. We plan to construct Gag, PR, and RT expression plasmids so that mature-sized Gag, PR, RT, and IN can be independently expressed within the same cells to evaluate the requirements for IN degradation. IN and PR will be coexpressed in one experiment as a direct test of PR catalyzed degradation of IN. In additional experiments, Gag, PR, RT and IN will be coexpressed in several combinations to establish the minimal requirements to reproduce the regulated degradation of IN. If IN regulation can be observed in one of these combinations, the requirement for PR will be evaluated.

The independent expression of all of the mature Tf1 proteins will present an opportunity to determine if particle assembly requires intermediates composed of primary translation products. One model for particle assembly predicts that the attachment of Gag to the Pol proteins in the primary translation product may be required to localize RT and IN to the sites of particle formation. Alternatively, the result that Tf1 proteins are completely processed into mature products that appear to form particles before RT and IN are degraded suggests that the independent expression of Gag, RT and IN may result in functional particles. The ability of independently expressed mature-sized proteins to form functional particles will be tested by expressing Gag from a Tf1-*neo* transcript that has a frame shift placed before PR.

4. The study of particle stability to measure the structural integrity of log phase particles compared to stationary phase particles.

The Tf1 protein isolated from stationary phase cells was found to sediment in sucrose gradients as particles that contained 50-fold more Gag than IN [Atwood, 1995 #1576]. Particles isolated from log phase cells have very different properities including the association of a much greater amount of IN and RT [Atwood, 1995 #1576]. We wish to probe these two forms of Tf1 particles for evidence of a maturation process that could result in greater structural integrity. The stability of the log and stationary phase particles will be evaluated by their resistance to perturbations such as treatment with denaturing agents and proteases. Particles from log and stationary phase cells will be isolated in sucrose gradients. The strength of the Gag and IN association in these particles will be tested in various concentrations of denaturing agents (e.g. tween, urea and guanidinium hydrochloride) to evaluate the stability of log-phase particles compared to stationary phase particles. After the denaturing treatments, the particles will be refractionated on sucrose gradients and the fractions analyzed on immunoblots to determine the effect of the treatments on particle integrity. The structure of log phase and stationary phase particles will also be probed by partial protease treatments. The sensitivity of Gag and IN to proteases will also be monitored by immunoblot analysis.

C. The primer cleavage activitiy of RNaseH.

Two different mutations in the RNaseH domain of RT were identified that caused a sharp drop in transposition activity despite the results of DNA blot analysis that showed these strains produced normal levels of full-length reverse transcript. This was an unexpected result because previous studies of RNaseH mutants have indicated that the synthesis of mature double-stranded cDNA requires RNaseH activity to release single stranded DNA intermediates from their RNA templates [Champoux, 1993 #1442; Blain, 1995 #1602]. One likely explanation of these results is that the mutants specifically affect the ability of RNaseH to cleave the residule RNA primers off the 5' ends of the cDNA without reducing the enzyme's general ability to degrade RNA annealed to DNA. The result of this defect would be to allow RT to product completed cDNA but the presence of the RNA primers would inhibit the integration reaction. Although this primer cleavage activity has been observed *in vitro* [Champoux, 1984 #1603; Rattray, 1987 #1606; Furfine, 1991 #1604; Pullen, 1992 #1605], no evidence has yet been reported indicating the two RNaseH activities have different active site requirements. We will test this hypothesis using a primer extension method and a DNA blot technique to detect the cDNA primers. If the mutants exhibit normal primer removal, we will consider alternative causes of the reduced transposition such as participation of RNaseH in the integration reaction. This issue could be addressed using the type of *in vitro* integration assays developed for other retrotransposons [Kirchner, 1995 #1474; Braiterman, 1994 #1492; Braiterman, 1994 #1493].

D. The characterization of host gene function in Tf1 retrotransposition.

1. *hop1.*

The observation that *hop1-1* mutant cells do not accumulate Tf1 protein could be due to a defect in translation or an increase in degradation. We have now assembled a set of Tf1 expression plasmids that contain lacZ fused to several different regions of Tf1. These plasmids will be expressed in *S. pombe* to determine whether the Tf1 5' untranslated region or the Tf1 coding sequence must be fused to *lacZ* for beta-galactosidase activity to be reduced in *hop1-1* cells. We have also constructed a plasmid containing the Tf1 ORF fused to the *nmt1* promoter with the *nmt1* 5' untranslated region. This plasmid will be transformed into wild type and *hop1-1* cells to test the stability of normal Tf1 proteins expressed from this heterologous transcript. Any effect of the *hop1-1* mutant on Gag levels expressed from this

plasmid would suggest that Tf1 protein levels drop due to increased degradation. The results produced using all the fusion plasmids will allow additional plasmids to be produced that will help to further define which sequences of Tf1 are most effected by *hop1-1*.

We will seek additional information about the function of *hop1* by continuing our effort to clone the gene. We now have one 6 kb genomic clone that suppresses the *hop1-1* phenotype and is geneticlly linked to the site of the original *hop1* mutation. Should this 6 kb insert contain the *hop1* gene, we will analyze its sequence for information related to its function.

2. *hop2.*

Strains with the *hop2-1* mutation have an 8-fold transposition defect and a 4-fold drop in their homologous recombination frequency. Because the phenotype of the *hop2* strains is similar to *hop1* cells in that the levels of Tf1 protein accumulation was low, we are testing these strains for defects in translation or protein stability using the same *lacZ* fusions developed for characterizing *hop1*. We will also attempt to isolate the *hop2* gene using an *S. pombe* genomic library to identify complementing clones.

3. *hop3.*

The *hop3-1* mutation caused a 10-fold drop in both the transposition and homologous recombination assay frequencies. Unlike the *hop1* and *hop2* mutations, *hop3* cells were able to produce wild type levels of mature Tf1 proteins in log phase cultures. However, 1 day after *hop3-1* cells reached stationary phase, they exhibited a sharp drop in Gag levels suggesting a loss of protein stability. Although we observed the *hop3-1* cells contained normal levels of reverse transcript, the loss of Gag and possibly IN could result in lower IN activity or less efficient cDNA presentation to the nucleus. To reveal the cause of low Tf1 protein accumulation in stationary phase cells we are testing these strains for defects in translation or protein stability using the same *lacZ* fusions developed for characterizing *hop1*. Preliminary results indicate that the Gag protein in stationary phase *hop3-1* cells is absent even when expressed from a plasmid with the *nmt1* 5' untranslated region fused to the Tf1 ORF. This suggests that the lack of Gag is due to degradation, not a defect in translation. We will therefore attempt to identify which Tf1 proteins are specificly destablized in *hop3-1* cells. We also plan to isolate the *hop3* gene using the genomic library to complement the lack of transposition exhibited by *hop3-1* cells.

4. *hop5*

Cells with the *hop5-1* mutation transpose with frequencies 12-fold lower than wild type strains. Results from the homologous recombination assay also indicated a 12-fold defect. As observed for *hop3-1* cells, DNA blots made from *hop5-1* strains showed normal levels of mature reverse transcript were produced. Immunoblots indicated that this mutation had no effect on the levels of Tf1 protein accumulation. We speculate that the defect caused by *hop5-1* may occur in the presentation of the cDNA:IN complex to the nucleus. Alternatively, the transposition and recombination defect could be caused by a mutation in a general recombination factor that contributes to both processes. To test this possibility, we will measure the efficency of homologous recombination between plasmid and genomic copies of *ade6* in both wild type and *hop5-1* cells. We will also measure the frequency of switching at the mating type cassette as an independent measure of a homologous recombination process.

Additional information about the function of *hop5* will be sought by the isolation and characterization of its sequence.

Publications

Atwood A, Lin J, Levin H. The retrotransposon Tf1 assembles virus-like particles with excess Gag due to specific degradation of Pol protein, Mol Cell Biol 1995; in press.

Levin HL. A novel mechanism of self-primed reverse transcription defines a new family of retroelements, Mol Cell Biol 1995;15:3310-17.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD01010-01

PERIOD COVERED

October 1, 1994 through September 30, 1995

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

Regulation of Eukaryotic Protein Synthesis

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

PI:	Thomas E. Dever	Unit Head	LMG:NICHD
Others:	Kobayashi, Makiko	Visiting Fellow	LMG:NICHD
	Locke, Emily	Biologist	LMG:NICHD
	Ung, Tekly	Volunteer	LMG:NICHD

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Genetics

SECTION

Unit on Translational Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.4	1.9	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 regulation of protein synthesis in mammalian cells under stress conditions as well as the regulation of GCN4-specific translation in the yeast Saccharomyces cerevisiae under amino acid starvation conditions is mediated by phosphorylation of the eukaryotic translation initiation factor (eIF)-2. Phosphorylation of serine-51 on the α subunit of eIF-2 by the mammalian PKR kinase inhibits general translation, while phosphorylation of serine-51 by the yeast GCN2 kinase is required to increase expression of GCN4. We have been studying how these protein kinases specifically recognize and phosphorylate eIF-2α on serine-51. Over 100 mutations throughout the eIF-2α protein have been identified that prevent induction of GCN4 expression when GCN2 is activated. Among the residues immediately flanking serine-51, the positions -1 and -2 appear most important for regulation. Examination of eIF-2α phosphorylation in vivo in strains carrying various mutant alleles reveals that some of the substitutions markedly affect the ability to phosphorylate serine-51. We have also been studying the vaccinia virus K3L protein, a pseudosubstrate inhibitor of PKR. We have established a system to suppress the toxicity of PKR expression in yeast by co-expressing K3L. Using this system both loss-of-function and increased activity K3L mutants have been isolated. Residues near the carboxyl-terminus of K3L, that are conserved in eIF-2α (residues 73-83), are critical for K3L activity. In addition, the corresponding residues in eIF-2α are required for proper regulation of GCN4 expression. Finally, we have begun characterization of a novel eIF-2α kinase inhibitor from the baculovirus Autographa californica.

