STUDIES ON CHROMOSOME REPLICATION IN *ESCHERICHIA COLI*

By

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The mode of replication of the chromosome of Escherichia coli has been investigated by measuring the frequencies of chromosomal regions. Known chromosome regions, in the form of the DNA of several F merogenotes purified from Proteus mirabilis F' hybrids, were used to assay region frequencies by DNA reannealing techniques. Both quantitative reannealing procedures and kinetics of renaturation were used to determine the frequencies of chromosomal regions corresponding to the regions present on the F merogenotes.

When all of the data for region frequencies in exponentially growing cells were compared to all reasonable theoretical curves, the data most closely fit a model for chromosome replication having the point of origin near minute 70 on the E. coli genetic map, a simultaneous bidirectional mode of replication in each chromosome, and a terminus near minute 25. The results obtained with E. coli strains K12, B, B/r, C, and 15 were consistent with this model. Detailed measurements with E. coli K12 in the region between minute 40 and minute 70 strongly support this model. Two lines of evidence showed that inte-

grated sex factors did not alter the point of origin or the direction of replication of the chromosome.

Examination of region frequencies in chromosomes of E. coli growing at various growth rates supports the findings of others that initiation of chromosome replication occurs less frequently in cells having longer generation times.

In the past, chromosomes synchronized by one of a number of methods have been used to study the origin and direction of chromosome replication in E. coli. The effectiveness of several of these procedures in terminating chromosome replication was determined. The relative frequencies of a chromosome region near the origin of replication and of a region near the terminus were used to test for the completion of chromosome replication in cells of E. coli K12 subjected to the various synchronizing procedures. Cells subjected to the following were found to have completed chromosome replication without reinitiation: (1) growth of cells to stationary phase; (2) treatment of cells with phenethyl alcohol; (3) treatment of cells with chloramphenicol; (4) treatment of cells with L-valine, which leads to starvation for L-isoleucine. However, allowing cells to deplete a limiting concentration of a required amino acid during growth did not align chromosomes in a completed state, although the method gave reasonable cell division synchrony when cells were subsequently released from the xynchronizing procedure.

LITERATURE REVIEW AND INTRODUCTION

Literature Review

Initiation of Chromosome Replication

The genome of Escherichia coli is organized into a circular chromosome of double-stranded DNA whose synthesis is semi-conservative and sequential (Meselson and Stahl, 1958; Cairns, 1963a; Cairns, 1963b; Bleecken et al., 1966).

Maaløe and Hanawalt (Maaløe and Hanawalt, 1961; Hanawalt et al., 1961) have proposed that protein synthesis is required in order to initiate replication of DNA. This hypothesis is based on the amount of DNA synthesized after protein synthesis is halted and on the sensitivity of amino acid-starved cells to thymineless death. Resistance of thy cells to exponential death in the absence of thymine is taken as an indication of the completion of DNA synthesis.

Chromosome replication is terminated in a specific region of the chromosome in cells which are starved for amino acids. This was determined by the use of radioactive and density labels. DNA labeled immediately after release of cells from amino acid starvation can be "recalled" by repeating the starvation and release procedures (Lark et al., 1963; Pritchard and Lark, 1964).

Various agents may be used to inhibit the initiation of new rounds of DNA synthesis while allowing chromosomes to complete replication which has already begun. Blocking RNA synthesis by treatment with actinomycin (Sŏska and Lark, 1966) or by starvation for uracil (Maaløe

and Hanawalt, 1961; Soška and Lark, 1966) produces effects similar to the blockage of protein synthesis.

A model for the control of DNA replication has been proposed by Jacob, Brenner, and Cuzin (1963). In this model, a unit of replication, or a "replicon" (Jacob and Brenner, 1963), consists of a gene or genes directing the synthesis of an initiator protein or complex which interacts with a locus or origin on the chromosome, initiating a new round of DNA synthesis.

The model was based on the isolation of temperature-sensitive mutants of the fertility factor, F, of E. coli in which replication of F was blocked at the restrictive temperature (Cuzin and Jacob, 1967). It should be noted that replicons may differ in sensitivity to inhibitors even when the replicons inhabit the same cells. Such is the case in the curing of F by blocking its replication with acridine dyes (Hirota, 1960). Moreover, chromosomal mutants affecting the chromosomal replication apparatus or replication of a specific plasmid may allow the replication of other replicons (Hirota, Ryter, and Jacob, 1968). Mutants which cannot initiate DNA synthesis at elevated temperatures have been isolated (Mendelson and Gross, 1967; Gross et al., 1968; Hirota and Jacob, 1966; Kohiyama, 1968; Kuempel, 1969; Abe and Tomizawa, 1971).

The putative initiator appears to accumulate during the division cycle, and presumably initiation occurs only when a fixed amount of initiator accumulates in the cell (Nakada, 1960; Maaløe, 1963; Pritchard and Lark, 1964; Pritchard, 1966; Maaløe and Kjeldgaard, 1966; Ehret and Trucco, 1967; Helmstetter et al., 1968; Yoshikawa and Hass, 1968; Cooper, 1969). Moreover, the amount of initiator required to initiate

chromosome replication appears to be the same for all physiological states (Donachie and Masters, 1969; Helmstetter, 1969). The rate of synthesis of initiator depends on the rate of protein synthesis (and therefore, growth rate) of the cells.

At generation times of 20-60 minutes, about 40 minutes are required to replicate the complete chromosome in E. coli (Cairns, 1963a; Clark and Maaløe, 1967; Helmstetter, 1967; Bird and Lark, 1968; Cooper and Helmstetter, 1968; Helmstetter and Cooper, 1968; Helmstetter et al., 1968), and replication is regulated solely by the frequency of initiation. At generation times greater than 70 minutes, the rate of travel of replication forks decreases, and the time span for replication of the entire chromosome increases (Lark, 1966; Eberle and Lark, 1967; Bird and Lark, 1968; Ccoper and Helmstetter, 1968; Helmstetter et al., 1968). It is assumed by most investigators that replicating forks, once initiated, travel at a constant velocity to a terminus (Cairns and Davern, 1967; Clark and Maaløe, 1967; Cooper and Helmstetter, 1968). Once a round of DNA synthesis has started, the rate of synthesis is insensitive to changes in nutritional environment (Neidhardt and Magasanik, 1960; Schaechter, 1961; Helmstetter et al., 1968; Yoshikawa and Hass, 1968; Cooper, 1969).

It is thought that completion of a round of chromosome replication is required in E. coli for cell division to take place (Clark, 1968; Helmstetter and Pierucci, 1968), although some evidence indicates that a DNA/mass ratio specific for the growth rate is required for septum formation (Donachie and Hobbs, 1967; Donachie et al., 1968; Donachie and Masters, 1969). Mutants of E. coli having DNA replication uncoupled from cell division have been isolated. These mutants undergo abnormal divisions which give rise to cells having no DNA (Hirota and

Jacob, 1966; Adler et al., 1967; Gross et al., 1968; Hirota, Jacob, Ryter, Buttin, and Nakai, 1968; Hirota, Ryter, and Jacob, 1968; Inouye, 1969). A similar mutant has been described in Salmonella typhimurium (Shannon and Rowbury, 1972). In Bacillus subtilis, however, blockage of DNA synthesis with naladixic acid or thymine starvation does not prevent cell division, and cells containing no DNA are produced under these conditions (Donachie et al., 1971).

Essentially, very little has been learned of the coupling of replication to division by studying mutants. The mutants uncoupling division from replication map at several loci on the E. coli genetic map and have diverse phenotypes (Hirota, Ryter,and Jacob, 1968). The initiation complex has been studied with more success. Phenethyl alcohol (PEA) has been shown to inhibit DNA replication (Berrah and Konetzka, 1962) by inhibiting initiation of new rounds of replication (Treich and Konetzka, 1964). It has been shown that two proteins, one sensitive to low concentrations of chloramphenicol (CAM), the other resistant to CAM but sensitive to PEA, are required for initiation of chromosome replication (Lark and Lark, 1966). It was suggested by these workers that the two types of protein may correspond to membrane-bound structural and/or cytoplasmic initiator protein(s) (Lark and Lark, 1964; Lark, 1966; Lark and Lark, 1966) as proposed by the replicon hypothesis (Jacob et al., 1963). The synthesis of the two types of protein may be separated in the division cycle (Ward and Glaser, 1969a; Lark and Renger, 1969) and the third process, suggested by Lark and Renger (1969), may be the requirement for the synthesis of RNA as a structural entity (Lark, 1972). The synthesis of RNA,which is not necessarily used for protein synthesis, has also

been implicated in the conversion of single-stranded DNA of bacteriophage M13 to the double-stranded replicative form (Brutlag et al., 1971).

The existence of several initiation factors is suggested by the work of Cuzin and Jacob (1965), showing that temperature-sensitive replication mutants of the fertility factor exist in several complementation groups. Similarly, regulatory mutants of DNA replication map at several loci (Hirota, Ryter,and Jacob, 1968; Karamata and Gross, 1970; Wechsler and Gross, 1971; Gross, 1971).

Briefly, then, a model has been proposed in which initiator of DNA synthesis accumulates in cells at the same rate as total protein. When a specific amount of initiator accumulates, a new round of DNA synthesis begins, presumably at a fixed origin, and replication forks proceed at a constant velocity to a terminus. After a constant time period, cell division takes place. Such a model is consistent with data reported on amounts of DNA, protein and mass per cell (Schaechter et al., 1958), as well as the results of nutritional shifts (Kjeldgaard et al., 1958; Kjeldgaard, 1961; Sud and Schaechter, 1965; Kjeldgaard, 1967; Schleif, 1967; Cooper, 1969).

Procedures which block replicating forks on the DNA molecule lead to large increases in the rate of DNA synthesis after release from inhibition. Such bursts of DNA synthesis have been observed following a period of thymine starvation (Barner and Cohen, 1956; Nakada, 1960; Pritchard and Lark, 1964; Hardy and Binkley, 1967), treatment with ultraviolet light (Swenson and Setlow, 1966),or treatment with naladixic acid (Boyle et al., 1967; Ward et al., 1970). Evidence has indicated that the increased rate of DNA synthesis is the result of a high rate of reinitiation of DNA synthesis at the

presumed origin. It appears that this explanation holds true for the release of cells from thymine starvation (Pritchard and Lark, 1964; Kallenbach and Ma, 1968).

It has been reported that 5-bromouracil (BU) induces new initiations of DNA synthesis at the origin (Abe and Tomizawa, 1967; Wolf, Newman, and Glaser, 1968). An explanation of this result is given by the continued synthesis of initiator and decreased rate of travel of replicating forks in the presence of BU (Yoshikawa and Haas, 1968; Pierucci, 1969).

In certain temperature-sensitive DNA replication mutants, blocked in the travel of replicating forks at the restrictive temperature, release from inhibition resulted in reinitiations of DNA synthesis from the origin (Stein and Hanawalt, 1969; Worcel, 1970; Schwartz and Worcel, 1971) as seems to be the case whenever replication forks are inhibited in their travel. Similarly, treatment with ultraviolet light resulted in increased DNA synthesis from the origin defined by amino acid starvation (Hewitt and Billen, 1964; Hewitt and Billen, 1965), as did naladixic acid treatment (Boyle et al., 1967; Ward et al., 1970).

One explanation of the above observations may be that such treatments do not "induce" replication but that "induction" is the result of normal accumulation of initiator during the inhibition of normal DNA synthesis. In such cases, new initiations would occur before the previous replication forks had traveled their normal distances on the chromosome.

A perplexing observation is the occurrence of continued chromosome replication in the absence of protein synthesis following a

previous treatment to inhibit travel of replication forks. A stable replication apparatus seems to be formed allowing continued linear synthesis of DNA on chromosomes chosen randomly from the accumulating chromosome pool. The continued replication can occur for as long as 20 hours (Kogoma and Lark, 1970).

Heterogeneity in the time required for individual cells to re-initiate chromosome replication after amino acid starvation (Lark et al., 1963; Billen and Hewitt, 1966) has been explained as the heterogeneity in amounts of initiator in individual cells in the cell population. It is expected that individual cells would vary in the time necessary to accumulate sufficient initiator for replication.

It seems reasonable that cells growing with a generation time less than the chromosome replication time would initiate replication prior to the completion of the previous round of synthesis, giving rise to chromosomes having multiple replicating forks. Evidence confirming this prediction has been gathered using a variety of techniques (Yoshikawa et al., 1963; Oishi et al., 1964; Yoshikawa and Haas, 1968; Helmstetter and Cooper, 1968; Bird and Lark, 1968; Helmstetter, 1968; Caro, 1970). Similarly, gaps between successive rounds of DNA synthesis were predicted for cells growing with generation times greater than the chromosome replication time. Such gaps in DNA synthesis have been demonstrated experimentally (Helmstetter, 1967; Lark, 1966; Kubitschek et al., 1967).

Origin and Direction of Replication

A prediction of the replicon hypothesis (Jacob et al., 1963) is that chromosomes initiate replication from an origin. If DNA synthesis proceeds from a fixed origin, genetic markers proximal to the

origin will be present in more copies during replication than markers near the terminus since replicating forks are present at different chromosomal positions in an exponentially growing cell population and genetic markers which have been replicated are present in two copies whereas markers not yet replicated are present in only one copy. The frequency of genetic markers in a randomly dividing population will follow an age distribution function (Sueoka and Yoshikawa, 1965; see Mathematical Appendix of this dissertation) similar to distributions of cell ages in a randomly dividing population (Powell, 1956). The unnormalized frequency of genetic markers in a random population of cells may be given as

$$g_n(X) = 2^{n(1-X)}$$

where "X" is the distance of the marker from the origin for replication, the origin having a value of zero and the terminus a value of one, with "n" the average number of rounds of replication occurring on each chromosome. This relationship has been exploited in B. subtilis, using transformation frequency as a measurement of marker frequencies. A comparison of marker frequencies has allowed the construction of a replication map by application of the above formula. Marker frequencies obtained with DNA isolated from exponentially growing populations were compared to marker frequencies obtained with non-replicating DNA isolated from stationary phase cells or spores (Sueoka and Yoshikawa, 1963; Yoshikawa et al., 1963; Yoshikawa and Sueoka, 1963). The replication map has been confirmed by following the order of increases in marker frequencies during synchronous spore germination (Wake, 1963; Yoshikawa et al., 1963; Oishi et al., 1964; O'Sullivan and

Sueoka, 1967) or after dilution from stationary growth phase (Sueoka and Yoshikawa, 1963; Yoshikawa and Sueoka, 1963). The two strains of B. subtilis studied by Sueoka and his co-workers have a common origin and direction of replication, although strain 168 differs from W23 in that chromosomes of strain 168 accumulate in a partially replicated form on entry of the cell population into stationary growth phase.

In E. coli, density transfer experiments referenced previously did not unambiguously demonstrate that an origin was the same in all cells in a population, nor that the origin was the same as that used during exponential growth. It has been shown, however, that one origin is used for exponential growth within a single cell for several consecutive generations (Nagata and Meselson, 1968).

The earliest attempts to localize an origin for DNA synthesis in E. coli were performed on synchronously dividing populations of cells derived by a fractional filtration procedure (Maruyama and Yanagita, 1956). Nagata (1962 and 1963) reported that, in synchronously dividing Hfr cells of E. coli K12, the ratio of copies of prophage genomes changed during the division cycle in a manner consistent with chromosome replication initiated at the integrated F factor and with replication in a direction opposite the direction of conjugal transfer. In synchronized F^- strains, however, the ratio of the prophages did not change during the division cycle, implying that replication was randomized in female strains. The data reported by Nagata are consistent with the data of Abbo and Pardee (1960), who reported that induced β-galactosidase synthesis increased exponentially during one division cycle of E. coli B. Nagata's results were confirmed by Nishi and Horiuchi (1966), using induced enzyme synthesis for three enzymes

as a measure of gene frequency. Nishi and Horiuchi found that levels of induced enzyme synthesis increased abruptly in synchronized E. coli K12 Hfr populations in a manner consistent with an origin for DNA replication at the integrated F factor and a direction of replication opposite the direction of conjugal transfer. In F⁻ strains, no abrupt increases in induced enzyme synthesis were noticed. However, markers present on a merogenote were shown to give abrupt increases in induced enzyme synthesis during the division cycle. Rudner, Rejman, and Chargaff (1965), using the same synchronization procedure as Nagata and Nishi and Horiuchi, observed two peaks of pulse labeled RNA in the division cycle of Hfr strains of E. coli K12. The two peaks of presumed ribosomal RNA synthesis could be best explained by a pattern of replication with an origin at the integrated F factor and a direction of synthesis in the same direction as conjugal transfer.

Vielmetter, Messer, and Schütte (1968) have taken a novel approach to the study of the location of the origin for chromosome replication. They studied the segregation of mutational heterozygotes induced by nitrosoguanidine treatment of exponentially growing cells. The authors reasoned that cells in which nitrosoguanidine had induced mutants in a non-replicated portion of the chromosome would segregate pure mutant colonies at the first cell division, while cells in which mutations had been induced in the replicated portion of the chromosome would not segregate pure mutant colonies until the second cell division. Mutations induced by ^{32}P were also studied. The chromosomes of exponentially growing cells were labeled with ^{32}P-phosphate, and the cells were frozen a sufficient length of time for several decimal reductions of the viable cell count to occur, a process referred to as ^{32}P suicide.

The authors argued that, in surviving viable cells, only one of the daughter chromosomes arising from the completion of replication would be mutant and viable if a double-stranded break had been induced by ^{32}P decay in the portion of the chromosome already replicated. A double-stranded break in the non-replicated portion of the chromosome would yield two non-viable chromosomes upon replication and hence a non-viable cell. Therefore, ^{32}P induced mutations in the replicated portion of the chromosome would give rise preferentially to heterozygous mutant colonies when plated. ^{32}P induced mutations in the non-replicated portion of chromosome would give rise preferentially to homozygous mutant colonies when plated. The results of the experiments in which the fraction of homozygous mutant colonies to total mutant colonies was determined for a number of genetic markers located at various loci on the chromosome, indicated that chromosomes of both Hfr and F^- populations were replicated in the manner suggested by Nagata (1962 and 1963).

In contrast to the above reports of random origins for chromosome replication in F^- strains, synchronously dividing cell populations prepared by sedimentation (Mitchison and Vincent, 1965) showed periodic increases in rates of induced enzyme synthesis of several enzymes for both Hfr (Kuempel et al., 1965) and F^- (Donachie and Masters, 1966) strains of *E. coli* K12. *E. coli* B/r was shown to have increases in rates of induced enzyme synthesis similar to those of *E. coli* K12. A strain of genotype *F-lac$^+$/lac$^+$* showed two distinct increases in the rate of induced enzyme synthesis of β-galactosidase (Donachie and Masters, 1966).

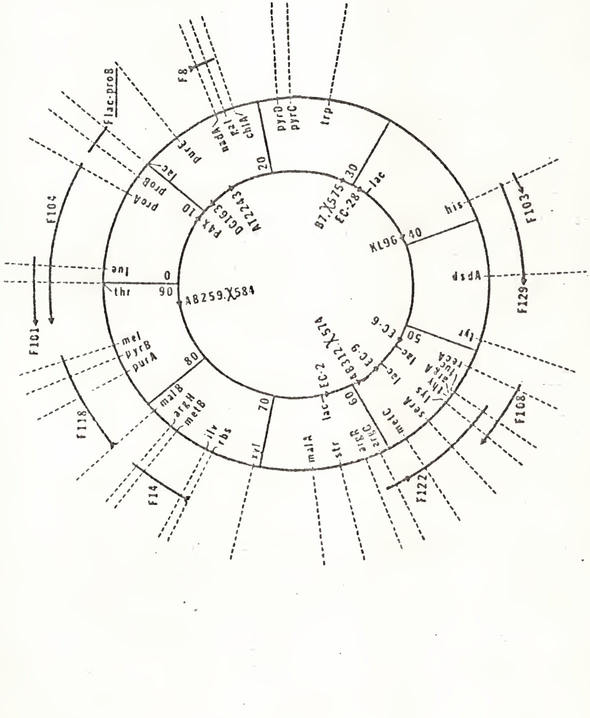
Cutler and Evans have used transfer of stationary growth phase

cells into fresh media to sychronize cell populations with respect to cell division (Cutler and Evans, 1966) and chromosome replication (Cutler and Evans, 1967a). E. coli DNA segments, pulse labeled with BU during the division cycle, were hybridized with RNA of λ and 424 bacteriophages. The data indicated that phage λ was replicated before phage 424 in E. coli K12 strain 3000 (HfrH). The results indicated a clockwise direction of replication in relation to the genetic map of E. coli (Cutler and Evans, 1967b). No origin may be deduced from the data, but the direction of replication was opposite that predicted by the hypothesis of Nagata (1962 and 1963).

An attempt to locate the origin of chromosome replication in E. coli K12 after transfer of exponentially growing cells to BU has been made (Abe and Tomizawa, 1967). Phage P1 transducing lysates were prepared on thymine requiring Hfr and F^- cells growing exponentially after various times of uptake of BU. The authors showed evidence that BU induced the initiation of new rounds of replication from the origin as defined by amino acid starvation. Assuming that the appearance of a genetic marker in phage particles of hybrid density was evidence of the replication of the marker, it was concluded that lys was replicated as an early marker and his as a late marker (see genetic map, Fig. 1) with a clockwise direction of replication.

A number of attempts to locate the origin of chromosome replication have been made using transducing particles prepared on cells in which chromosomes had been aligned by amino acid starvation. In these experiments, origin DNA was labeled with BU after release from amino acid starvation (Espardellier et al., 1967; Caro and Berg, 1968; Wolf, Newman, and Glaser, 1968; Wolf, Pato, Ward, and Glaser, 1968; Caro and

Figure 1. Genetic map of E. coli K12. The positions of genes are from the map of Taylor (1970). The map is divided into 90 minutes. The origins and directions of transfer of Hfr strains are taken from Hayes (1968) and from Dr. Brooks Low (personal communication). The locations and extents of merogenotes were those of Low (1968 and personal communication). The location and transfer properties of transposition Hfr strains (EC-2, EC-6, EC-9, and EC-28) were taken from Beckwith et al. (1966).



Berg, 1969). P1 transducing phage were then prepared on these cells having either the origin or terminus labeled with BU, and the genetic markers carried by transducing particles of hybrid density were determined. Data from such experiments have indicated the origin to be between either argG and ilv (see genetic map, Fig. 1) (Espardellier et al., 1967), lys and xyl (Wolf, Newman,and Glaser, 1968; Wolf, Pato, Ward,and Glaser, 1968) or argG and xyl (Caro and Berg, 1968; Caro and Berg, 1969).

E. coli strains K12 and 15T$^-$, synchronized by a period of amino acid starvation followed by thymine starvation, have been assayed for replications patterns by examining mutants induced by pulse mutagenesis of the replicating fork with nitrosoguanidine (Cerdá-Olmedo and Hanawalt, 1968). The data indicated an origin between tyr and argG (see genetic map, Fig. 1) with a clockwise direction of replication. Similar pulse mutagenesis experiments on E. coli B/r synchronized by a membrane filter technique (Helmstetter and Cummings, 1965) have indicated an origin between argA and his (Wolf, Pato, Ward,and Glaser, 1968; Ward and Glaser, 1969b). In support of an origin near minute 55 on the genetic map (see Fig. 1), Botstein and Jones (1969) have presented evidence that nitrosoguanidine induced mutations in stationary growth phase,E. coli K12 were found predominantly in the 45- to 75-minute region of the genetic map (see Fig. 1), while mutants induced during exponential growth were random.

E. coli B/r, synchronized by the elution of newly divided cells from filters (Helmstetter and Cummings, 1965), were examined for gene dosage by comparing levels of induced enzyme synthesis during subsequent synchronous growth (Helmstetter, 1968; Pato and Glaser, 1968; Wolf, Pato, Ward ,and Glaser, 1968). The data supported the hypothesis of

a chromosome origin in the region of 55 minutes on the E. coli map, with a clockwise direction of synthesis.

Similarly, E. coli B/r, synchronized by release from naladixic acid inhibition of DNA synthesis, but with nitrosoguanidine pulse mutagenesis as a measure of gene replication, showed an apparent origin for DNA synthesis between his and argA, with a clockwise direction of replication (Ward et al., 1970).

Schwartz and Worcel (1971) have measured gene replication by both nitrosoguanidine pulse mutagenesis and enzyme induction in cells synchronized by temperature shift and have concluded that chromosome replication was initiated slightly counterclockwise to lysA on the E. coli genetic map, proceeding with a clockwise direction of replication.