Project Description:

Objective: To understand the mechanism and regulation of protein biosynthesis in eukaryotic organisms, especially focusing on the phosphorylation of eIF-2α by the yeast Saccharomyces cerevisiae GCN2 kinase and by the mammalian PKR kinase, and to understand how viruses circumvent this regulatory network.

Major Findings:

1. Identification of eIF-2α mutants defective for GCN4 translational regulation.

The yeast GCN2 kinase phosphorylates eIF-2α on serine-51 in response to an amino acid starvation. Phosphorylated eIF-2 acts as a competitive inhibitor of eIF-2B, the guanine nucleotide exchange factor for eIF-2. Thus, phosphorylation of eIF-2 results in an inhibition of general translation. In yeast, this phosphorylation of eIF-2 and subsequent inhibition of eIF-2B activity not only limits general translation, but also specifically stimulates GCN4 expression. Increased expression of GCN4 is required for yeast strains to grow under amino acid starvation conditions. Therefore, yeast mutants that are unable to phosphorylate eIF-2α or which fail to inhibit eIF-2B cannot induce GCN4 expression and are thus sensitive to amino acid starvation conditions. To identify how the eIF-2α kinases, such as GCN2, specifically recognize serine-51 on the α subunit of eIF-2 we have begun a mutagenic analysis of eIF-2α. The residues immediately flanking serine-51 were randomly mutated and the mutant alleles were introduced into yeast in place of the wild-type eIF-2α. Pools of mutants were screened to identify eIF-2α mutations that block the growth of the yeast cells under amino acid starvation conditions. Following the identification of the mutants sensitive to starvation conditions, the plasmids carrying the mutant eIF-2α alleles were isolated and sequenced to identify the amino acid substitution in eIF-2α that blocks the translational regulation of GCN4 expression. The sequence flanking serine-51 from residue 48 (-3) to residue 54 (+3) is: $SELS_{51}RRR$. No loss-of-regulation mutations were identified at residues S48, R52 or R53. Only a single mutation was identified at the +3 position: R54G. However, 10 mutations at position -1 (L50) and all 19 possible substitutions at position -2 (E49) blocked the regulation. There are at least two possible mechanisms by which a mutation in eIF-2α could block regulation of GCN4 expression: (1) the mutation could block the ability of GCN2 to phosphorylate eIF-2α or (2) the mutation could prevent phosphorylated eIF-2 from regulating eIF-2B. One-dimensional polyacrylamide isoelectric focusing slab gels can be used to examine eIF-2α phosphorylation in yeast cells. To date, only one of the loss-of-regulation mutations, a leucine to proline change at position 50 (L50P), has been found to severely inhibit phosphorylation of eIF-2α by GCN2.

In addition to examining the residues immediately flanking serine-51, we have also mutated other residues based on their conservation in the vaccinia virus K3L protein. The vaccinia virus K3L protein is an 88 amino acid pseudosubstrate inhibitor of the mammalian anti-viral PKR kinase. K3L shares 28% amino acid sequence identity with the amino-terminal 90 residues of eIF-2α. This homology is most striking between residues 72 and 83 of eIF-2α where 10 of 12 residues are conserved between K3L and human eIF-2α. Based on the strong sequence conservation and the fact that both K3L and eIF-2α bind to PKR we hypothesized that the conserved residues may be important for the kinases to recognize and phosphorylate eIF-2α. To test this prediction the residues in yeast eIF-2α that are conserved in K3L were randomly mutated and the mutant pools screened to identify eIF-2α alleles that block regulation. To date, 14 residues have been examined and 72 loss-of-regulation mutations have been identified. It appears that for several of the residues in the conserved block between positions 72 and 83 any mutation will obstruct regulation. Preliminary analysis of several of these mutations using the isoelectric focusing gels suggests that they do not impede the ability of GCN2 to phosphorylate eIF-2α. Therefore, these mutations are probably affecting the inhibition of eIF-2B by phosphorylated eIF-2. Since both K3L and eIF-2α bind to the eIF-2α kinases

it seems reasonable to suggest that the residues conserved between K3L and eIF-2α are probably important for this interaction. However, the mutational and isoelectric focusing analysis of some of these conserved residues suggests that they are affecting the interaction between eIF-2 and eIF-2B. Taken together these results suggest a model in which the kinase recognition and eIF-2B recognition surfaces on eIF-2α overlap.

2. Mutational analysis of the vaccinia virus K3L protein.

As introduced above, the vaccinia virus K3L protein is a pseudosubstrate inhibitor of the eIF-2α kinase PKR, a component in the mammalian antiviral defense mechanism. To increase our understanding of how the eIF-2α kinases recognize their substrate we have carried out an analysis of the K3L protein. First, we obtained from Dr. Patrick Romano a yeast strain in which the PKR kinase, expressed under the control of a GAL promoter, was integrated into the yeast genome. This strain grows well on glucose medium, however the strain is dead on galactose medium due to severe inhibition of translation caused by phosphorylation of eIF-2α. The vaccinia virus K3L protein gene was cloned into the vector pEMBLyex4 such that K3L expression was also under GAL control. When the K3L expression plasmid was introduced into the yeast strain expressing PKR we found that K3L could suppress the lethality caused by high level expression of PKR in yeast. These results established the yeast system as a tool to study K3L.

Two types of mutational analyses have been conducted on K3L: a random mutational analysis to identify superactive K3L alleles and a site-directed mutational analysis to assess the importance of residues conserved between K3L and eIF-2α. For the random mutational analysis the K3L gene was amplified under low-fidelity conditions using the PCR. The mutant pool of K3L genes was subcloned into pEMBLyex4 and the plasmids were introduced into the yeast strain containing the GAL-PKR construct. The colonies were screened to identify K3L mutants that were better suppressors of PKR. Five such mutants have been identified and one, which is significantly better than the others, has been more extensively characterized. This latter K3L upmutant gene was sequenced and found to contain two nucleotide substitutions. One was a silent mutation that did not alter the amino acid sequence, and the second caused a histidine to arginine change at residue 47 (K3L-H47R). While wild-type K3L yielded a partial suppression of the slow-growth phenotype due to high level expression of PKR in yeast, the K3L-H47R mutant afforded almost complete suppression of the toxic effects associated with PKR. Immunoblot analysis reveals that the K3L-H47R protein is not expressed to higher levels than the wild-type protein nor is PKR expression lower in the K3L-H47R strain than in the wild-type K3L strain. Thus the increased suppression is not due to trivial reasons like increased K3L expression or decreased PKR levels. One hypothesis we are currently pursuing is that K3L-H47R will bind tighter to PKR than wild-type K3L. We have been able to co-immunoprecipitate K3L and K3L-H47R with PKR. Currently, we are altering the wash conditions to see if we can detect a difference in the strength of the association between PKR and K3L-H47R versus wild-type K3L. A point of interest relating back to the first project is that the K3L-H47R mutation makes K3L more similar to eIF-2α. In fact, the H47 residue in K3L aligns with R52 in eIF-2α immediately adjacent to the phosphorylation site at serine-51. A possible interpretation of these results is that by increasing the similarity between K3L and eIF-2α around the serine-51 region, K3L will interact more avidly with PKR.

The site-directed mutational analysis of K3L has focused on the carboxyl-terminal region. As stated above, this region of K3L contains a stretch where 10 out of 12 residues are perfectly identical to mammalian eIF-2α. The sequence KGYID found between residues 74 and 78 in K3L is perfectly conserved in all eIF-2α and K3L proteins that have been identified. As mentioned previously mutations in this region of yeast eIF-2α block the regulation of GCN4 expression. Mutation of K74, Y76 or D78 of K3L to alanine blocks the ability of either wild-type K3L or the upmutant K3L-H47R to suppress PKR. In addition, immunoblot analysis reveals that the Y76A mutation does not alter K3L protein levels ruling out the trivial possibility

that the loss of suppression is due to lower K3L protein levels. A second set of site-directed mutations were introduced to determine the carboxyl-terminal boundary of K3L required for its PKR inhibitory activity. Truncation of the 88-residue K3L protein at residue 73, eliminated the KGYID sequence and the ability to suppress PKR. Truncation of only the last five residues had no effect on K3L activity. While after truncation of three additional residues (8 total), the ability of K3L or K3L-H47R to inhibit PKR was lost. Recent results reveal that after removal of the carboxyl-terminal 6 amino acids K3L-H47R retains its suppressing activity suggesting that the carboxyl-terminal boundary for K3L activity is either 6 or 7 residues from the end of the protein.

3. Identification of a baculovirus inhibitor of eIF-2α kinases.

Many viruses have evolved ways to counteract the antiviral defense mechanisms of eukaryotic cells. One of the defense mechanisms cells employ is phosphorylation of eIF-2α by the PKR kinase. As described above, vaccinia virus expresses the K3L protein, a pseudosubstrate inhibitor of PKR. Other viruses express different protein or RNA inhibitors of PKR and still others activate latent cellular inhibitors of PKR or even degrade PKR. Since our studies of the K3L protein have provided insights into how PKR recognizes eIF-2α, we have examined the literature closely to see if we could identify any new viral inhibitors of PKR that might offer new insights into this mechanism of translational regulation. When the sequence of the baculovirus Autographa californica was reported late in 1994 an open reading frame designated ORF123 encoding a truncated protein kinase termed pk2 was identified. The amino acid sequence of pk2, though truncated, was most similar to the eIF-2α kinase family. Since many viruses encode inhibitors of the eIF-2α kinases, we reasoned that pk2 might be an inhibitor of PKR. To test this hypothesis the pk2 gene was isolated from baculovirus using the PCR and the gene was inserted into the vector pEMBLyex4. Introduction of the pk2 expression plasmid into the yeast strain that expresses PKR under the control of the GAL promoter revealed that pk2 could suppress the toxicity of PKR in yeast. Not only could pk2 suppress PKR toxicity, but pk2 was also able to suppress both wild type and hyperactive alleles of the yeast eIF-2α kinase GCN2. These results demonstrate that pk2 is an eIF-2α kinase inhibitor, and since baculovirus is an insect virus this is the first indication that insects may also use phosphorylation of eIF-2α as a means to block viral infection.