Eberle (1970) has measured replication of integrated λ phage (by a DNA-DNA reannealing technique) in synchronized cells after release from amino acid starvation. Her conclusion, that there was an origin for DNA synthesis 55 per cent of the length of the chromosome from λ, is in agreement with an origin for chromosome replication near argG. A similar experiment (Abe and Tomizawa, 1971) has been performed, using DNA from four different prophages. The terminal regions of chromosomes of a mutant,temperature sensitive for DNA initiation, were labeled and the various phage DNA's were used to determine,by a DNA-DNA reannealing technique, the relative amounts of each prophage labeled during the termination of chromosome replication. The results indicated an origin between 40 and 50 minutes on the E. coli genetic map.

In an experiment with methodology similar to that developed for

this dissertation, Yahara (1971), to detect specific chromosome regions, used DNA isolated from three Proteus mirabilis F' hybrids and a DNA reannealing assay to measure amounts of three regions of the chromosome which were labeled during and after amino acid starvation. The data were consistent with an origin between lys and ilv; however, only the amounts of these three regions were measured. Yahara used a membrane-bound DNA-DNA reannealing assay (Denhardt, 1966), and no corrections were introduced for F factor homology with E. coli DNA. The data favored a bidirectional mode of replication; however, the observations made by the author may also be ascribed to failure of chromosomes to terminate a round of chromosome replication during amino acid starvation.

In none of the experiments described above, for E. coli synchronized by various procedures, was it shown unambiguously that an origin demonstrated by synchrony was the same as one used during exponential growth. In many experiments, the data did not preclude an origin with replication in both directions. Indeed, many authors have honestly reported this alternative explanation of the data.

Three papers have reported experiments using bacteriophage P1 transduction frequencies as a measure of gene frequency in lysates prepared on exponentially growing populations of cells. In these experiments, it was assumed that P1 transduces chromosomal genes in a random fashion. With this assumption Berg and Caro (1967) found no difference in the transduction frequencies of several genes using phage lysates prepared on several different Hfr cultures, each having different F integration sites. (The donor cultures were growing rapidly in exponential growth phase). Therefore, it was concluded

that integrated F factors are not the origin for chromosome replication in Hfr strains of E. coli K12. Another approach has been to take advantage of the different numbers of replicating forks present in chromosomes of cells growing at fast and at slow growth rates. Transducing lysates were prepared on E. coli growing in rich medium and on E. coli growing in minimal medium. By comparing transducing frequencies of donor markers from fast-growing cells to those of slow-growing cells, a curve with a maximum for the ratio at the origin to a minimum for the ratio at the terminus was predicted. Caro and Berg (1969) presented curves obtained for two strains of E. coli K12 in which the origin was determined to lie between argG and xyl with a clockwise direction of replication. In addition, the ratio of transducing frequencies of xyl to argG in rich medium was shown to be approximately four to one for nine sublines of E. coli K12.

Conflicting data presented by Masters (1970) and by Masters and Broda (1971), who have performed the same experiment on two strains of E. coli, B/r and K12, indicated that in both strains chromosome replication was bidirectional with an origin between argG and malA and a terminus near his. The experiments of Caro and Berg (1969) and of Masters (1970) and Masters and Broda (1971) appear to be directly comparable in methods, but the conflicting data cannot be reconciled by this writer. Unfortunately, the reports above have provided the only published data on marker frequencies in exponentially growing populations of E. coli.

Recently, experiments measuring the frequency of phage Mu-1, integrated in various loci on the E. coli chromosome, have been performed on exponentially growing E. coli K12 (Bird, R. E., Louarn, J.,

Martuscelli, J., and Caro, L. G., The Origin and Sequence of Chromosome Replication in Escherichia coli, submitted for publication, J. Mol. Biol., 1972). The authors prepared strains which were lysogenized with λ and which had phage Mu-1 integrated at one of a number of several different positions located around the E. coli chromosome. DNA isolated from exponentially growing doubly-lysogenic E. coli was bound to membrane filters (Denhardt, 1966) and reannealled with a mixture of sheared and denatured ^{14}C labeled λ DNA and ^{3}H labeled Mu-1 DNA. The ratio of the fraction of added ^{14}C label bound to the filter to the fraction of ^{3}H label bound to the filter was taken as the frequency of phage Mu-1 on the replicating chromosome. The data strongly indicated that replication proceeds from an origin close to ilv in both directions on the chromosome to a terminus near trp. This result indicated that replication is symetrical, that is, the origin and terminus are located mid-way around the chromosome from one another. This is in contradiction to the data of Masters and Broda (1971) indicating asymmetric replication. Bird et al. also presented evidence indicating that chromosomes complete a round of chromosome replication during amino acid starvation and that the sequence of genes replicated after release from amino acid starvation is bi-directional.

Although the more recent papers described above reported that integrated sex factors did not control chromosome replication, under certain conditions integrated episomes such as F (Nishimura et al., 1971) and bacteriophage P2 (Lindahl et al., 1971) have been suspected of serving as origins for DNA replication. In certain temperature-sensitive mutants, blocked in the initiation of DNA synthesis,

integrated episomes can correct the temperature-sensitive defect in a phenomenon which has come to be called "integrative suppression."

Chromosome replication has been studied in organisms other than B. subtilis and E. coli. For example, Salmonella typhimurium, like E. coli, is a member of the Enterobacteriaceae and may be expected to replicate its chromosome in a similar manner. Nishioka and Eisenstark (1970) have performed experiments in which chromosome termini were labeled with BU during amino acid starvation. Similarly, chromosome origins were labeled with BU after the cells were released from amino acid and thymine starvation. Generalized transducing phage P22 were prepared on the BU-labeled cells. The data obtained from transducing particles of hybrid density indicated an origin near ilv on the genetic map of S. typhimurium. The genetic maps of S. typhimurium and E. coli are very similar (Sanderson, 1970). The data of Nishioka and Eisenstark (1970) do not distinguish between bi-directional replication and a failure of chromosome replicating forks to travel to the terminus during amino acid starvation. Fujisawa and Eisenstark (1970) have reported an experiment on S. typhimurium similar to the experiment of Botstein and Jones (1969) on E. coli. Their data on nitrosoguanidine mutagenesis of stationary phase cells of S. typhimurium indicated the origin to be between lys and cysG on the Salmonella map. Data from pulse mutagenesis of synchronized cultures showed that $cysG^+$ revertants were induced early while lys^+ revertants were induced later in the replication cycle. Recently, Fujisawa and Eisenstark (1972) have reported on an analysis of P22 transduction frequencies using phage prepared on rapidly growing and an slowly growing S. typhimurium. These techniques were similar to

those described above for E. coli. The data indicated an origin in the region between metC and ilv with bidirectional replication to a terminus near trp.

It is pertinent to make a comment on two virus systems, phage λ and phage P2. Phage λ has been reported to have a bidirectional mode of replication from a single origin (Schnös and Inman, 1970; Makover, 1971; Stevens et al., 1971). However, phage P2 appeared to replicate its genome from a single origin, in one direction (Schnös and Inman, 1971).

The many conflicting results obtained for the origin and direction of DNA replication in E. coli require comment. One major objection to much of the work on E. coli is the meaning of synchrony. To my way of thinking, an answer should be sought with experiments using exponentially growing populations of cells, because synchronizing procedures, no matter how gentle, may disrupt the physiology of the cell. The filter pile synchronizing procedure of Maruyama and Yanagita (1956) has been shown to yield cells in which there is exponentially increasing DNA synthesis within the division cycle of E. coli K12 (Nagata, 1962 and 1963) and E. coli B (Abbo and Pardee, 1960). In contrast, when the elution of newly divided cells from membrane filters is used, as a synchronizing procedure for E. coli B/r (Helmstetter and Cummings, 1965) the rate of DNA synthesis at each replication fork is constant throughout the division cycle. Abrupt changes in the rate of DNA synthesis occurred at the termination of a round of chromosome replication and at the initiation of new rounds of replication (Helmstetter, 1967; Helmstetter and Cooper, 1968; Cooper and Helmstetter, 1968; Helmstetter et al., 1968; Ward and Glaser, 1969b). Similar step-wise

increases in rate of DNA synthesis occur in E. coli synchronized by sucrose density gradient centrifugation (Donachie and Masters, 1969). Unfortunately, the membrane filter method of Helmstetter and Cummings (1965) has been used to select synchronous populations of only E. coli B/r. Recently, a modification of the method has been shown to give synchronized populations of E. coli K12 Hfr and F⁻ strains (Cummings, 1970). The modified synchronizing procedure has not yet been employed in the search for the origin and direction of chromosome replication in E. coli K12. The amino acid starvation procedure used by numerous workers to align chromosomes in a completed state is thought by some to prevent the completion of chromosome replication to a terminus in most cells of a population (Caro and Berg, 1968; Caro and Berg, 1969). In B. subtilis, the results of marker frequency analysis by transformation have shown that amino acid starvation does not lead to termination of chromosome replication at the origin-terminus in strains 168 (Copeland, 1969; Copeland, 1972) and W23 (Copeland, 1970; Copeland, 1971). Further, the effect in Bacillus depends strongly on the amino acid used for starvation.

5-bromouracil incorporation, thymine starvation, naladixic acid treatment, and nitrosoguanidine treatment have in common the property of altering the macromolecular metabolism of the cell. Each of these treatments can lead to cell death.

It should be pointed out that the methods used by most workers to measure gene frequency (that is transduction, enzyme induction, mutagenesis) in E. coli are indirect. Only the nucleic acid reannealing procedures, used by a relatively few workers, measure gene content directly.

Rates of induced enzyme synthesis have been used as an indirect measure of gene content. However, the rates of induced or derepressed enzyme synthesis are affected strongly by the internal concentrations of regulatory molecules. Steps must be taken to insure that genes measured are completely derepressed or completely induced. During outgrowth of B. subtilis spores, blocking DNA synthesis with 5-flourodeoxyuridine did not prevent the periodic synthesis of ornithine transcarbamylase under conditions where the enzyme was not induced (Masters and Donachie, 1966). However, under conditions where induced levels of enzyme synthesis were measured, blockage of DNA synthesis completely blocks the increases in induced enzyme synthesis of a number of enzymes (Donachie and Masters, 1966; Pato and Glaser, 1968).

Bacteriophage P1 has been thought by many to transduce regions of the chromosome randomly. If this assumption is correct, then P1 transduction should be a good measure of frequency without resorting to normalizing data by comparing transduction frequencies obtained with rapidly growing cells to those of slowly growing cells. If one examines critically the paper of Masters and Broda (1971), in particular the unnormalized transduction frequencies, it is seen that the ratio of origin markers to terminus markers was 8:1. At the growth rates of the cell populations used by Masters and Broda, E. coli is expected to have no more than a 4:1 ratio of origin markers to terminus markers. In contrast, Caro and Berg (1969) consistently obtained origin to terminus marker ratios between 3:1 and 4.5:1 for cells growing in rich medium with generation times similar to those used in the experiments of Masters and Broda (1971). Furthermore, the experiments

of Caro and Berg (1969) consistently showed xyl and ilv as early markers, his as a marker in the middle of the replicating chromosome, and lys and argG as markers near the terminus. The experiments reported by Masters (1970) and Masters and Broda (1971) showed his to be a terminus marker, with thy, lys, and argG as markers near the origin for chromosome replication.

The preceding discussion has pointed out the contradictions and confusion in the current state of knowledge of chromosome replication. Clearly, a more "reliable" technique is needed to resolve the issues.

Introduction

It was the purpose of this dissertation to apply a direct measurement of gene frequency to the age distribution formula for gene frequency in exponentially growing cells (Sueoka and Yoshikawa, 1965; also see Mathematical Appendix of this dissertation). Our procedure was to isolate specific regions of DNA in both labeled and unlabeled form from a series of Proteus mirabilis-E. coli F' hybrids, and to use these to measure frequencies of homologous chromosomal regions by specific DNA-DNA reannealing techniques. Results are presented which were obtained with two different, but related, methods. The first method was to use a quantitative assay procedure in which chromosomal DNA, labeled over many generations of exponential growth, was reannealed with unlabeled E. coli DNA segments from Proteus F' hybrids. The amount of reannealing of labeled DNA from E. coli with unlabeled DNA from stationary phase P. mirabilis F' hybrids is proportional to the size and frequency of repetition of the chromosomal segment located on the F merogenote. The proportion is valid if appropriate controls are included for the reannealing of unlabeled

P. mirabilis DNA and F DNA (obtained from a P. mirabilis F' hybrid having no genes on the merogenote in common with the E. coli chromosome) with labeled E. coli DNA.