The pk2 protein is similar to the carboxyl-terminal 1/2 of an eIF-2α kinase domain. This raised the possibility that the corresponding fragment from an authentic eIF-2α kinase domain may also act in a dominant negative manner. The amino acid sequences from GCN2 and pk2 were aligned and oligonucleotides were identified to amplify the corresponding region of the GCN2 kinase using the PCR. Following cloning into pEMBLyex4, the truncated gcn2 kinase domain was introduced into various strains. High level expression of the truncated gcn2 kinase domain was found to inhibit the toxicity associated with the hyperactive $GCN2^c$ kinases and thus reveal that this region of an eIF-2α kinase can act in a dominant negative manner.

4. Examination of substrate specificity of the eIF-2α kinases.

Most protein kinases can be classified into one of two classes either serine/threonine specificity or tyrosine specificity. However, a few kinases have been identified with dual specificity that will phosphorylate both tyrosine and serine/threonine residues. To date the only known physiologically relevant substrate of the eIF-2α kinases is eIF-2α, however recent reports suggest that PKR may have alternative substrates. While the eIF-2α kinases have only been reported to phosphorylate serine residues the alternative

phospho-acceptors threonine and tyrosine have not been examined. Intriguingly, the mouse PKR kinase was originally identified as TIK in a screen for tyrosine kinases. However, in this study there was no evidence that PKR was phosphorylated on or that PKR could phosphorylate other proteins on tyrosine residues. To further investigate the substrate specificities of the eIF-2α kinases and as a possible means to examine the differences between the serine/threonine and tyrosine kinases we mutated the serine-51 residue in eIF-2α to threonine and tyrosine. Yeast cells expressing the eIF-2α-S51T allele were practically indistinguishable from wild-type cells with a good growth rate and the ability to grow under amino acid starvation conditions, thus indicating that GCN2 can phosphorylate the threonine at residue-51 on the eIF-2α-S51T protein. In addition, high level expression of PKR in the eIF-2α-S51T strain was lethal again demonstrating that the eIF-2α kinases can phosphorylate a threonine residue, and that the phosphothreonine residue maintains the normal regulation of eIF-2B.

When serine-51 was replaced with tyrosine initial results suggested that tyrosine was not a substrate. Yeast strains expressing GCN2 and carrying the eIF-2α-S51Y allele are sensitive to amino acid starvation conditions suggesting that GCN2 cannot phosphorylate eIF-2α-S51Y. In addition high level expression of PKR in the eIF-2α-S51Y strain is not lethal. Both of these results are consistent with the eIF-2α kinases being unable to phosphorylate tyrosine residues. However, upon closer inspection a very modest slow-growth phenotype was observed in eIF-2α-S51Y strains expressing high levels of PKR. In addition, these strains expressing PKR to high levels could grow under amino acid starvation conditions suggesting that PKR may be phosphorylating the tyrosine residue and inducing GCN4 expression. To provide convincing evidence that PKR can in fact phosphorylate eIF-2α-S51Y, isoelectric focusing analysis was performed. A signal consistent with phosphorylation of eIF-2α was observed in strains expressing PKR and either wild-type eIF-2α or eIF-2α-S51Y, but not eIF-2α-S51A. In addition, the phosphorylation of eIF-2α-S51Y was dependent on wild-type PKR and was not observed with an inactive mutant of PKR. To confirm that the eIF-2α-S51Y protein was indeed phosphorylated on tyrosine the immunoblot from the isoelectric focusing gel was probed with anti-phosphotyrosine antibodies. A signal was specifically detected in the strains expressing both PKR and eIF-2α-S51Y, but not wild-type eIF-2α. Thus PKR can phosphorylate a tyrosine residue in vivo. Surprisingly, $GCN2^c$ kinases were also found to phosphorylate eIF-2α-S51Y when examined on the isoelectric focusing gels despite the lack of any noticeable phenotypes. The finding that PKR can phosphorylate a tyrosine residue in vivo raises the possibility that alternate substrates for PKR or any of the eIF-2α kinases may be phosphorylated on tyrosine residues rather than exclusively on serine residues.

Proposed Course of Project:

1. eIF-2α mutants defective for translational regulation. We plan to continue our analysis of eIF-2α mutants identifying alleles that fail to properly induce GCN4 expression under amino acid starvation conditions. More importantly, we will examine the phosphorylation of serine-51 in the mutant proteins using the isoelectric focusing gels. While only a single mutation has been identified that severely impairs phosphorylation, a few mutations have been identified that appear to reduce phosphorylation. By constructing chimeric eIF-2α proteins containing combinations of mutations that alone modestly impair phosphorylation, we hope to obtain additional alleles that are severely impaired for phosphorylation by GCN2 in vivo. The eIF-2α alleles that are defective for phosphorylation by GCN2 in vivo will also be examined both in vivo and in vitro with PKR. The ability of these alleles to suppress the toxicity of high level PKR expression in yeast will be monitored, and the ability of PKR to phosphorylate these proteins will be examined both in vivo (using the isoelectric focusing gels) and in vitro. The in vitro kinase assays will employ either recombinant PKR or PKR immunoprecipitated from yeast and the substrate will be

recombinant eIF-2α alleles expressed using bacterial expression systems. Alleles of eIF-2α that are defective for phosphorylation by PKR will be used in a reversion analysis to identify PKR mutants with altered substrate specificity. Pools of mutant PKR genes will be introduced into yeast cells expressing the mutant eIF-2α alleles, and PKR mutants that restore phosphorylation will be identified as causing a slow-growth phenotype or increased resistance to amino acid starvation conditions.

2. Analysis of the vaccinia virus K3L protein. The first priority is to determine if the superactive K3L mutants bind to PKR with greater avidity. As discussed above, K3L can be co-immunoprecipitated with PKR and we are now varying conditions to see if K3L-H47R binds tighter than wild-type K3L. Similarly, the loss-of-function mutants in K3L will also be examined using the co-immunoprecipitation assay to determine if these mutations alter the binding affinity of K3L for PKR. A new screen for additional K3L upmutants will be performed. Through the identification of key residues in the K3L protein we hope to uncover the recognition elements the kinases use when modifying eIF-2α. In a second screen we will identify PKR mutants that are resistant to K3L. The K3L upmutant K3L-H47R will be introduced into a yeast strain under the control of a GAL promoter. Wild-type PKR will be non-toxic in this strain due to inhibition by K3L. The PKR gene also under GAL control will be randomly mutated and a pool of mutant PKR genes will be introduced into the yeast strain expressing K3L-H47R. PKR mutants resistant to K3L-H47R will be identified as causing a slow-growth or no-growth phenotype on galactose medium. These PKR mutants will also be examined to determine their resistance to the various K3L upmutants uncovered in the other screens. The identification of PKR mutants resistant to K3L will help identify the substrate binding region in PKR; and through the examination of the various K3L and PKR mutants we may be able to map the interacting surfaces in PKR and K3L. These results should help identify how PKR and the other eIF-2α kinases interact with the authentic substrate eIF-2α.

3. Analysis of the baculovirus pk2 protein. A primary goal for this study is to determine how pk2 inhibits the eIF-2α kinases. First, we will examine eIF-2α phosphorylation in yeast cells expressing pk2 and various eIF-2α kinases. Based on preliminary results we expect pk2 to lower eIF-2α phosphorylation levels. Two models can be proposed for the suppressing activity of pk2: (1) pk2 binds to eIF-2 and prevents the eIF-2α kinases from gaining access to their substrate, or (2) pk2 heterodimerizes with the eIF-2α kinases and the heterodimers are less active. According to the first model it would be expected that overexpressing eIF-2 or eIF-2α alone would reverse the effects of pk2. While overexpression of GCN2 or another eIF-2α kinase would be expected to counteract pk2 according to the second model. High level expression of pk2 causes a slow-growth phenotype in yeast cells independent of any eIF-2α kinases. The cause of this slow-growth phenotype is unknown, however suppressors of the slow-growth may provide insights into proteins which interact with pk2. To identify dosage-dependent suppressors the pk2 gene under the control of the GAL promoter will be introduced into a yeast cell. This strain will subsequently be transformed with a high copy-number library of yeast genes and fast growing transformants will be identified. Prior to this random screen for dosage-dependent suppressors, various candidate genes (for example, eIF-2α and GCN2) on high copy number vectors will be tested. While it is not known if the slow-growth phenotype caused by high-level expression of pk2 is related to the ability of pk2 to suppress the eIF-2α kinases, the identification of proteins that interact with pk2 is of interest since these proteins may also interact with the eIF-2α kinases. Another means to identify proteins that interact with pk2 is to perform co-immunoprecipitation reactions and examine if candidate proteins such as eIF-2 can be co-precipitated with pk2. To facilitate this analysis we have engineered a c-myc epitope tag on the carboxyl-terminus of pk2.

In addition to examining authentic pk2, we will continue our examination of the related domain from the eIF-2α kinases. As reported above, the corresponding region of the GCN2 kinase will act to suppress at least two different GCN2^c kinases. Similar regions of PKR and HRI have also been examined, however

these proteins fail to suppress either PKR or GCN2. A caveat in these results is that the amino-terminal boundary is not exactly the same for the various constructs, therefore we will express several different PKR and HRI constructs varying the amino-terminus of the protein to see if any of these truncated kinase domains can inhibit the activity of a wild-type eIF-2α kinase (GCN2 or PKR).