A second method of measuring frequencies was by DNA renaturation kinetics. The renaturation of DNA is a second-order kinetic reaction (Britten and Kohne, 1966; Wetmur and Davidson, 1968; Britten, 1968), and it is expected that regions of the chromosome will renature at rates proportional to their concentrations. In this case the number of copies of the region per replicating chromosome was normalized for the amount of DNA per replicating chromosome. For example, a region whose frequency is an average of two copies per replicating chromosome will renature twice as fast as a region whose frequency is an average of one copy per replicating chromosome (see Mathematical Appendix for a more thorough treatment). In our experiments, labeled chromosomal regions were isolated from Proteus F' hybrid and renatured with unlabeled DNA from exponentially growing E. coli. Each region of the chromosome was expected to renature with a rate proportional to its frequency on the replicating chromosome. The frequency should be a function of the position of the region on the replicating chromosome as predicted by the replicon hypothesis (Jacob et al., 1963).

MATERIALS AND METHODS

Media

Complex Media

L broth (Luria and Burrous, 1957)

Tryptone	10.0 gm/liter
Yeast Extract	5.0 gm/liter
NaCl	10.0 gm/liter
Glucose	1.0 gm/liter

pH adjusted to 7.1-7.2 with NaOH

The above medium was solidified by the addition of agar to 2.0% for solid medium. L broth was routinely used when complex medium was desired.

MacConkey agar

MacConkey agar (Difco) containing 1% carbohydrate was used to test fermentation capabilities.

Urease test medium

Urease production medium (BBL) was used to test *P. mirabilis* and *P. mirabilis* hybrids for the production of urease.

Synthetic Media

Minimal medium (Davis and Mingioli, 1950)

K_2HPO_4	7.0 gm/liter
KH_2PO_4	3.0 gm/liter
Na_3-citrate$\cdot 3H_2O$	0.5 gm/liter
$MgSO_4 \cdot 7H_2O$	0.1 gm/liter
$(NH_4)_2SO_4$	1.0 gm/liter

pH 7.0-7.2

Carbon and energy sources (sterilized separately) were added to the above medium at a concentration of 0.5%. For the study of fermenta-

tions, in Proteus mirabilis, citrate was omitted from the above medium, as P. mirabilis has the ability to use citrate as both a carbon and energy source. For growth of P. mirabilis in liquid minimal medium, casamino acids were added at a concentration of 0.003%. Minimal medium was solidified with agar to a final concentration of 2% for solid medium. For sublines of P. mirabilis capable of swarming, 5% agar was used.

Amino acid supplements

Minimal medium was supplemented with amino acids at 50 µg/ml with respect to the L-isomer.

Vitamin supplements

Vitamins were supplied at a concentration of 0.2 µg/ml. Nicotinic acid (niacin) was supplied to all cultures of P. mirabilis.

Purine and pyrimidine supplements

Purine and pyrimidines were added to the medium at a concentration of 50 µg/ml where required. In labeling experiments with radioactive thymine, the total thymine was at a concentration of 2 µg/ml.

Bacterial Strains

Escherichia coli

Strains of E. coli were obtained from several sources and are listed in Table 1. The position of pertinent genetic markers, transfer origin of Hfr strains and segments of chromosomes carried on merogenotes are shown in Figure 1.

Proteus mirabilis

An IlvE mutant of P. mirabilis strain PM-1 (AC3145) was obtained from Dr. D. E. Duggan. A prototrophic revertant of AC3145, PF2, was

Table 1. Strain list

Strain	Sex	Genotype	Source	Remarks
		Escherichia coli		
χ574	Hfr[a]	thy_2,[b] Δlac-proB	Dr. Roy Curtiss, III Oak Ridge, Tenn.	Isogenic with χ575, χ583, χ584, and χ593
χ575	Hfr	thy_2, Δlac-proB	Same as above	
χ583	F⁻	thy_2, Δlac-proB	Same as above	
χ584	Hfr	thy_2, Δlac-proB	Same as above	
AB1514	F⁻	ilv	Dr. D. E. Duggan University of Florida	
AB264	F⁺	ara	Same as above	
F_{T62}lac/ $2{,}000_{X111}$	F'	Flac⁺, proB⁺/ Δlac-proB	Dr. J. R. Beckwith Harvard University	Flac-proB is temperature-sensitive for replication
$2{,}000_{X111}$	F⁻	Δlac-proB	Lac⁻ segregant of F_{T62}lac/$2{,}000_{X111}$ grown at 42°C	
X7026	F⁻	Δlac-proB	Dr. J. R. Beckwith Harvard University	Isogenic with EC-2, EC-6, EC-9, and EC-28
EC-2	Hfr	Flac⁺, proB⁺/ Δlac-proB	Same as above	Hfr having F_{T114} integrated into chromosome
EC-6	Hfr	Δlac-proB	Same as above	Same as above
EC-9	Hfr	Δlac-proB	Same as above	Same as above
EC-28	Hfr	Δlac-proB	Same as above	Same as above

Table 1. Continued

Strain	Sex	Genotype	Source	Remarks
AB259	Hfr	thi	Dr. D. E. Duggan University of Florida	Hayes type Hfr
F118/ KL132	F'	F*pyrB*$^+$/*pyrB*, *thi*, *thr*, *leu*, *pro*, *his*, *lac*, *mal*, *xyl*, *recA1*	Dr. Brooks Low Yale University	Carries F118^c
W4520	F'	F*gal*$^+$/*gal*, *met*	Dr. P. Gemski	Carries F8
F101/ AB2463	F'	F*thr*$^+$, *leu*$^+$,/*thr*, *leu*, *proA*, *thi*, *argE*, *his*, *str*, *mal*, *mtl*, *ara*, *lac*, *gal*, *recA13*	Dr. Brooks Low	Carries F101
F104/ AB2463	F'	F*thr*$^+$, *leu*$^+$, *proA*$^+$/*thr*, *leu*, *proA*, *thi*, *argE*, *his*, *str*, *mal*, *mtl*, *ara*, *lac*, *gal*, *recA13*	Same as above	Carries F104
F103/ KL110	F'	F*his*$^+$/*his*, *metB*, *leu*, *argG*, *str*, *mal*, *xyl*, *lac*, *recA1*	Same as above	Carries F103
F129/ KL250	F'	F*his*$^+$/*his*, *metB*, *leu*, *proA*, *trp*, *argE*, *thi*, *thy*, *str*, *mal*, *mtl*, *ara*, *lac*, *gal*, *rec*	Same as above	Carries F129
F108/ MA50	F'	F*lysA*$^+$, *cysC*$^+$/ *lysA*, *cysC*, *thr*, *leu*, *lac*, *mal*, *xyl*, *mtl*, *thi*	Same as above	Carries F108
F122/ KL110	F'	F*argG*$^+$/*argG*, *his*, *leu*, *metB*, *xyl*, *str*, *mal*, *lac*, *recA1*	Same as above	Carries F122

Table 1. Continued

Strain	Sex	Genotype	Source	Remarks
Flac/ $2{,}000_{X111}$	F'	$Flac^+$, $proB^+$/ Δlac-proB	PF51 X $2{,}000_{X111}$	Carries Flac-proB
AB312	Hfr	thr, leu, str	Dr. D. E. Duggan University of Florida	
AT2243	Hfr	metB, purE, thi	Dr. A. L. Taylor University of Colorado	Cavelli type Hfr
DG163	Hfr	met, argA, thy	Dr. B. Wolf University of California, Berkley	
KL96	Hfr	thi	Dr. Brooks Low Yale University	
P4X	Hfr	met	Same as above	
B7	Hfr	met	Same as above	
555-7	F^-	thy_2, trp, arg, met	Dr. D. Billen University of Florida	E. coli 15
B	F^-		Dr. E. Previc University of Florida	E. coli B
C	F^-		Dr. P. M. Achey University of Florida	E. coli C
		Proteus mirabilis[d]		
PF1	F^-	ilvE	Dr. D. E. Duggan	Received as AC3145
PF2	F^-		Plating of PF1 on minimal medium	Prototroph

Table 1. Continued

Strain	Sex	Genotype	Source	Remarks
PF7	F^-	his	Dr. E. A. Adelberg Yale University	Received as AC2627
PF8	F^-	leu	Mutagenesis of PF2 with ethyl methane sulfonate	
PF12	F^-	argF	Mutagenesis of PF2 with nitrosoguanidine	
PF17	F^-	lys	Same as above	
PF38	F^-	gal	Same as above	
PF40	F^-	xyl	Same as above	
PF49	F^-	thy_2, lys	Selection of Thy_2 from PF17 by use of trimethoprim	
PF51	F'	$Flac^+$/lac	Dr. I. Rosen University of Florida	Carries Flac-proB
PF50	F'	$Flac^+$/lac, ilvE	$Flac/2{,}000_{X111}$ X PF1	Carries Flac-proB
PF53	F'	$Fara^+$, leu^+/ara, leu	F101/AB2463 X PF8	Carries F101
PF54	F'	$Fara^+$/gal	W4520 X PF38	Carries F8
PF55	F'	$Filv^+$/ilv	Dr. E. A. Adelberg Yale University	Received as AC2634, carries F14
PF56	F'	$Ffuc^+$, lys^+/fuc, lys	F108/MA50 X PF17	Carries F108
PF57	F'	$Fhis^+$/his	F103/KL110 x PF7	Carries F103

Table 1. Continued

Strain	Sex	Genotype	Source	Remarks
PF58	F'	F*his*$^+$/*his*	F129/KL250 X PF7	Carries F129
PF59	F'	F*mel*$^+$/*mel*	F118/KL132 X PF2	Carries F118
PF61	F'	F*ara*$^+$, *argF*$^+$/ *ara*, *argF*	F104/AB2463 X PF12	Carries F104
PF63	F'	P*fuc*$^+$, *lys*$^+$, *thy*$^+$/*fuc*, *lys*, *thy*	F122/KL110 X PF49	Carries F104
PF74	F'	F*ara*$^+$, *argF*$^+$/ *ara*, *argF*, *thy*$_2$	Selection of Thy$_2^-$ from PF61 with trimethoprim (TMP)	Carries F104
PF75	F'	F*gal*$^+$/*gal*, *thy*$_2$	Selection of Thy$_2^-$ from PF54 with TMP	Carries F8
PF77	F'	F*his*$^+$/*his*, *thy*$_2$	Selection of Thy$_2^-$ from PF58 with TMP	Carries F129
PF78	F'	F*his*$^+$/*his*, *thy*$_2$	Selection of Thy$_2^-$ from PF57 with TMP	Carries F103
PF79	F'	F*mel*$^+$/*mel*, *thy*$_2$	Selection of Thy$_2^-$ from PF59 with TMP	Carries F118
PF80	F'	F*lac*$^+$/*lac*, *ilvE*,	Selection of Thy$_2^-$ from PF50 with TMP	Carries F*lac*-*proB*
PF82	F$^-$	*xyl*, *thy*$_2^-$	Selection of Thy$_2^-$ from PF40 with TMP	

Table 1. Continued

Strain	Sex	Genotype	Source	Remarks
PF84	F'	F*ilv*$^+$/*ilv*, *thy*$_2^-$	Selection of Thy$_2^-$ from PF55 with TMP	Carries F14

[a] The origins and directions of transfer in Hfr strains are shown in Figure 1.

[b] Thymine auxotroph with thymine requirement satisfied by 2 μg/ml.

[c] Positions and extents of merogenotes are shown in Figure 1.

[d] *P. mirabilis* is naturally *lac*, *mtl*, *mal*, *ara*, *mel*, *rha*, *fuc*, *man*, *nic*.

used for induction of further auxotrophs by mutagenesis with ethyl methanesulfonate (Eastman, Rochester, New York), (Strauss, 1962) or with nitrosoguanidine (Aldrich Chemical Co., Inc., Cedar Knolls, New Jersey), (Adelberg et al., 1965). Thymine requiring mutants were selected with the use of trimethoprim (Stacey and Simon, 1965).

Proteus mirabilis-Escherichia coli F' Hybrids

E. coli F' strains were mated with appropriate mutants of P. mirabilis using the methods described by Falkow, Wohlhieter, Citarella and Baron (1964). Donor E. coli F' cultures were grown in minimal medium maintaining selection for merogenote linked genetic markers. Recipient P. mirabilis strains were grown in L broth. Donors and recipients in exponential growth phase were mixed by dilution into fresh L broth to final cell concentrations (cells/ml) of 2×10^8 and 5×10^8, respectively. Matings were allowed to proceed at 37°C without shaking for six hours. Mating mixtures were harvested and washed twice with the salt solution of the minimal medium. The centrifuge pellet was resuspended in 1/10 of the original volume in minimal salt solution. Dilutions were plated on minimal medium, selecting for genetic markers on the merogenote. Occasionally, matings were performed directly on plates of selective media. Hybrids were identified by their characteristic segregation of F-linked markers when grown on non-specific media. Hybrids showed the ability to propagate male specific phage (Horiuchi and Adelberg, 1965), with the exception of all of the F14 hybrids derived from AC 2634. All hybrids were positive for the production of urease. The hybrid strains pertinent to this dissertation are listed in Table 1. The extent of chromosomal genes on the merogenotes is shown in Figure 1.

Growth of Strains

Large quantities of cells for the isolation of unlabeled DNA were grown in 20-liter carboys by sparging 15 liters of medium with filtered air. A growth temperature of 37°C was used for E. coli strains and for P. mirabilis grown in complex medium. P. mirabilis F' hybrid strains were grown in minimal medium at 27°C. This temperature was used for consistency since some Proteus strains and hybrids, notably those carrying F118, grew poorly at 37°C.

E. coli strains, in which chromosome replication states were to be determined with the use of unlabeled DNA, were grown in 3-liter Erlenmayer flasks. The media (2.5 liters), inoculated with a low population density of cells, were sparged with filtered air at 37°C. At intervals, samples were taken for cell counts, turbidity readings, DNA content,and protein determination in order to follow growth.

Strains for the isolation of labeled DNA were grown in 125 ml nephelometer flasks on a shaking water bath at 37°C (for E. coli) or 27°C (for P. mirabilis and P. mirabilis F' hybrids).

Use of Labeled Materials

Thymine, labeled specifically in the 5-methyl residue, with either ^{3}H or ^{14}C, was purchased from Schwarz-Mann, Orangeburg, New York. Labeled materials were diluted with unlabeled thymine to the required specific activity prior to use.

Assays of Macromolecules

DNA was assayed by the formation of the colored product with indole (Keck, 1956) using Salmon Sperm DNA (Calbiochem, Los Angeles, California) as a standard.

Protein was determined by the assay for tyrosyl residues using phenol reagent (Lowry et al., 1951) using Bovine Serum Albumin (Fraction V, Armour Laboratories, Kankakee, Illinois) as a standard.

Concentrations of purified DNA were estimated by the absorbance at a wavelength of 260 nm. A_{260} of a 1 µg/ml solution of DNA in a 1 cm path length was taken to be 0.02.

Determination of Growth Parameters

Culture Turbidity

Culture turbidities on 5 ml samples of growing cultures were estimated with a Klett-Summerson colorimeter (Klett Manufacturing Company, Inc., New York), using a No.66 Klett filter.

Total Cell Counts

Cell counts were performed on samples from growing cultures by means of a Petroff-Hausser (C. A. Hausser and Son, Philadelphia, Pennsylvania) counting chamber, under a phase contrast microscope. The samples (0.9 ml) were added to 0.1 ml of 40% formaldehyde and refrigerated in screw cap tubes until counted.

Treatment of Samples for Assays of DNA and Protein from Unlabeled Cultures

Sufficient trichloroacetic acid (TCA) was added to 20 ml samples of growing culture to bring them to 5% TCA concentration, and the samples were then chilled. After at least one hour, samples were centrifuged, and the pellets were washed twice with 10 ml portions of cold 5% TCA and were then resuspended in 2 ml of distilled water and frozen until portions were removed for analysis of DNA and protein by methods outlined earlier.

Treatment of Samples for Assays of DNA from Labeled Cultures

A portion of the culture was pipetted into cold TCA to a final concentration of 5% and refrigerated for at least an hour. Samples were then filtered on to 24 mm nitrocellulose filters (type B-6, Schleicher and Schuell, Keene, New Hampshire) and washed with three 10 ml portions of 5% TCA followed by three 10 ml portions of 70% ethanol (EtOH). Filters were dried overnight at 70°C and counted in a Packard 3380 Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Company, Downers Grove, Illinois), using the toluene-based scintillation fluid, Omnifluor (Packard Instrument Company, Downers Grove, Illinois). In order to decrease non-specific binding of labeled thymine to the filters, thymine was present in all TCA solutions at a concentration of 500 µg/ml.

Harvesting Cells for DNA Preparation

Macromolecular synthesis in bacteria was halted by pouring cells into 0.5 volumes of frozen 3X concentrated $NETN_3$ buffer to achieve the following concentration of ingredients: 0.1 M NaCl; 0.05 M ethylenediaminetetracetic acid (EDTA); 0.05 M 2-amino-2-methyl-1, 3-propanediol (Tris); 0.001 M NaN_3, pH 8.2. The cells were centrifuged and washed once with the above buffer and finally resuspended in NET buffer (NET is the same as the above buffer, only lacking NaN_3) at a concentration of approximately 10-20 volumes of buffer per volume of cell pellet and then frozen until used.

Isolation of Unlabeled DNA (Brenner et al., 1969)

The following procedure for DNA isolation was used:

1) Cells in NET were defrosted in a 60°C water bath and

lysed by the addition of sodium lauryl sulfate to a concentration of 0.5%

2) Pronase (Grade B, Calbiochem, LaJolla, California) was added to a concentration of 100 µg/ml and the lysate was incubated overnight at 37°C.

3) Sodium lauryl sulfate was added to 1% concentration. An equal volume of liquified phenol (previously saturated with NET buffer) was added, and the mixture was shaken by hand for 15 minutes at room temperature. All subsequent procedures through step 7 were performed at room temperature.

4) The resulting emulsion was centrifuged and the entire aqueous phase, including the interface, was removed to an Erlenmayer flask and brought to a concentration of 1 M $NaClO_4$ to disassociate nucleic acids from proteins.

5) One volume of chloroform-isoamyl alcohol (24:1) was added to the aqueous phase and the mixture was shaken by hand for 15 minutes. The chloroform denatured protein and removed phenol while isoamyl alcohol reduced foaming.

6) The emulsion was broken by centrifuging, and the aqueous layer was removed to a clean Erlenmayer flask, leaving the denatured protein interface behind.

7) Steps 5 and 6 were repeated until very little denatured protein appeared at the interface following centrifugation.

8) Two volumes of cold (5°C) 95% EtOH were added to the aqueous phase, and the nucleic acids were collected on a glass stirring rod as the EtOH and aqueous phases were mixed by swirling the flask.

9) The nucleic acid fibers were dissolved in 0.15 M NaCl-0.015 M Na_3 citrate, pH 7.0 (SSC buffer) either overnight at room temperature or 1/2 hour with shaking.

10) The nucleic acids were again precipitated with two volumes of EtOH and dissolved in SSC.

11) After precipitation once more with EtOH, the fibers were dissolved in NET.

12) Heat treated (100°C/15 min) RNA-ase (Worthington Biochemical Corp., Freehold, New Jersey) was added to a concentration of 50 µg/ml and the solution incubated at 60°C for 1-2 hours.

13) Sodium lauryl sulfate was added to 0.5% concentration and steps 2 through 9 were repeated.

14) The purified DNA was precipitated from EtOH 2-3 times and dissolved in 0.3 M Na-acetate, 10^{-4} M EDTA (pH 7) to supply the correct ionic environment for the selective precipitation of DNA with isopropyl alcohol.

15) DNA was precipitated by the addition of 0.54 volumes of isopropyl alcohol.

16) The DNA fibers were washed in 70% EtOH and stored in the freezer in 70% EtOH.

The final yield of DNA prepared by this procedure was 50% to 80%.

Isolation of Labeled DNA

Labeled DNA was isolated by a hydroxylapatite fractionation method (Britten et al., 1970) as follows:

1) Cells were suspended in 8 M urea, 0.24 M phosphate buffer (consisting of equal molar amounts of Na_2HPO_4 and NaH_2PO_4, pH 6.8), 1% sodium lauryl sulfate, 0.01 M EDTA.

2) Lysates were blended in a sealed, filled container with a Vir-Tis tissue homogenizer at low speed for 2 minutes.

3) Blended lysates were passed over hydroxylapatite.

4) Blended lysates were washed with several volumes of 8 M urea, 0.24 M phosphate buffer (PB).

5) Blended lysates were washed with several volumes of 0.014 M PB to remove the urea.

6) DNA was eluted from hydroxylapatite with several volumes of 0.4 M PB, and frozen until use.

The yield of DNA prepared by this procedure was approximately 70%.

Purity of DNA Preparations

The absorbancies of DNA preparations were measured in a Gilford 2400 Spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). The A_{260}:A_{280} was found to be 1.8 to 2.0 in purified DNA preparations. Hyperchromicity was measured in unlabeled DNA preparations after alkali denaturation. All of our preparations showed

hyperchromicities between 1.25 and 1.35. In a few instances, protein content was measured as described above. Less than 1% of protein, by weight of DNA, was demonstrated in the unlabeled DNA preparations examined. Amounts of labeled DNA were too small to allow a determination of contaminating materials.

Preparation for Melting and Reannealing of DNA

DNA samples, prepared by chemical precipitation, were dissolved in 0.14 M PB. All DNA preparations were dialyzed against 0.14 M PB before use.

Shearing of DNA was accomplished with a Vir-Tis 45 Tissue Homogenizer (The Virtis Company, Inc., Gardiner, New York) run at full speed for one hour. This procedure reduces DNA to a molecular weight of less than 500,000 daltons (Dr. R. J. Britten, personal communication).

Denaturation and Reannealing of DNA

Denaturation

Labeled DNA was mixed with an excess of unlabeled DNA in 0.14 M PB (greater than a 1,000-fold excess of unlabeled DNA was used for quantitative experiments, and a greater than 100-fold excess was used for the kinetics to be described) and denatured by boiling for 15 minutes.

Reannealing

Samples were removed from the boiling water bath to controlled temperature water baths and reannealed by incubation for the required time periods. Samples removed at various time intervals for renaturation kinetics were frozen until analyzed by hydroxylapatite chromatography.

Hydroxylapatite Chromatography of DNA

Hydroxylapatite (Biogel H. T., Bio-Rad Laboratories, Richmond, California) was boiled 15 minutes in 0.14 M PB prior to use, in order to reduce binding of single-stranded DNA.

Hydroxylapatite separation of renatured DNA from unrenatured DNA was accomplished by a batch procedure (Brenner et al., 1968). Defrosted 2 ml samples in 0.14 M PB were added to hydroxylapatite pellets in centrifuge tubes. 1 ml of packed hydroxylapatite was allowed for each 100 µg of DNA to be fractionated.

Samples were held for 20 minutes in a water bath held at the temperature at which the DNA was renatured, The hydroxylapatite was dispersed with a glass stirring rod and centrifuged for three minutes at 70% of maximum speed in a Sorvall NSE centrifuge with an SPX centrifuge head (Ivan Sorvall, Inc., Norwalk, Connecticut). The centrifuge was housed in an incubator held at 60°-70°C. Brenner et al. have shown that the temperature change during centrifugation does not affect the experimental results. The supernatants were poured into appropriately labeled tubes. The hydroxylapatite pellets were washed 5 times with fresh 0.14 M PB in volumes equal to twice the volume of packed hydroxylapatite pellet. Washes were performed at the temperature at which the DNA was renatured. The pooled washes from 0.14 M PB constitute the single-stranded fraction of DNA of the sample. The pellet was then brought to 0.4 M in PB by addition of 0.6 M PB equal to two pellet volumes. Centrifugation and washes were repeated 4 times in the same fashion as for single-stranded washes, with the exception that 0.4 M PB was used for washing. The pooled 0.4 M PB washes constitute the double-stranded DNA fraction of the sample.

Pooled double-stranded and pooled single-stranded DNA washes were precipitated separately by the addition of TCA to a final concentration of 10%. 100 µg of total yeast RNA (Miles Laboratories, Inc., Elkhart, Indiana) had been added as a carrier before precipitation with TCA. Samples were placed in ice for at least one hour, filtered onto nitrocellulose B-6 filters (24 mm) and washed with three 5 ml portions of cold 5% TCA followed by three 5 ml washes with 70% EtOH. Samples were then dried in an incubator overnight at 70°C and counted in the scintillation counter using a toluene-based scintillation fluid.