4. Substrate specificity of the eIF-2α kinases. This project is close to completion. A quantitative measure of the ability of the various combinations of kinases and substrates to activate GCN4 expression will be obtained by measuring the expression from a GCN4-lacZ allele. In addition, to even more convincingly show that the eIF-2α-S51Y protein is phosphorylated on a tyrosine residue, extracts from various cells will be treated with phosphotyrosine-specific phosphatases prior to analysis using the isoelectric focusing gels. The phosphotyrosine phosphatase should specifically affect eIF-2α-S51Y but not the wild-type protein. Mutant alleles of the GCN2 or PKR kinase that more efficiently phosphorylate eIF-2α-S51Y could be identified to increase our understanding of the structural differences that distinguish the tyrosine-specific from the serine/threonine class of protein kinases, however, these projects are not planned for the immediate future.

5. Molecular analysis of yeast FUN12. The recent report of the DNA sequence of the entire yeast chromosome I identified a novel gene termed FUN12 that shares amino acid sequence similarity to bacterial IF-2 proteins. This protein is not the yeast mitochondrial IF-2 because this latter gene has already been identified elsewhere in the genome. IF-2 in bacteria functions to deliver the Met-tRNA$_i^{Met}$ to the ribosome, the same role performed by eIF-2 in eukaryotes. Since yeast already contains eIF-2, the function of FUN12 is unknown. The translational regulation of GCN4 is exquisitely sensitive to translation initiation levels and especially the levels of Met-tRNA$_i^{Met}$, so the regulation of GCN4 expression is an ideal system to study FUN12.

Preliminary results have revealed that overexpression of FUN12 will suppress the slow-growth phenotype of at least one of the GCN2^c kinases. This early result suggests that FUN12 functions in cytoplasmic translation initiation in yeast. To further explore the role of FUN12 in translation initiation, the FUN12 gene will be randomly mutated and the mutants will be screened to identify alleles that alter GCN4 translational regulation. We will screen for both alleles that block induction of GCN4 expression under amino acid starvation conditions as well as alleles that lead to high unregulated levels of GCN4 even in non-starvation conditions. The identification of either class of mutation will confirm that FUN12 functions in translation initiation. During this screen we will also identify any temperature sensitive (ts$^-$) alleles of FUN12. The biochemical analysis of FUN12 ts$^-$ mutants should help define the step of translation at which FUN12 acts. If the mutant screens fail to identify any interesting FUN12 alleles, the suppression of the GCN2^c kinases can be used in an analysis of the structural domains of the FUN12 protein. In addition to random mutants, site-directed mutations will be introduced into the FUN12 GTP-binding motif. Mutations in this region of bacterial IF-2 have been identified that lead to a slow-growth phenotype, we will examine the effects of the corresponding mutations in yeast FUN12. The identification of a protein in yeast showing homology to bacterial IF-2 proteins is quite surprising, through molecular genetic analysis of this gene in yeast we hope to identify the role for this protein in eukaryotic translation. These results take on added significance with our recent identification of an expressed sequence tag (EST) from mouse that bears striking homology to FUN12, raising the possibility that this protein is conserved in humans as well.

Significance to Biomedical Research and the Program of the Institute.

The eIF-2α kinases play a key role in the antiviral and stress responses of mammalian as well as yeast cells. The ability of cells to alter gene expression under stress conditions is essential to the survival of the organism. In addition, the genetic characterization of substrate recognition by the eIF-2α kinases should provide insights into the mechanism of substrate recognition by other protein kinases. Finally, increasing our understanding of viral inhibitors of the eIF-2α kinases will not only provide insights into the molecular mechanisms regulating these kinases, but may also identify ways to defeat these viral defense mechanisms.

Publications:

Dever TE, Yang W, Astrom S, Bystrom AS, Hinnebusch AG. Modulation of $tRNA_i^{Met}$, eIF-2 and eIF-2B expression shows that GCN4 translation is inversely coupled to the level of the eIF-2•GTP•Met-$tRNA_i^{Met}$ ternary complexes, Mol. Cell. Biol. (In Press).

LME-FY95

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01900-05 LME

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Developmental Regulation of Differential Gene Expression

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

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INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
15	15	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.)*

Our work is focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogeneis. Progress has been achieved in the following key areas:

1. We have determined that nucleosome mobility has a key role in facilitating transcription of chromatin templates and that linker histones repress transcription by restricting this mobility.

2. We have determined that chromatin structure has an essential role together with Xenopus heat shock transcription factor in regulating Xenopus hsp70 gene transcription in Xenopus oocytes.

3. We have determined that DNA methylation does not influence the association of linker histones with DNA, and that incorporation of HMG14 and 17 into nucleosomes during chromatin assembly does influence chromatin compaction.

4. We have found that nucleosome dilution in vitro can facilitate transcription factor access to nucleosomal DNA in vitro.

5. We have examined the nucleic acid binding specificities of two proteins capable of interacting with either DNA or RNA. TFIIIA which binds specifically to 5S rRNA and 5S DNA, does not interact specifically with RNA-DNA heteroduplexes countaining 5S RNA and DNA sequences. FRGY2 is found to recognize specific RNA sequences via the highly conserved cold shock domain.

Objectives:

The object of this project is to determine the molecular processes responsible for establishing and maintaining stable states of gene activity during vertebrate development.

Methods Employed:

High resolution gel electrophoresis is used to resolve DNA sequence at the nucleotide level, various chemical and enzymatic probes determine the nature and pattern of transcription factor binding to DNA. Transcription factors and RNA polymerase are fractionated and purified by standard chromatographic procedures. High efficiency in vitro transcription assays are used to assess the function of transcription factor binding to specific DNA sequences and the function of transcription factor interactions with other transcription factors. These interactions are quantitated further by competitive footprinting and gel retardation assays. Chromatin structure of active and inactive genes is assessed using enzymatic and chemical probes, followed by gel electrophoresis and sucrose gradient sedimentation. Histones and transcription factors associated with chromatin are monitored by electrophoresis and immunoblotting using polyclonal antibodies. Sub-cellular localization is assessed using immofluorescence and microscopy. Chromatin is assembled in vitro using cell-free preparations of Xenopus eggs and embryos. Microinjection of oocytes and embryos is used to assess the functional role of DNA sequence and protein-nucleic acid interactions in vivo. mRNA is isolated, cDNA synthesized and fusion proteins expressed in bacteria. Mutant proteins are constructed by reverse genetic techniques and their functions assayed in vitro as described above.

Major Findings:

GENE REGULATION DURING EARLY EMBRYOGENESIS

Constraints on transcriptional activator function contribute to transcriptional quiescence during early Xenopus embryogenesis

We have examined the cause of transcriptional quiescence prior to the mid-blastula transition (MBT) in Xenopus laevis. We have found distinct requirements for transcription of class II and class III genes. An artificial increase of the amount of DNA present within the embryo over the amount of DNA present within the embryo over that found at the MBT allows precocious transcription of tRNA genes, but not of the adenovirus E4 or human cytomegalovirus (CMV) promoters. Thus titration of an inhibitor by exogenous DNA determines class III but not class II gene activation. We have demonstrated that the action of the inhibitor depends on the association of core histones with DNA. The addition of exogenous TBP, together with an increase in the amount of DNA within the embryo, allows significant basal transcription of class II genes prior to the MBT, whereas it does not increase transcription of tRNA genes. To examine the activation of transcription above basal levels, we used a defined minimal promoter containing five Gal4 binding sites and the activator Gal4-VP16. Precocious transcriptional activation is directed by Gal4-VP16 prior to the MBT, demonstrating that a functional transcriptional machinery exists at this early developmental stage. Furthermore, since this activation can occur in the absence of exogenous TBP or chromatin titration, a transcription factor that can penetrate chromatin is

sufficient for recruitment of this machinery to a promoter. Our results support the hypothesis that the temporal regulation of transcription during early embrogenesis in Xenopus reflects not only a titration of inhibitors by DNA, but also a deficiency in the activity of transcriptional activators prior to the MBT.

The heat shock response in Xenopus oocytes, embryos and somatic cells: an essential regulatory role for chromatin

The heat shock response in Xenopus laevis has been reported to be developmentally regulated at the transcriptional level. We find that the heat shock response of an exogenous Xenopus hsp70 gene introduced into Xenopus oocytes, embryos, and somatic cells is dependent on the transcriptional assay conditions employed. Under conditions of efficient chromatin assembly, transcription from the Xenopus hsp70 gene promoter is repressed in oocytes and embryos, yet the promoter responds to heat shock by activating transcription. Under conditions of inefficient chromatin assembly, the Xenopus hsp70 gene is constitutively active in oocytes and somatic cells. Our results resolve previous controversy concerning the existence of a heat shock response for the hsp70 promoter in oocytes and illustrate the importance of considering chromatin assembly as a contributory factor in reconstructing the developmental control of gene expression.

The role of chromatin and Xenopus heat shock transcription factor (XHSF1) in the regulation of transcription from the Xenopus hsp70 promoter in vivo

Xenopus oocytes activate transcription from the Xenopus hsp70 promoter within a chromatin template in response to heat shock. We have isolated and characterized a cDNA encoding Xenopus heat shock transcription factor (XHSF1) causes the activation of the wild type hsp70 promoter within chromatin. XHSF1 activates transcription at normal growth temperatures (18°C), but heat shock (34°C) facilitates transcriptional activation. Titration of chromatin in vivo leads to constitutive transcription from the wild type hsp70 promoter. The Y-box elements within the hsp70 promoter facilitate transcription in the presence or absence of chromatin. The presence of the Y-box elements prevents the assembly of canonical nucleosomal arrays over the promoter and facilitates transcription. In a mutant hsp70 promoter lacking Y-boxes, exogenous XHSF1 activates transcription from a chromatin template much more efficiently under heat shock conditions. Activation of transcription from the mutant promoter by exogenous XHSF1 correlates with the disappearance of a canonical nucleosomal array over the promoter. Chromatin structure on a mutant hsp70 promoter lacking Y-boxes can restrict XHSF1 access, however on both mutant and wild type promoters chromatin assembly can also restrict the function of the basal transcriptional machinery. We suggest that chromatin assembly has a physiological role in establishing a transcriptionally repressed state on the Xenopus hsp70 promoter in vivo.

Nuclear localization and transcriptional activation by Xenopus heat shock transcription factor (XHSF1): the definition of dominant negative mutants that inhibit the heat shock response in Xenopus oocytes

We have defined regions of the Xenopus heat shock transcription factor XHSF1 that are essential for nuclear accumulation of HSF and transcriptional activation of the Xenopus hsp70 promoter within Xenopus oocyte nuclei. In addition we have made use of mutant XHSF1 proteins to establish that distinct molecular mechanisms exist capable of inhibiting heat inducible transcription from the Xenopus

hsp70 promoter in Xenopus oocytes. Deletion of the carboxyl (C)-terminal 38 amino acids of XHSF1 prevents nuclear accumulation and transcriptional activation, however expression of this truncated protein does not impede the endogenous heat shock response. Deletion of a further segment of HSF including the C-terminal hydrophobic domain, but retaining the amino (N)-terminal DNA-binding and trimerization domains creates proteins that exert a dominant negative influence on the heat shock response. Inhibition of heat inducible transcription by these truncated proteins depends on their capacity to retain wild type HSF in the cytoplasm. In contrast, deletion of the N-terminal 23 amino acids disrupts DNA binding and transcriptional activation but does not influence the capacity of HSF to localize to the oocyte nucleus. This N-terminal deletion mutant also functions as a dominant negative inhibitor of the heat shock response. Thus mutant HSF proteins can exert dominant negative effects through two independent pathways that operate either in the nucleus or cytoplasm.

CHROMATIN STRUCTURE AND FUNCTION

A positive role for nucleosome mobility in the transcriptional activity of chromatin templates: restriction by linker histones

We have established a model system for investigation of the selective repression of transcription from nucleosomal templates by linker histones. We make use of physiologically spaced dinucleosomal templates in which it is possible to assay both chromatin structural and transcriptional characteristics. We find that nucleosome mobility is a characteristic of transcriptionally competent chormatin templates. Reconstitution of chromatin with linker histones restricts nucleosome mobility and locks the nucleosome into a unique position. This fixation of histone-DNA contacts is concomitant with transcriptional repression. Thus, stable states of gene expression can be established at the nucleosomal level.

Disruption of reconstituted nucleosomes: the effect of particle concentration, $MgCl_2$ and KCl concentration, the histone tails and temperature

We have found that reconstituted nucleosome cores containing specific DNA sequences dissociate on dilution. This disruption of histone-DNA contacts leading to the release of free DNA is facilitated by the presence of the core histone tails, $MgCl_2$ (5mM), KCl (60mM), and temperatures above 0°C. Under reaction conditions that are commonly used to assess trans-acting factor access to nucleosomal DNA, histone-DNA contacts are on the threshold of instability. We have found that dilution of reconstituted nucleosomes containing a TATA box can facilitate TBP access to DNA.

Methylation at CpG sequences does not influence histone H1 binding to a nucleosome including a Xenopus borealis 5S rRNA gene

We have found that methylation of the 12 dinucleotide CpGs within a GC-rich DNA fragment containing a Xenopus borealis 5S rRNA gene does not influence histone H1 binding to naked or nucleosomal 5S DNA. Thus a simple mechanism in which histone H1 selectively associates with nucleosomes containing methylated CpG cannot explain the repressive effects of methylation on gene activity.

Incorporation of chromosomal proteins HMG-14/HMG-17 into nascent nucleosomes induces an extended chromatin conformation and enhancers the utilization of active transcription complexes

In collaboration with M. Bustin (NCI) we have examined the role of chromosomal proteins HMG-14 and HMG-17 in the generation of transcriptionally active chromatin in a Xenopus laevis egg extract which supports complementary DNA strand synthesis and chromatin assembly. Chromosomal proteins HMG-14/HMG-17 enhanced transcription from a chromatin template carrying a 5S rRNA gene, but not from a DNA template. The transcriptional potential of chromatin was enhanced only when these proteins were incorporated into the template during, but not after, chromatin assembly. HMG-14 and HMG-17 stimulate transcription by increasing the activity, and not the number, of transcribed templates. They unfold the chromatin template without affecting the nucleosomal repeat or decreasing the content of histone B4. We suggest that HMG-14/HMG-17 enhance transcription by inducing an extended conformation in the chromatin fiber, perhaps due to interactions with histone tails in nucleosomes. By disrupting the higher order chromatin structure HMG-14/HMG-17 increase the accessibility of target sequences to components of the transcriptional apparatus.

NUCLEIC ACID BINDING SPECIFICITIES OF THE MULTIFUNCTIONAL XENOPUS OOCYTE PROTEINS TFIIIA AND FRGY2

The interaction of TFIIIA with specific RNA-DNA heteroduplexes

We have examined the association of transcription factor TFIIIA with RNA-DNA heteroduplexes containing sequences from the Xenopus borealis 5S rRNA gene. Under conditions where TFIIIA selectively binds to 5S rRNA or to the internal control region of the 5S rRNA gene, no specific association of TFIIIA with RNA-DNA heteroduplexes containing either strand of 5S DNA could be detected. Our results exclude specific models of TFIIIA recognition of the internal control region in an A-type DNA configuration or of DNA-RNA hybrids during the process of transcribing the 5S rRNA gene.

Sequence specific RNA recognition by the Xenopus Y-box proteins: an essential role for the cold shock domain

The Xenopus Y-box protein FRGY2 has a role in the translational silencing of masked maternal mRNA. We have determined that FRGY2 will recognize specific RNA sequences. The evolutionarily conserved nucleic acid-binding cold shock domain is required for sequence-specific interactions with RNA. However RNA-binding by FRGY2 is facilitated by amino and carboxyl terminal regions flanking the cold shock domain. The hydrophilic carboxyl-terminal tail domain of FRGY2 interacts with RNA independent of the cold shock domain, but does not determine sequence-specificity. Thus, both sequence-specific and non-specific RNA recognition domains are contained within the FRGY2 protein.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01901-01 LME

PERIOD COVERED

October 1st, 1994 to September 30, 1995

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

Gene Regulation by Thyroid Hormone during Tissue Remodeling

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

PI: Yun-Bo Shi, Senior Staff Fellow LME/NICHD

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Melissa Stolow, Adjunct Scientist LME/NICHD
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COOPERATING UNITS *(if any)*

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Section of Mol. Biol., LME, NICHD (A. Wolffe)

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Unit on Molecular Morphogenesis

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-5455

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.8	4.8	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 research in the Unit of Molecular Morphogenesis is focussed on the understanding of the molecular mechanism of amphibian metamorphosis. The control of this developmental process by thyroid hormone (TH) offers a unique paradigm in which to study genes that are important for post-embryonic organ development. We began to study metamorphosis by choosing the remodeling of the tadpole intestine in Xenopus laevis as a model system. The tadpole intestine is a simple tubular structure consisting of primarily a single layer of primary epithelial cells. During metamorphosis, it is transformed into a multiply folded adult epithelium with elaborate connective tussue and muscles through specific cell death and selective all proliferation and differentiation. We have isolated and identified many TH-response genes in the intestine during this transition. Among them are the TH receptor (TR) β genes. Analysis of the receptor gene expression in different tissues shows that not only TRβ but also TRα and RXR genes are regulated in an organ-dependent manner during metamorphosis. RXRs are known to be able to form heterodimers with TRs. Indeed, Xenopus TR/RXR heterodimers can bind to their binding site even in a chromatinized template and activate or repress transcription in the presence or absence of TH, respectively. Thus, our results strongly suggest TR/RXR heterodimer are the functional complexes mediating the effect of TH during metamorphosis. In addition, we have also obtained evidence that two NFI genes, which are also regulated by TH, are involved in the adult organ development. Finally, a putative morphogen, hedgehog, and the matrix metalloproteinase, stromelysin-3, appear to participate in the regulation of cell-cell and cell-ECM interactions during intestinal remodeling.

Project Description:

Objectives:

To understand the molecular mechanism of gene regulation by thyroid hormone and the roles of thyroid hormone response genes, especially those influence cell-cell and cell-ECM interactions, during frog organ development.

Methods Employed:

In vitro biochemical studies of protein function and nucleic acid manipulation; functional investigation of transcription factors in oocytes, in situ hybridization and immunohisto-chemical analyses of gene expression.

Major findings:

Regulation and function of TH receptors (in collaboration with Dr. Alan Wolffe)

TH is known to regulate gene expression by controlling the transcription of target genes directly through thyroid hormone receptors (TRs). Work from other laboratories on TRs in birds and mammals has shown that TRs belong to the superfamily of the steroid hormone receptors. These receptors are sequence specific DNA binding proteins that regulate the transcription of genes containing their binding sites (TREs or thyroid hormone response elements for TRs) in a ligand dependent manner. While TRs can bind to TREs as monomers and homodimers, they most likely function as heterodimers formed with RXRs (9-cis retinoic acid receptor).