Recovery of label from hydroxylapatite chromatography and precipitation was complete.

RESULTS AND DISCUSSION

Determination of Gene Frequency by Quantitative DNA-DNA Reannealing

In the first experiments, I have tested the hypothesis that replication of DNA is initiated at an integrated F factor. To eliminate the unpredictable effects of strain differences on the origin of replication, I carried out this first study with five isogenic strains of *E. coli* K12, three Hfr's, an F^+, and an F^-. The relative frequencies of various chromosomal regions in exponentially growing cells were used as a measure of the origin and direction of chromosome replication in order to distinguish which of several models is correct, as described below. The frequencies of these regions were measured by DNA reannealing techniques using *E. coli* F' DNA from *Proteus mirabilis* - *E. coli* F' hybrids as the reagent to reanneal specifically with, and therefore quantitate, defined regions of the replicating *E. coli* chromosomes. In the same experiment, the frequencies of sex factor regions in the three isogenic Hfr strains were determined, as described below, by reannealing with sex factor DNA, as a measure of direction of replication.

If DNA replication in *E. coli* is initiated at a single point on the chromosome and if one or more replicating forks proceed at a constant rate in a single direction around the chromosome to a terminus, then one can predict that regions of the chromosome near the origin should be present in more copies than those near the terminus. The

frequency of the regions, $f_n(X)$, should follow an age distribution:

$$f_n(X) = 2^{n(1-X)} \; n \; \ln 2/(2^n-1)$$

as described by Sueoka and Yoshikawa (1965; also see Mathematical Appendix to this dissertation). In this equation, "n" represents the average number of rounds of replication proceeding on a single chromosome measured from the origin (0) to the terminus (1). Here $f_n(X)$ is a normalized frequency function, since the expression $(2^n-1)/n\ln 2$ is the relative amount of DNA per replicating chromosome compared to the non-replicating chromosome which has a value of unity.

If chromosome replication begins at the same origin in each of these strains and travels in the same direction, then $f_n(X)$ will be the same in each strain regardless of sex. However, if chromosome replication begins at the integrated F factor, then $f_n(X)$ for each region of the chromosome will be a function of the site of integration of the fertility factor and the direction of chromosome transfer in each individual Hfr strain. If the frequencies of chromosomal segments on replicating chromosomes of the various Hfr strains are compared to those of an F^- strain growing exponentially with same growth rate, the ratio of $f_n(X)$'s, for Hfr:F^-, will be unity if the former hypothesis is correct. If the latter hypothesis is correct, however, the ratio for each Hfr strain will follow the age distribution given previously with the origin-terminus at the site of the integrated F factor.

In these experiments, as mentioned above, the relative number of copies of several regions of the various replicating chromosomes were determined by reannealing techniques. Labeled DNA from exponentially growing E. coli K12 strains was reannealed with segments of the E. coli

chromosome which are carried in Proteus strains as one of several large F merogenotes. The frequencies of chromosome segments of F^- and Hfr strains were compared and expressed as a ratio of $f(X)_{hfr}/f(X)_{F^-}$.

The frequency of copies of the integrated sex factor in the replicating Hfr chromosome can be used to estimate the origin and direction of chromosome replication. The frequency of such sex factor regions can be measured by the extent of reannealing of labeled Hfr DNA to sex factor DNA present in unlabeled DNA isolated from a Proteus hybrid carrying an E. coli merogenote.

In order to determine the relative frequency of segments of the replicating E. coli chromosome, it was necessary to make corrections for non-specific reannealing by both the sex factor DNA and the Proteus DNA present in the hybrids. These corrections were simplified by the choice of strains used in the study, each harboring a deletion of the lac-proB region of the E. coli chromosome.

The origins and directions of chromosome transfer in the isogenic Hfr strains χ574, χ575, and χ584 are shown in Figure 1. The thymine requirement of these strains is satisfied by 2 μg/ml. Each Hfr strain, the parental F^+ strain (χ593), and the F^- strain (χ583) carry an identical lac-proB deletion. This deletion was of advantage as described below. The P. mirabilis-E. coli F' hybrid strains PF50, PF53, PF56, and PF58 are described in Table 1. The region of the E. coli chromosome present in these hybrids as an F merogenote is shown in Figure 1. The extents of the merogenotes are not known exactly, but the positions are known.

All labeled DNAs from replicating chromosomes were obtained from E. coli K12 strains carrying a lac-proB deletion. The extent of

reannealing of DNA from this strain with unlabeled DNA from PF50, a *P. mirabilis Flac-proB* hybrid strain, provided the data correcting for the non-specific reannealing of labeled *E. coli* DNA with unlabeled *P. mirabilis* and sex factor DNA's. The reannealing values so obtained were normalized by comparing them to reannealing values obtained with a common unlabeled *E. coli* DNA. The ratio of frequencies of chromosome regions, recorded in Table 3, is calculated as follows:

$$\text{Ratio of frequencies} = f(X)_{male} / f(X)_{female}$$

$$\text{where } f(X) = \frac{A-B}{C}$$

where A = percent reannealing of labeled K12 DNA with each test *Proteus* F' hybrid DNA, measuring reannealing of labeled *E. coli* DNA with unlabeled *Proteus* DNA, with unlabeled sex factor DNA, and with unlabeled DNA corresponding to the chromosomal segment present on the merogenote

where B = percent reannealing of labeled K12 DNA with PF50 hybrid DNA, measuring reannealing of labeled *E. coli* DNA with unlabeled *Proteus* DNA and with unlabeled sex factor DNA

where C = percent reannealing of labeled K12 DNA with KL96 DNA.

The factor "B" corrects for reannealing of both *Proteus* and sex factor DNA with labeled *E. coli* DNA; factor "C" normalizes the reannealing of all strains to that of one strain, KL96.

Frequencies of the regions of F factors integrated at different chromosomal locations in Hfr's were similarly calculated as one measure of the direction of replication. In this case, the reannealing of *P. mirabilis* DNA (from PF2) and of DNA from the hybrid PF50, which carries *Flac-proB*, with labeled DNA from exponentially growing *E. coli* was used for these calculations as follows:

$$\text{F factor homology} = \frac{B-D}{C}$$

where "B" and "C" are as in the previous expression and "B" = percent reannealing of labeled E. coli DNA with unlabeled P. mirabilis PF2 DNA.

E. coli DNA obtained from cells of the isogenic strains labeled[1] during exponential growth (with a generation time of 60 minutes). The percent renaturation of this labeled DNA with unlabeled DNA from P. mirabilis PF2, from E. coli HfrKL96, and from each Proteus hybrid was determined for each labeled DNA preparation. Nine ml of sheared unlabeled DNA, in 0.14 M PB at a concentration of 1.1 mg/ml, was mixed with 1.0 ml of ^{3}H labeled E. coli DNA containing 2-4 µg of labeled DNA in 0.14 M PB (approximately 50,000 cpm/µg) and denatured by boiling for 15 minutes. DNA mixtures were allowed to renature at 75°C for 30 hours, when renaturation was terminated by the addition of four volumes of ice cold 0.14 M PB. Samples were frozen until processed further. Preliminary experiments showed that E. coli DNA at these conditions of time and temperature would self-reanneal to approximately 85% while the nonspecific reannealing of labeled E. coli DNA with unlabeled P. mirabilis DNA was on the order of 1% or less.

Samples of the renaturation mixture were thawed and passed over hydroxylapatite at 75°C by the centrifuge procedure described in Materials and Methods. Fractionated DNA was precipitated and counted as described in Materials and Methods.

The results of these experiments, shown in Table 2, show no difference in region frequencies among Hfr, F^{+}, and F^{-} sublines of the

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[1]The culture was grown for five generations in the presence of ^{3}H labeled thymine present at 5 µc/2 µg/ml.

Table 2. Renaturation of Proteus mirabilis F' hybrid DNA with labeled DNA from exponentially growing E. coli

DNA from E. coli Strain	Percent Renaturation of Unlabeled Proteus F' Hybrid DNA with Labeled E. coli DNA			Percent Reannealing of F DNA
	PF53 (F101)	PF58 (F129)	PF56 (F108)	
583 (F^-)	1.86 ± 0.09	1.98 ± 0.38	2.70 ± 0.47	2.52 ± 0.18
575 (Hfr)	2.16 ± 0.59	1.95 ± 0.29	2.64 ± 0.18	3.65 ± 0.12
574 (Hfr)	1.86 ± 0.19	2.28 ± 0.56	2.54 ± 0.51	2.66 ± 0.22
584 (Hfr)	2.01 ± 0.18	2.07 ± 0.12	2.68 ± 0.17	4.60 ± 0.19
593 (F^+)	2.00 ± 0.26	2.10 ± 0.16	2.66 ± 0.19	4.04 ± 0.19

The percent renaturation of unlabeled Proteus mirabilis F' hybrid DNA with ^3H labeled DNA from exponentially growing E. coli was determined as described in the text. The percent renaturation of merogenote DNA with labelled E. coli DNA has been corrected for the renaturation of F DNA with E. coli DNA and has been extrapolated to 100% renaturation (that is, the reported values (columns 2-4) are equivalent to the expression, $\frac{A-B}{}$, in the text). Similarly, the percent reannealing of F DNA $\frac{}{C}$ has been corrected for the renaturation of Proteus DNA with labeled E. coli DNA and has been extrapolated to 100% renaturation. The reported values (column 5) are equivalent to the expression $\frac{B-D}{C}$ in the text. Errors are given as two standard errors.

isogenic E. coli strains used in this work. The data of Table 2 have been used to calculate, by the methods described above, the ratio of $f_n(X)_{male}/f_n(X)_{female}$ reported for each male strain in Table 3. The ratios are close to a value of unity. There are, however, differences in F factor reannealing values (Column 5, Table 3). The data show that the frequencies of sex factor DNA decrease in the three Hfr strains in the following order:

$$\chi584 > \chi575 > \chi574.$$

The data of Table 3 are plotted in Figure 2 together with theoretical curves of f(X) for each of three possible modes of chromosome replication. If chromosome replication is initiated at an integrated F factor with a direction of replication which is either the same as that of transfer or opposite that of transfer, the expected value of $f(X)_{male}/f(X)_{female}$ are shown by curve A and curve B, respectively in Figure 2. The third model of replication, all strains having the same origin and direction for replication, yields a curve of unity (curve C, Figure 2) for a plot of $f(X)_{male}/f(X)_{female}$. As may be seen in Figure 2, the data best fit curve C indicating that frequencies of chromosome regions are the same for replicating chromosomes of a series of isogenic Hfr, F^+, and F^- strains.

The percent reannealing of sex factor DNA with labeled DNA from Hfr strains (Table 3) suggests an origin for chromosome replication between the F integration sites of Hfr's $\chi584$ and $\chi574$ and a clockwise direction of replication.

The frequencies of each of the chromosomal regions carried on the merogenotes could be determined directly from the reannealing of merogenote DNA with labeled DNA from exponentially growing cultures

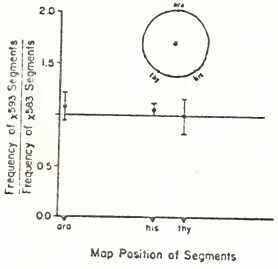
Table 3. Male:female frequency ratios and reannealing of F DNA in exponentially growing E. coli

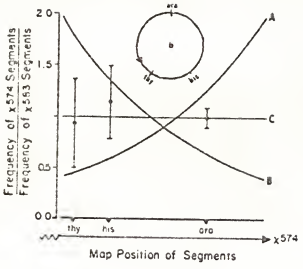
Male E. coli strain	Ratio of Male Regions:Female Regions			Percent reannealing of F DNA
	PF53 (F101)	PF58 (F129)	PF56 (F108)	
x584 Hfr	1.08 ± 0.05	1.05 ± 0.20	0.99 ± 0.17	2.08 ± 0.26
x575 Hfr	1.16 ± 0.32	0.98 ± 0.23	0.98 ± 0.17	1.13 ± 0.22
x574 Hfr	1.00 ± 0.10	1.15 ± 0.35	0.94 ± 0.44	0.14 ± 0.29
x593 F+	1.08 ± 0.14	1.06 ± 0.06	0.99 ± 0.17	1.52 ± 0.27

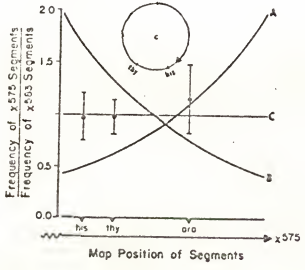
The ratio of the frequency of chromosome regions of male cells to the frequency of chromosome regions in female cells was determined as described in the text. Each value is the average of eight determinations. For the determinations of percent reannealing of F DNA, (column 5) the reannealing of unlabeled PF50 DNA with labeled E. coli x583 DNA has been subtracted from the reannealing values obtained with labeled DNA from the male strains. Errors are given as one standard error.

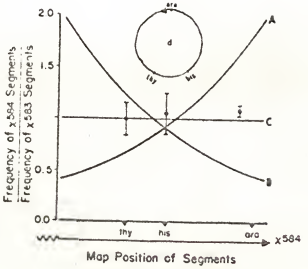
Figure 2. Frequencies of chromosomal regions of E. coli expressed as the ratio of male frequency: female frequency, measured as described in the text. Chromosomes have been opened at the F factor for ease of interpretation. Curve A is the theoretical curve for replication from an integrated F factor and travelling in the direction of transfer. Curve B is a theoretical curve for replication from an integrated F factor with replication forks travelling in a direction opposite the direction of transfer. A frequency ratio of unity (curve C) is expected as a theoretical result, if both male and female strains replicate their chromosomes in the same fashion. Curves A and B are plots of $f_n(X) = 2^n(1-X)\ n\ \ln 2/(2^n-1)$ for $n = 2.25$. This value of "n" was chosen on the basis of the measured generation time and the chromosome replication time for the concentration of thymine (2 μg/ml) used in the experiment. Errors shown in the figure are one standard error.

a. Ratio of χ593 regions:χ583 regions
b. Ratio of χ574 regions:χ583 regions
c. Ratio of χ575 regions:χ583 regions
d. Ratio of χ584 regions:χ583 regions


Frequency of x593 Segments
Frequency of x583 Segments
2.0
1.5
0.5
0.0
ora
his
thy
Map Position of Segments


Frequency of x574 Segments
Frequency of x563 Segments
2.0
1.5
0.5
0.0
A
B
C
thy
his
ora
x574
Map Position of Segments


Frequency of x575 Segments
Frequency of x563 Segments
2.0
1.5
0.5
0.0
A
B
C
his
thy
ora
x575
Map Position of Segments


Frequency of x584 Segments
Frequency of x583 Segments
2.0
1.5
0.5
0.0
A
B
C
thy
his
ora
x584
Map Position of Segments

if the sizes of the merogenotes were known. Since I do not know the physical size of the merogenotes used in the quantitative experiments described above, I have been limited to comparing the extent of re-annealing of merogenote DNA with labeled DNA from various E. coli strains.

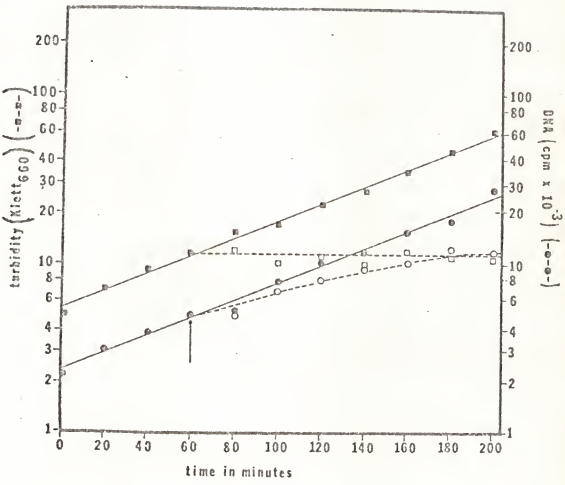
The theoretical curves in Figure 2 are plotted assuming the number of replicating forks, "n" is 2.25, as predicted by Zaritsky and Pritchard (1971) for E. coli thy strains growing in limiting concentrations of thymine. This value of "n" was calculated from the data of Zaritsky and Pritchard for the generation time (60 minutes) and thymine concentration (2 µg/ml) used in my experiments. In order to confirm the value of "n" used in calculating the theoretical curves, the following experiment was performed: Chloramphenicol (150 µg/ml) was added to cells of E. coli K12 strain χ583 growing exponentially in the presence of ^{3}H labeled thymine at 5 µc/2 µg/ml under conditions identical to those used in our DNA labeling experiments. Residual incorporation of labeled thymine into TCA insoluble material was approximately 140% (see Figure 3). The residual DNA synthesis required to complete rounds of replication already in progress may be given as (Sueoka and Yoshikawa, 1965; also Mathematical Appendix):

$$\text{percent increase in DNA} = \frac{2^{n-} \frac{2^{n}-1}{n\ln 2}}{2^{n}-1)/n\ln 2} \times 100.$$

In my experiments, the percent increase of DNA after blocking initiation of new rounds of synthesis, approximately 140%, gives a calculated value for "n" of approximately 3.

The quantitative technique for determining gene frequency was

Figure 3. Replication of DNA and increase in turbidity of E. coli strain χ583 in the presence of chloramphenicol. A culture of E. coli χ583 growing exponentially in minimal medium supplemented with 1% casamino acids, 0.5% glucose, and ^{3}H labeled thymine (5 μc/2 μg/ml was treated with 150 μg/ml chloramphenicol at the time indicated by the arrow. Samples were analyzed for turbidity increase and incorporation of ^{3}H counts into TCA insoluble material.


turbidity (Klett$_{600}$) (-▫-▫-)
DNA (cpm x 10^{-3}) (-●-●-)
time in minutes
200
100
80
60
40
30
20
10
8
6
4
3
2
1
0
20
40
60
80
100
120
140
160
180
200

useful only to compare two or more E. coli strains in order to see if replication was the same in these strains, and required the use of a lac-proB deletion in each E. coli strain to be tested. This limits the amount of information which may be obtained, and led to a search for a more encompassing assay procedure for determining frequencies.

Determination of Gene Frequency by DNA Renaturation Kinetics

Preparation of Labeled Merogenote DNA

The regions of the chromosome vary in their frequency dependent on their position on the replicating chromosome (see Figure 15, Mathematical Appendix). The differences in frequency will be reflected as differences in renaturation rate for each of the regions (see Mathematical Appendix of this dissertation). Renaturation kinetics as a measure of region frequencies was carried out, since it would allow the determination of frequencies in any strain of E. coli growing under any of a wide variety of conditions.

Partial purification of merogenote DNA by thermal elution

In order to perform renaturation kinetics experiments, it was first necessary to separate and purify defined regions of the chromosome in labeled form. Merogenote DNA was separated from Proteus F' hybrids by two methods; the first involving the removal of labeled Proteus DNA from merogenote DNA by selective denaturation of the Proteus DNA while leaving the merogenote DNA in native form, the second involving the removal of labeled Proteus DNA and labeled F factor DNA by two successive renaturations with a vast excess of un-

labeled DNA from a Proteus F' hybrid different from those used for the preparation of labeled merogenote DNA.

The Proteus hybrids PF74, PF75, PF77, PF78, PF79, PF80, and PF84 and P. mirabilis PF82 were grown to stationary phase in 10 ml of minimal medium containing ^{3}H labeled thymine present at 100 µc/2 µg/ml. E. coli χ583 was grown to stationary phase in 10 ml of minimal medium with ^{14}C labeled thymine present at 1 µc/2 µg/ml. Cells were harvested and DNA purified by the chemical procedure outlined in Materials and Methods after the addition of a total of 1 mg of unlabeled P. mirabilis PF2 DNA to each batch of labeled cells as a carrier. Purified ^{14}C labeled E. coli χ583 DNA was mixed with purified ^{3}H labeled Proteus PF82 (in order to follow purification, see Table 4) and to all Proteus F' hybrid DNA's at a ratio of ^{14}C counts : ^{3}H counts of 1:200 and sheared. The addition of a ^{14}C bulk labeled E. coli DNA to the ^{3}H labeled Proteus and Proteus F' hybrid DNA preparations served two purposes. First, the ^{14}C labeled E. coli DNA has the same properties as the merogenote DNA in the Proteus F' hybrid DNA preparations and was used as a marker to follow the purification of ^{3}H labeled merogenote DNA from Proteus F' hybrids. Second, in renaturation experiments, the ^{14}C labeled E. coli DNA served as an internal standard for the renaturation kinetics of the bulk E. coli chromosomal DNA.

Advantage was taken of the difference in melting temperatures of the DNA to selectively denature the Proteus DNA in the Proteus F' hybrids while leaving the merogenote DNA in double-stranded form. The procedure was based on data from a preliminary experiment in which a mixture of ^{14}C labeled E. coli χ583 DNA and ^{3}H labeled P. mirabilis PF82 DNA was fractionated on hydroxylapatite by stepwise temperature

Table 4. Purification and characterization of ^{3}H labeled merogenote DNA

DNA preparation		DNA unfractionated	DNA after thermal elution	DNA after first renaturation purification
PF74	ratio: $^{3}H/^{14}C$	131.4	32.4	17.8
	percent ^{14}C	0.76	3.0	5.3
PF75	ratio: $^{3}H/^{14}C$	118.0	18.3	4.3
	percent ^{14}C	0.84	5.2	18.9
PF77	ratio: $^{3}H/^{14}C$	116.5	20.1	9.7
	percent ^{14}C	0.85	4.7	9.4
PF78	ratio: $^{3}H/^{14}C$	110.8	17.8	7.8
	percent ^{14}C	0.90	5.3	11.4
PF79	ratio: $^{3}H/^{14}C$	191.7	59.1	35.6
	percent ^{14}C	0.52	1.67	2.7
PF80	ratio: $^{3}H/^{14}C$	128.0	16.2	4.1
	percent ^{14}C	0.78	5.8	19.6
PF84	ratio: $^{3}H/^{14}C$	140.1	22.3	10.6
	percent ^{14}C	0.71	4.3	8.6
PF82	ratio: $^{3}H/^{14}C$	130.9	16.4	3.9
	percent ^{14}C	0.76	5.8	20.4
Percent recovery of ^{14}C labeled DNA in PF82 preparation		100	53	84

Table 4. Continued

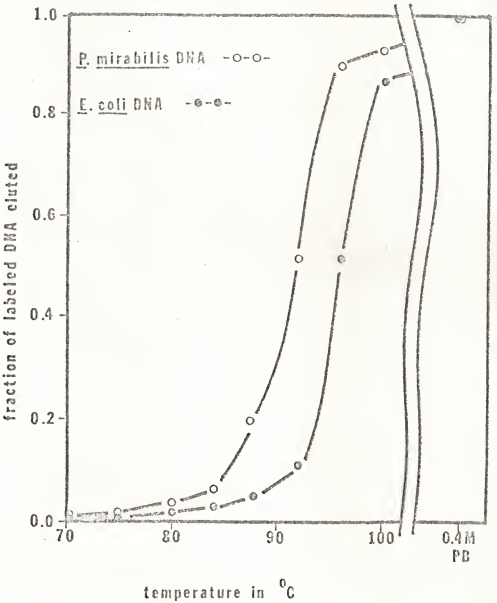
DNA after second renaturation purification	Percent reannealing with unlabeled AB259 (Hfr) DNA	Percent reannealing with unlabeled AB1514 (F^-) DNA	Percent purity (average of two previous columns)
14.3 6.5	89.97 ± 0.19	87.55 ± 0.39	88.76 ± 0.57
2.3 30.4	45.81 ± 1.22	40.28 ± 1.60	43.04 ± 1.53
6.6 13.2	81.43 ± 0.06	76.68 ± 1.50	79.05 ± 1.26
6.0 14.3	86.01 ± 0.79	84.55 ± 0.64	85.28 ± 0.77
31.4 3.1	89.54 ± 0.49	84.51 ± 0.75	87.03 ± 1.19
2.5 28.6	62.75 ± 0.51	58.88 ± 1.09	60.43 ± 1.13
8.1 11.0	89.58 ± 0.50	85.94 ± 0.99	87.76 ± 1.15
2.9 25.6	-	-	-

increases. Proteus DNA (^{3}H labeled, 20,000 cpm) and E. coli DNA (^{14}C labeled, 1,000 cpm) were mixed and fractionated on 1 ml packed volumes of hydroxylapatite by the centrifuge method described in Materials and Methods. Samples were heated at temperatures which were increased in a stepwise manner with increments of several degrees until 100°C was reached. Single-strands were eluted by three 2 ml washes with 0.14 M PB at each temperature. As the temperature was raised, fractions of the DNA would dissociate into single-strands when their melting temperature was exceeded. The single-stranded washes at each temperature were precipitated and counted. The small amounts (4-5%) of DNA still bound after three washes at 100°C was eluted in three washes of 0.40 M PB. The results of the stepwise thermal denaturation are shown in Figure 4. It may be seen that the melting points of E. coli DNA and P. mirabilis DNA differed by approximately 4-5°C. From the shape of the thermal elution profile I concluded that Proteus DNA could be partially separated from the E. coli DNA carried on merogenotes in Proteus F' hybrids by incubating and fractionating the mixed DNA's on hydroxylapatite at a temperature which denatures P. mirabilis DNA while leaving E. coli DNA as a double-stranded molecule. Accordingly, I have partially purified merogenote DNA by the following procedure:

1) 10 ml samples of ^{3}H labeled Proteus F' hybrid DNA mixed with 0.5% ^{14}C labeled E. coli χ583 DNA in 0.14 M PB were loaded into two 18 mm centrifuge tubes, each containing 5 ml of packed hydroxylapatite and allowed to equilibrate in a water bath held at 91°C for 20 minutes.