Toward the first step to investigate the roles of TR/RXR during metamorphosis, we have determine the mRNA levels of TR (TRα and TRβ) and RXR (RXRα and RXRγ) genes during metamorphosis in different organs, the limb, tail, and intestine, which undergo very contrasting changes, i.e. total resorption, de novo development, and remodeling, respectively. In general, TR and RXR genes are coordinately regulated in all organs. Thus, high levels of their mRNA are present during early stages of limb development when morphogenesis occurs and in the tail toward the end of metamorphosis when it is being resorbed. In the intestine, moderate levels are present throughout the remodeling period. Furthermore, by introducing TRs and/or RXRs through microinjection of their mRNAs into Xenopus oocytes, we have established an in vivo TH-response system. By using as a reporter the promoter of Xenopus TRβA genes, which we have shown previously to be regulated by TH, we have demonstrated that TRs or RXRs alone have little or no effect on the activity of the promoter. On the other hand, coinjection of TR and RXR mRNAs lead to transcriptional repression of the basal activity in the absence of TH and strong activation of the promoter when TH is present. In vitro DNA binding experiments indicate that only TR/RXR heterodimers bind strongly to the TRE in the TRβA promoter. Thus, while we have not analyzed the levels of TR and RXR proteins during development, our analysis of mRNA levels and functional studies of TRs and RXRs provide strong evidence that TRs function as heterodimers with RXRs during metamorphosis.

To investigate the mechanism of transcription regulation by TR/RXR heterodimers, we again made use of the oocyte system. In collaboration with Dr. A. Wolffe, we have reconstituted in vivo the TH-regulated TRβA promoter in chromatin and studied the effect TR/RXR on the promoter activity. Dr Wolffe's group has shown previously that when a double stranded (ds) promoter plasmid is injected

into oocytes, it are chromatinized such that the promoter is highly active. In contrast, when a single stranded (ss) promoter plasmid is injected into oocytes, it is rapidly replicated and undergoes replication-coupled chromatin assembly to produce a template of a very low basal promoter activity. We have found that heterodimers of TR and RXR within the chromatin templates, generated by injection of either ds or ss DNA containing the TRβA promoter can alteratively repress or active transcription dependent upon the absence or presence of TH. Efficient transcriptional repression requires the presence of unliganded TR/RXR heterodimers during replication-coupled chromatin assembly. In vivo DNaseI footprinting directly demonstrate that TR/RXR heterodimers can bind to the TRE in chromatinized templates independently of TH. Such binding also occurs in vitro on a reconstitate nucleosome containing the TRE. While TR/RXR binding alone does not affect the nucleosome in vitro or chromatin structure in vivo, the addition of TH, which results in active transcription in vivo, leads to strong chromatin disruption as revealed by micrococcal nuclease digestion of the chromatin templates. Interestingly, this disruption is dependent on TH but can occur even when transcription is blocked by α-amanitin. While the nature of this disruption is unclear, these results indicate that the additon of TH leads to a conformational change in the TR/RXR heterodimer to allow either the recruitment of other factors and/or conformational changes in transcriptional machinery, which in turn causes chromatin structural alterations and activation of the promoter.

Regulation of NFI genes during organ development

We have identified two of the TH-response genes in the intestine as members of the nuclear factor I (NFI) family. Mammalian NFIs are transcription factors that are believed to also participate in DNA replication. The two Xenopus NFI genes are highly homologous to mammalian and avian NFI genes. Their deduced amino acid sequences are over 84-95% identical to their respective counterparts in birds and mammals. In contrast, the two Xenopus NFIs are much less homologous to each other, sharing only 58% homology which largely resides in the DNA binding domain at the amino terminus. However, both NFIs can bind to a consensus NFI binding site and activate the transcription of a promoter bearing the binding site. Northern blot analysis reveals that both NFI genes are regulated in a tissue- and developmental stage-dependent manner. They are first activated, independently of thyroid hormone, to low levels at stages 23/24, around the onset of larval organogenesis. After stage 54, their mRNA levels are dramatically up-regulated by endogenous TH. High levels of their mRNAs are present during tail resorption, limb morphogenesis, and intestinal remodeling, even though each of which occurs at very developmental different stages. Furthermore, gel mobility shift assay shows that proteins recognizing the NFI binding site are present in different organs and their levels are regulated similarly to NFI mRNA levels. These results thus strongly suggest that NFIs play important roles during post-embryonic organ development, in contrast to the general belief that NFIs are ubiquitous transcription factors.

Cell-cell and cell-extracellular matrix interactions during tissue remodeling (in collaboration with Dr. Atsuko Ishizuya-Oka)

Each metamorphosing organ consists of many different cell types. Some of them are in direct contact with each other. Others are separated by extracellular matrices (ECM). Proper interactions among these cells are likely to be important for tissue remodeling. Thus, it is not supprising that among the TH-response genes are those encoding a matrix metalloproteinase and a putative morphogen, hedgehog.

The hedgehog gene was first cloned as a segment polarity gene in Drosophila. Its homologs in mammals and birds are capable of functioning as morphoges in processes such as neural induction and limb morphogenesis. We have found that the Xenopus hedgehog gene is activated by neurula stage during embryogenesis. Whole mount in situ hybridization analysis showed that at these early stages, the hedgehog gene is expressed in the notochord and floor plate similar to that observed in other animal species. During metamorphosis, hedgehog mRNA was found to be high in the intestine during the period when adult epithelial morphogenesis takes place. Thus, Xenopus hedgehog seems to play a role in organ development during both embryogenesis and metamorphosis.

Cell-cell interactions can also be regulated through the modification ECM. We have previously reported the characterization of Xenopus stromelysin-3 (ST-3) gene, which encode a putative matrix metalloproteinase (MMP). ST-3 is highly expressed in both the tail and intestine when extensive cell death takes place. In the limb, it is expressed at low levels during limb morphogenesis when cell death occurs in the interdigital region. However ST-3 is activated prior to any noticeable cell death. In contrast to several other MMP genes. Its mRNA is present only in the fibroblastic cells but not in the epithelial cells that will undergo apoptosis. Thus, if ST-3 plays a role in epithelial degenration, it is likely through alteration of cell-cell and/or cell-ECM interactions. In support of this, in collaboration with Dr. Ishizuya-Oka, we have found by in situ hybridization that ST-3 is highly expressed in the fibroblastic cells surrounding the epithelium at the time when basal lamina, which separates the epithelium and the connective tissue, becomes thick. Although the basal lamina is thick at this time, it allows extensive cell-cell contact and cell-migration through it, in contrast to the thin but apparently less permaeble pre- or post-metamorphic basal lamina. While the role of ST-3 in the ECM modifciations remains to be established, such ECM changes are likely to be important of larval epitheial cell death and adult epitheial proliferation and differentiation.

Proposed course of projects:

Gene regulation by thyroid hormone receptors

Our studies so far have shown that TR/RXR heterodimer can regulate transcription from a chromatinized template. The activation in the presence of TH leads to chromatin disruption. We plan to identify regions of TR and RXR that are important for chromatin disruption and transcription regulation. We would also like to determine the mechanism of this disruption. Do TR and RXR interact with any factors to bring about chromatin structural changes? What are these factors, if they do? Are these also the same factors that are involved in promoter activation?

In our analysis of the receptor function in vivo, we found that TRαB, encoded by one of the two TRα genes in Xenopus, failed to bind to a TRE or regulate transcription. Sequence comparison showed several amino acid differences between TRαA and TRαB. We would like to determine if any of these changes are responsible for the inactivity of TRαB and whether it is due to the failure to bind a TRE or inability of the receptor to interact with transcriptional machinery.

Finally, we are also interested to study TR/RXR function in developing embryos. We will approach this by introducing TR/RXR into fertilized eggs together with a reporter promoter. Alternatively, we can analyzed the expression of endogenous genes in these embryos with over-expressed TR/RXR or their mutants.

Cell-cell and cell-ECM interactions

We plan to investigate further how hedgehog and stromelysin-3 participate in intestinal remodeling. We intend to obtain antibodies against both proteins and analyzed the temporal and spatial distribution of the proteins by Western blot and immunohistochemistry. To study the biological functions we intent to establish primary intestinal epithelial cell and/or organ cultures under conditions such that they will respond to TH just like in intact tadpoles. At same time, we will try to generate functional hedgehog and stromelysin-3 proteins by stable cell transfection. Once these are achieved, we can directly investigate the function of hedgehog and stromelysin-3 by adding over-produced proteins to organ or cell cultures or using antibodies to block the function of these proteins and examining the response of the cells or organs to TH.

ECM is a complex structure made of many components. It is very likely that MMPs other than stromelysin-3 are involved. We plan to investigate this by analyzing the mRNA or protein levels of several other MMPs by Northern and Western blot analysis. Their regulation and function will be studied in comparison to these of stromelysin-3.

Significance to biomedical research and the program of the Institute

While metamorphosis is unique to lower organisms, the basic developmental process is similar to that in mammals. This is especially true for post-embryonic organ development. The regulation of the process by TH offers an unique opportunity to identify the genes which are important for organ development. The similarity in function of organs and in their developmental process between frogs and mammals suggest that the same genes which are involved in metamorphosis will likely be important for mammalian organogenesis. In addition, gene regulation by hormones is present in all vertebrate organisms. TH regulation is an extreme example of hormonal regulation during development. Understanding the mechanism of how TH controls the transcriptional state of target genes, especially in a chromatin context, will be of general interest research to fields on hormones, transcriptional regulation and the effects of chormatin on gene expression.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD01902-01 LME

PERIOD COVERED

October 1, 1994 to September 30, 1995

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

Analysis of the S phase checkpoint in higher eukaryotes

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

PI: Mary Dasso, Senior Staff Fellow LME/NICHD

OTHERS:
Hisato Saitoh, Visiting Associate LME/NICHD
Kathy Steinmann, Adjunct Scientist LME/NICHD
Robert Pu, IRTA Fellow LME/NICHD

COOPERATING UNITS *(if any)*

Dept. of Cell Biology, Johns Hopkins Medical School, Baltimore, MD (W. Earnshaw, Ph.D.); Dept. of Molecular Biology, Holland Laboratories of the American Red Cross, Rockville, MD (W.H. Burgess, Ph.D.); Dept. of Mol. Biol., Grad.

LAB/BRANCH

Laboratory of Molecular Embryology

SECTION

Unit on Cell Cycle Regulation

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892-5430

TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	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.)*

The eukaryotic nucleus is a highly ordered structure that carries out an assortment of complex functions. In order for the nuclear tasks to be carried out properly, they must be temporally and spatially coordinated amongst themselves and with respect to the other functions of the cell. We are investigating how cells send and interpret signals that coordinate these activities with each other and with the cell cycle. Among the proteins that are thought to be important for maintaining nuclear integrity, two proteins that appear to be essential for the spatial and temporal order of the nucleus are Ran and RCC1. These two proteins interact enzymatically with each other, and they are required for almost every nuclear process including RNA transcription and processing, nuclear transport, DNA replication, and cell cycle control. Ran is a small, very abundant Ras-like GTPase that is mainly nuclear, while RCC1 is a chromatin-bound protein that acts as a guanine nucleotide exchange factor (GEF) for Ran. Our goal is to understand RCC1 and Ran at a molecular level and to discover how they interact with other cellular components that regulate cell cycle progression and interphase nuclear activities. We have studied RCC1 and Ran in biochemical assays using purified components and in Xenopus laevis egg extracts. We have also begun to purify other proteins that interact with RCC1 and Ran from Xenopus egg extracts. We have thereby discovered a number of novel proteins that may be important for the activity of this GTPase pathway. This work is complemented by an analysis of chemical agents that bypass S phase cell cycle control in mammalian cells and by a search for the molecular target(s) of these agents. In particular, we are examining the regulation of mRNA stability by checkpoint regulatory systems. We have found a number of candidate mRNAs whose stability appears to be controlled by the replication state of the nuclear DNA and we are currently in the process of characterizing these messages further.

Objectives:

The objective of this unit is to discover the mechanism by which cells transmit and interpret signals that coordinate nuclear activities with each other and with the cell cycle. In particular, we have focussed on understanding the control of cell division with respect to the completion of DNA replication, since mitosis is inhibited by signals from the nucleus until DNA synthesis is complete. We are pursuing two molecular approaches to this problem: First, we are investigating the functions of RCC1 and Ran, two nuclear proteins that are essential for coordination of nuclear activities to the cell cycle. Second, we are characterizing cellular mRNAs whose stability is regulated by this checkpoint mechanism, and whose function is essential for the initiation of mitosis.

Methods Employed:

In vitro biochemical studies of protein function and nucleic acid manipulation; functional investigations of Ran GTPase pathway components in Xenopus laevis egg extracts; Molecular biological analysis of mRNAs in mammalian tissue culture cell lines.

Major Findings:

The RCC1 protein in interphase nuclear function (Collaboration with T. Nishimoto)

The Ran protein is a small GTPase that has been implicated in a large number of nuclear processes, including transport, RNA processing and cell cycle checkpoint control. A similar spectrum of nuclear activities has been shown to require RCC1, the guanine nucleotide exchange factor (GEF) for Ran. We have used the Xenopus laevis egg extract system and in vitro assays of purified proteins to examine how Ran or RCC1 could be involved in these numerous processes. We employed mutant Ran proteins to perturb nuclear assembly and function. The addition of a bacterially-expressed mutant form of Ran (T24N-Ran), which was predicted to be primarily in the GDP-bound state, profoundly disrupted nuclear assembly and DNA replication in extracts. We further examined the molecular mechanism by which T24N-Ran disrupts normal nuclear activity. We found that T24N-Ran binds tightly to the RCC1 protein within the extract, resulting in its inactivation as a GEF. The capacity of T24N-Ran blocked interphase extracts to assemble nuclei from demembranated sperm chromatin and to replicate their DNA could be restored by supplementing the extract with excess RCC1 and thereby providing excess GEF activity. Conversely, nuclear assembly and DNA replication were both rescued in extracts lacking RCC1 by the addition of high levels of wild-type GTP-bound Ran protein, demonstrating that RCC1 is not essential for any function beyond its role as a GEF. Taken together, these results indicate that GTP-Ran is essential for nuclear assembly and DNA replication in Xenopus extracts, and that these processes are blocked when nucleotide exchange is either inhibited by T24N-Ran mutant protein or absent because of RCC1-depletion.

RCC1- and Ran-associated protein in Xenopus extracts

It is currently unknown how the activity of RCC1 is regulated or whether interactions with other nuclear components facilitate its proper localization or control. It was originally thought that RCC1

binds to DNA directly, since it both binds to chromatin in vivo and to DNA cellulose in vitro. However, more recent evidence indicates that RCC1 associates to chromatin through interactions with other proteins. We have therefore been interested in discovering and characterizing proteins that interact with RCC1 and that may serve to regulate its distribution or activity. We sought to identify proteins that interact with RCC1 via their association with a glutathione-S-transferase-RCC1 fusion protein (GST-RCC1) in Xenopus laevis egg extracts. We found that at least four proteins bound specifically to GST-RCC1 in extracts. Consistent with the behavior of endogenous RCC1-containing complexes, magnesium and guanine nucleotides released these proteins from their association to GST-RCC1 protein. Three of these proteins have previously been identified: Ran, RanBP1, hsc70. The fourth protein has an apparent molecular weight of 340 kDa, and had not been previously described in association with RCC1.

We have pursued a similar strategy to examine Ran-interacting proteins (RIPs), looking especially for polypeptides that interact with both RCC1 and Ran. To do this, we incubated a glutathione S transferase-Ran fusion protein in Xenopus egg cytosol, as described for GST-RCC1 above. We found that a number of proteins were associated with the GST-Ran protein, some of which common to both the GST-Ran and GST-RCC1 associated fractions. The common bands had apparent molecular weights of 340 kDa, 72 kDa and 30 kDa. The 72 kDa and 30 kDa bands in the GST-RCC1 fractions had previously been identified as the heat shock cognate protein hsc70, and RanBP1, respectively. There were also bands that did not correspond to proteins associated with RCC1. These bands had apparent molecular weights of 180 kDa, 120 kDa, 92 kDa, 88 kDa, 18 kDa and 10 kDa. We are currently in the process of identifying these peptides and beginning a more complete biochemical analysis of their interactions with Ran.

We have gone on to perform a more extensive characterization of the p340 protein. We raised polyclonal antibodies against p340 and used immunogold electron microscopy (in collaboration with W. Earnshaw and C. Cooke) to localize this protein within Xenopus tissue culture cells. We found that this protein is associated with the cytosolic face of the nuclear pore, as well as with unidentified foci within the cytosol. Our results suggest that it is likely that p340 is a component of a series of fibers that electron microscopy has shown to emanate from the pore into the cytosol. These fibers may serve as docking sites for proteins that will be transported into the nucleus. Thus, it will be important to understand p340's activity in order to understand the mechanism of nuclear transport and how transport is regulated. Toward this end, we purified p340 and subjected it to peptide sequencing (in collaboration with W. Burgess). The peptides revealed that this protein has homology to nuclear pore proteins. Comparison of our sequences with the cDNA of a hamster protein (RanBP2) that was discovered by T. Nishimoto and colleagues suggests that these proteins are homologous. RanBP2 was found in a two-hybrid screen for proteins that interact directly with Ran, and antibodies directed against RanBP2 are capable of blocking nuclear import. The p340/RanBP2 cDNA contains sequence motifs that may provide clues regarding its function: The cDNA sequence contains RanBP1-like sequences (Ran-binding domains), a zinc finger domain and a cyclophilin-like region. Since p340 is a nuclear pore protein that is required for nuclear transport and that interacts with Ran and other nuclear pore components, an understanding of its activity would be a significant step toward understanding the nuclear pore as a whole.

Cloning and characterization of the Xenopus RNA1 and RanBP1 homologues

Like other small GTPases of the ras superfamily, Ran has a low intrinsic GTPase activity that can be activated by interacting with a GTPase activating protein, RanGAP1. RanGAP1 was isolated as a Ran GTPase activator that can increase the hydrolysis of GTP by three orders of magnitude. RanBP1 was purified as a Ran-binding protein and was shown to be able to increase RanGAP1 induced GTP hydrolysis of Ran-GTP. We have begun to examine the cellular roles of RanGAP1 and RanBP1 using the Xenopus egg extract system and a variety of molecular biological techniques. Our goal is to define how these proteins function biochemically, whether and how they are regulated, and how their activity impacts on the control of nuclear functions and cell cycle control. As a first step toward characterizing RanGAP1 and RanBP1's functions, we have cloned the Xenopus RanGAP1 and RanBP1. This was done using degenerate primers corresponding to the amino acid sequences that are conserved among the known RanGAP1 and RanBP1 homologues. These primers were used to perform polymerase chain reaction (PCR) using Xenopus cDNA library as the template to amplify DNA a fragment of putative RanGAP1 or RanBP1 sequence. After confirming the amplified DNA fragments encode RanGAP1 or RanBP1 by sequencing, these fragments were used as the probes to screen the same Xenopus cDNA library to identify clones. The cDNAs of Xenopus RanBP1 and RanGAP1 show that these proteins are well conserved between Xenopus and other species.

These cDNAs have been cloned in a bacterial expression vectors to allow production and purification of recombinant RanGAP1 and RanBP1. An epitope-tagged version of RanGAP1 and RanBP1 have also been produced, so that soluble proteins can be purified in a one-step column chromatography procedure. Such tagged proteins will allow the purification of RanGAP1 and RanBP1 interacting factors, in a manner similar to the purification of RCC1- and Ran-interacting proteins discussed above. The purified recombinant RanGAP1 and RanBP1 proteins that we have produced are currently being used to raise polyclonal antibodies in rabbits. We are making a series of mutants of the RanBP1 protein that will alter sequences that are suspected to be important for its function. We are now expressing these proteins for analysis in biochemical assays and in the egg extract. Another series of mutants is currently being made to allow the expression of mutant RanGAP1 proteins. These proteins and antibodies will be used in the functional studies described below.

The regulation of mRNA stability in the cell cycle

Despite the importance of the DNA replication checkpoint for maintaining genetic stability in eukaryotic cells, many of the intermediary components in the checkpoint pathway coupling the onset of mitosis to the completion of DNA replication remain to be identified. One mechanism by which mitotic onset is controlled is through changes in mRNA stability. At least some of the mRNA required for induction of mitosis that accumulates during S phase arrest in baby hamster kidney (BHK) cells is unstable as long as DNA replication is ongoing. This mRNA is stabilized by drugs that inhibit DNA replication (e.g., hydroxyurea), but becomes unstable again once the drugs are removed and replication resumes. These properties will allow the identification of these mRNAs by the technique of differential display. Using this strategy is important because it has the potential to identify new proteins that are required for mitosis and that are regulated by the DNA replication checkpoint.

The immediate goals of this work are to: a) Use differential display to identify mRNA sequences whose levels decrease when DNA replication resumes in S phase arrested BHK cells. b) Use Northern blot analysis first to confirm that mRNAs identified by differentially displayed sequences are present at higher levels in S phase arrested BHK cells than in cells undergoing DNA replication and then to identify the mRNAs whose differing levels in these two cell populations are the result of differences in mRNA stability. c) Sequence the differential display products to identify conserved sequences as well as sequences found in databases. We have been successful in identifying populations of candidate mRNAs that are regulated by ongoing DNA replication in BHK cells through differential display. We are presently in the process of confirming these data by Northern blot analysis, and we will then characterize the mRNAs by sequencing their cDNAs. Within the coming year, we hope to find mRNA sequences that serve as targets for destabilization by signals indicative of incomplete nuclear DNA replication.

Proposed course of projects:

Characterization of Ran-interacting proteins (RIPs) in Xenopus

We will continue the examination of Ran-interacting proteins (RIPs) by both characterizing unidentified proteins that interact with Ran in Xenopus extracts and by a more in-depth examination of the p340 protein. Unidentified proteins that associate with GST-Ran in Xenopus extracts include proteins of apparent molecular weights 180 kDa, 120 kDa, 92 kDa, 88 kDa, 18 kDa and 10 kDa. We have already examined whether these proteins associate with Ran in a nucleotide-dependent manner and whether they can bind to 32P-GTP bound Ran in an overlay blot assay. During the coming year, we intend to purify the proteins in sufficient quantity to obtain protein sequence data and to attempt to clone the proteins. We then intend to pursue an analysis of each protein that is analogous to the analysis underway for RanBP1 and RanGAP1. We believe that characterizing the proteins that interact with Ran is the best way to achieve a complete molecular picture of the Ran GTPase pathway.

We wish to examine the cellular role of p340/RanBP2 in three stages: First, in order to examine how p340 interacts with other extract proteins, including Ran, RCC1 and the other RIPs, we will express domains of the mammalian RanBP2 protein in E. coli as fusion proteins and purify these domains by affinity chromatography using the fusion peptide. It will be possible to map the interactions between p340 and the other proteins by examining the interactions between the fusion proteins and the p340-associated extract proteins. Using the isolated domains, it will also be possible to test for biochemical activities: For instance, it will be of interest to determine whether the cyclophilin homology domain of p340 has activity as a cis-trans-peptidylprolyl isomerase (PPIase). If it does, we would then seek to determine whether p340's activity can be blocked by cyclosporin A. In this case, a number of experiments will be performed to relate the pharmacological effects of this inhibitor to the activity of p340 in nuclear assembly and transport. Similarly, it will be of interest to determine whether the zinc-finger region of p340 can bind nucleic acids and whether this binding promotes RNA export.

The second stage of our studies on p340 is to undertake a direct analysis of p340's function using Xenopus extracts. This will be accomplished by immunodepletion of the endogenous p340 and examination of how nuclear assembly and transport are affected. We will further examine whether the fusion proteins containing isolated domains of p340 are able to restore activity for nuclear formation,

nuclear transport and DNA replication in p340-depleted extracts. We will also make use of the fusion proteins to determine whether they can act as dominant negative inhibitors of any of these processes. To do this, we will add the fusion proteins in excess prior to the beginning of the nuclear assembly reaction and then determine the effect on nuclear morphology, nuclear transport and DNA replication. Mutations that block biochemical activities of the individual domains will be used to extend this analysis.

Third, we will perform an ultrastructural analysis of the p340 protein. There is currently good evidence that at least part of p340 resides on the outer face of the nuclear pore. However, some antibodies against p340 also recognize the inner face of the pore. It is therefore of interest to determine how p340 is oriented within the pore. To do this, we will localize tagged fusion proteins by immunogold electron microscopy. We would simultaneously raise monoclonal antibodies against domains of the p340 protein and determine whether these antibodies give results that are consistent with the observations from tagged proteins. Not only would these experiments be important for considering structural and functional questions related to p340, but they could also be extended to examine how the distribution of p340 changes in response to nucleotide analogs and to other agents that disrupt nuclear transport. Such experiments could be expected to reveal whether the localization of p340 is dynamic during nuclear transport.

The roles of RNA1 and RanBP1 in cell cycle control and interphase nuclear function

During the coming year, we will investigate the cellular roles of RanBP1 and RanGAP1 in extract depletion studies and mutational studies. In the depletion studies, RanGAP1 or RanBP1 will be removed from extracts using the antibodies that we have recently generated. The depleted extracts will be used in the nuclear assembly, DNA replication and nuclear import assays in order to determine how these proteins are required for interphase nuclear functions. If depletion has a clear effect in one of these assays, purified RanGAP1 and RanBP1 will be used to restore the defects caused by the depletion treatment. [If the defect cannot be rescued, we would examine whether the depletion process removed some other component(s) that is required for nuclear function.] While we expect that such depletion will result in non-functional nuclei, it will be of interest to determine whether defects are solely in one of the nuclear functions, such as envelope formation or nuclear transport. If these depletion experiments are successful, they will also establish an assay system in which to analyze RanGAP1 and RanBP1 mutants.

Mutational studies on RanGAP1 and RanBP1 will be focused on defining the domains of these proteins. As mentioned above, constructs have been or will be made which will allow the expression of mutant proteins or of protein fragments. The bacterially expressed proteins will be used for two types of experiments. First, they will be used in depleted extracts to define the critical domains of each of these proteins. Second, they will be used as affinity reagents in experiments similar to those already discussed for RCC1 and Ran. The combination of these two approaches may simultaneously provide evidence of which domains of these proteins control their localization and function and of what other extract proteins interact with these domains. Finally, dominant negative mutants of both RanGAP1 and RanBP1 will be of great interest if they can be generated, since they would be useful for blocking individual steps in nuclear transport. Such mutant proteins could allow dissection the nuclear transport pathway into sequential steps and the identification proteins that are involved in each of the steps.

Systems regulating mRNA stability in the cell cycle

After confirming that the candidate mRNAs found in our differential screen are regulated in their stability by the replication checkpoint, we will determine the sequences of these mRNAs. We then wish to find regions of the mRNAs that confer instability. To do this, the cDNAs corresponding to these mRNAs will be expressed from expression vectors in BHK cells. We will confirm that the mRNAs expressed from these vectors are regulated in a manner similar to the endogenous mRNA, then we will map the instability region by deletion analysis.

After the sequences responsible for mRNA stability are determined, we will pursue two avenues of experimental investigation: First, we will seek to demonstrate what role the proteins encoded by these mRNAs play in the regulation of mitosis. Destabilization of these mRNAs has been shown to block caffeine-induced premature mitosis. A direct role in caffeine-induced premature mitosis could be demonstrated if premature mitosis does not occur when the expression of a particular protein is blocked through antisense RNA strategies or if premature mitosis is potentiated when we express a message lacking the mRNA destabilizing element. Proteins that override the DNA replication checkpoint control when inappropriately expressed would be expected to interact with or be key components of the checkpoint pathway. Second, we would seek to identify the mechanisms through which the destabilizing sequences confer regulation on these mRNAs. For instance, mRNA stability could be controlled by the regulated binding of a protein to the stability sequence. We would test whether this is also the case for checkpoint regulation of mRNA stability, and if so, we would seek to discover the identity of the binding protein and how it is regulated by the checkpoint pathway.

Significance to biomedical research and the program of the Institute

Orderly progression through the cell cycle is required for the fidelity of transmission of genetic material. Checkpoint controls insure that initiation of later cell cycle events depends on the completion of earlier events. In eukaryotic cells, these controls are not only essential to the timing of events in early development, but also critical for the regulation of cell cycle progression in somatic cells. Our work concerns the molecular mechanism of these controls. For reasons that are discussed above, we have chosen two points at which to investigate the S phase checkpoint: The Ran GTPase pathway and the regulation of mRNA stability. Investigations of the Ran GTPase pathway will contribute to our knowledge of the fundamental cell biology of the nucleus and nuclear transport. Eventually, we anticipate that we will find how these essential nuclear activities are related to the signals that are communicated between the nucleus and the cytoplasm in order to control the cell cycle. In this regard, it is worth noting that an increasing number of oncogenes have been found to be nuclear pore proteins, re-enforcing the notion that nuclear transport control may be critical to the regulation of cellular activity. In a similar manner, we anticipate that a fundamental understanding of the cytosolic mRNA targets of checkpoint control will contribute to our understanding of how the DNA replication checkpoint signaling pathway functions. Once these mRNAs are identified, we will not only be able to investigate downstream mitotic processes for which their protein products are required, but also upstream signal transduction pathways through which information from the nucleus is relayed to cellular components in the cytosol.

Increasing our knowledge of how this checkpoint operates will ultimately help determine whether DNA replication checkpoint failure contributes to disease states such as cancer. If so, this understanding of the cell cycle may contribute not only toward basic biological knowledge but also toward the development of diagnostic tests and treatments for cancer.

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