2) The DNA preparations were washed three times with 10 ml of 0.14 M PB at 91°C. The washes, consisting of DNA denatured at 91°C, contained mostly ^{3}H labeled DNA and very little ^{14}C labeled DNA. The single-stranded washes at 91°C were discarded and the double-stranded washes with 0.4 M PB (total 60 ml) were

Figure 4. Thermal elution profiles of E. coli DNA and P. mirabilis DNA. Single-stranded DNA fragments were eluted from hydroxylapatite, containing a mixture of ^{3}H labeled P. mirabilis DNA (20,000 cpm) and ^{14}C labeled E. coli DNA (1,000 cpm), at the indicated temperatures, by washing with 0.14 M PB. Labeled DNA removed at each temperature was counted. Label remaining bound to the hydroxylapatite after three washes with 0.14 M PB at 100°C was removed by three washes with 0.4 M PB.


1.0
0.8
0.6
0.4
0.2
0.0
P. mirabilis DNA -o-o-
E. coli DNA -●-●-
fraction of labeled DNA eluted
70
80
90
100
0.4M PB
temperature in °C

pooled and dialyzed against 10.0 liters of 25% polyethylene glycol (av. mol. wt. 15,000-20,000; Matheson, Coleman, and Bell, East Rutherford, New Jersey) in 0.14 M PB to a final volume of 5-10 ml.

3) The samples, brought to 10 ml with 0.14 M PB, were pooled and dialyzed exhaustively against 0.14 M PB and passed to the next purification step.

The extent of purification, given as the ratio of ^{3}H labeled hybrid DNA and as the percent ^{14}C labeled E. coli DNA in the mixture, and the percent recovery of ^{14}C labeled E. coli DNA are given in Table 4. As a means of following purification, a mixture of ^{14}C labeled E. coli DNA and ^{3}H labeled P. mirabilis PF82 DNA was carried through the procedures described. The amount of ^{14}C labeled DNA in the total labeled DNA increased from 0.76% to about 5.7%. The recovery of ^{14}C labeled E. coli DNA from this mixture in this step was 53%.

Removal of Proteus DNA and F DNA from merogenote DNA by renaturation

Partially purified merogenote DNA still contained, as the majority of its DNA, labeled Proteus and F factor DNA's. The level of the contaminating labeled Proteus DNA and F factor DNA was decreased in two steps as follows:

Partially purified ^{3}H labeled merogenote DNA (10 ml) was mixed with 10 ml of concentrated (1 mg/ml) unlabeled hybrid (PF56) DNA in 0.14 M PB and denatured by boiling. The mixtures were renatured for 10 hours at 60°C in order to convert labeled Proteus and F factor DNA's to double-strands. These conditions of time, temperature and DNA concentration had been shown in a preliminary experiment to result in the renaturation of approximately 80% of labeled Proteus DNA. DNA from the renaturation mixture was fractionated into single-strands and double-strands on 100 ml of packed hydroxylapatite, and the pooled single-stranded washes (approximately 300 ml) were dialyzed against

polyethylene glycol to reduce the volume to less than 10 ml. Volumes were brought to 10 ml by the addition of 0.14 M PB, and the preparations were dialyzed extensively against 0.14 M PB. The extent of purification, given as the ratio of ^{3}H labeled DNA to ^{14}C labeled DNA and as the percent ^{14}C labeled E. coli DNA in the mixture, and percent recovery of ^{14}C labeled DNA were again determined and are given in Table 4.

The DNA purified by renaturation with PF56 DNA was added to 10 ml of unlabeled PF63 DNA (1 mg/ml), and the renaturation step, fractionation, and concentration of the single-stranded washes were repeated as for PF56 DNA. The extent of purification, given as the ratio of ^{3}H labeled DNA to ^{14}C labeled DNA and as the percent ^{14}C labeled E. coli DNA in the mixture, and percent recovery were again determined and are given in Table 4.

The level of ^{14}C labeled DNA in the mixture of ^{3}H labeled Proteus (PF82) DNA and ^{14}C labeled E. coli DNA rose from 5.8% to 20.4% during the first renaturation purification step and from 20.4% to 25.6% during the second renaturation. The percent recoveries were 84% for the first renaturation and 77% for the second renaturation. Another measure of the purification of E. coli DNA is that the ratio of ^{3}H to ^{14}C, initially greater than 100:1 was reduced to a value of 2.9:1 following the final renaturation purification step. It may be seen that in the purification of DNA from hybrid strains carrying large merogenotes (PF74 and PF79) the level of ^{3}H labeled DNA remains high.

Assay of purity of merogenote DNA preparations

The two renaturation steps removed most of the labeled F factor and Proteus DNA's present as labeled contaminating DNA in the merogenote

DNA preparations by converting these unwanted portions to a double-stranded form during the renaturation step. The labeled merogenote preparations were then considered "purified" and were used in renaturation kinetics experiments. In order to use the labeled merogenote DNA preparations, it was necessary to demonstrate that F factor DNA was not a significant fraction of the labeled DNA, since F DNA would reanneal extensively with sex factor DNA from Hfr strains and therefore obscure the renaturation kinetics of the chromosomal DNA on the merogenote. In addition, it was necessary to know the proportion of labeled DNA which was present in the form of E. coli DNA. These parameters were measured by determining the extent of reannealing of labeled merogenote DNA with unlabeled DNA's from an Hfr strain and from an F^- strain of E. coli.

Merogenote DNA (1,000-5,000 cpm ^3H labeled DNA) was mixed with sheared, unlabeled DNA in 0.14 M PB from stationary phase cells of either AB259 (Hfr) or AB1514 (F^-) to a final concentration of 1 mg/ml of unlabeled DNA. If there were a significant amount of ^3H labeled F factor DNA present, it would have shown as a greater amount of reannealing of ^3H labeled DNA with unlabeled AB259 DNA than with unlabeled AB1514 DNA. The ^{14}C labeled E. coli DNA added to each of the ^3H labeled merogenote DNA preparations at the beginning of the purification procedures served as a measure of the reannealing of the unlabeled E. coli DNA. Samples, containing mixtures of labeled merogenote DNA with either unlabeled AB259 DNA or unlabeled AB1514 DNA, were boiled and renatured for 10 hours at 60°C and subsequently fractionated on hydroxylapatite. Pooled single-stranded (SS) washes and pooled double-stranded (DS) washes were precipitated, filtered, and counted as

described previously. The percent renaturation of the ^{3}H labeled merogenote DNA with unlabeled E. coli DNA was calculated as $(cpm_{DS}/cpm_{DS+SS}) \times 100$. Self-renaturation was determined from renaturation mixtures containing only labeled merogenote DNA, at concentrations of labeled DNA equivalent to those in the determinations using unlabeled E. coli DNA. The value of this self-renaturation control, varying between 2-5%, was subtracted from the determinations of percent reannealing of labeled merogenote DNA with unlabeled E. coli DNA. The percent renaturation of the ^{14}C labeled E. coli DNA with unlabeled AB259 or AB1514 DNA was used in calculations of the purity of ^{3}H labeled merogenote DNA as follows:

$$\text{Purity of merogenote DNA} = \frac{\text{renaturation of } ^{3}H \text{ labeled merogenote DNA}}{\text{renaturation of } ^{14}C \text{ labeled E. coli DNA}}$$

The results of these analyses are shown in columns 6, 7, and 8 of Table 4. In no case was the F factor DNA a large enough portion of ^{3}H labeled DNA to affect our interpretations. Percent purity of ^{3}H labeled merogenote DNA varied from 43% in F8, a small merogenote, to approximately 89% in F104, a large merogenote.

Renaturation Kinetics

The assay of frequency of the various chromosomal regions is based on the knowledge that the rate of reannealing of single-stranded DNA to form double-stranded DNA is directly proportional to the concentration of the single-stranded DNA species present in large excess (see Mathematical Appendix). The rate of reannealing is described by the relationship:

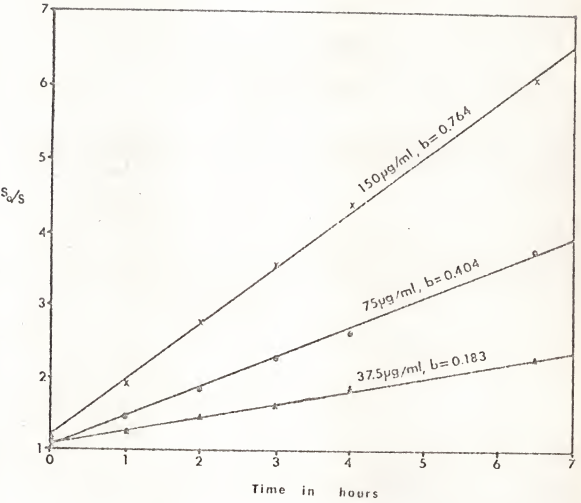
$$\frac{S_o}{S} = KtS_o + 1$$

where "S_o" is the concentration of single-stranded DNA at time zero,

"S" is the concentration of single-stranded DNA at any time "t", and "K" is the second-order rate constant. The apparent second order rate constant, "K'", is equal to the expression, "KS_o".

In a preliminary experiment to confirm the ability of renaturation kinetics to detect differences in DNA concentration, I have determined the apparent second order rate constant, as a measure of DNA concentration, for renaturation mixtures containing different concentrations of DNA. Unlabeled DNA from stationary phase cells of E. coli AB1514 was mixed with ^{14}C labeled DNA from stationary phase cells of E. coli χ583 to give a solution of 12,000 cpm/150 μg DNA/ml and then sheared. It was found that a solution of ^{14}C labeled DNA from χ583 contains approximately 12,000 cpm/μg DNA. From the sheared DNA, two additional renaturation mixtures were prepared by dilution of the above solution of DNA with 0.14 M PB so as to yield two additional concentrations of DNA: 6,000 cpm/75 μg/ml and 3,000 cpm/37.5 μg/ml. These three concentrations of DNA are in the same range as the concentration of DNA (100 μg/ml) used in renaturation kinetics experiments for the determination of frequencies of chromosomal regions. The three concentrations of DNA were heated at 100°C and then renatured at 60°C. At various times during the renaturation, 1 ml samples were removed and chilled in an ice bath. The samples were analyzed for ^{14}C counts in the single-stranded DNA fraction and in the double-stranded DNA fraction as described previously. The inverse fraction of single-stranded DNA (S_o/S) was calculated as $(cpm_{SS} + cpm_{DS})/cpm_{SS}$. The value of ($S_o/S$) was plotted versus time for each of the dilutions of DNA. As expected, the measured rate of renaturation, "K'", was proportional to the concentration of single-stranded DNA (Figure 5).

Figure 5. Renaturation rate as a function of DNA concentration. Dilutions from a mixture of unlabeled AB1514 DNA and ^{14}C labeled χ583 DNA, sheared at a concentration of 150 μg DNA/12,000 cpm/ml, were renatured at 60°C. The inverse fraction of single-stranded DNA (S_o/S) is plotted against time. "b" is the value of a linear regression of (S_o/S) on time.


S_0/S
Time in hours
150µg/ml, b=0.764
75µg/ml, b=0.404
37.5µg/ml, b=0.183
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7

Having established that I can measure differences in DNA concentrations in the concentration range used in experiments measuring region frequencies, I then measured the frequencies of chromosome regions as follows: Unlabeled DNA from the E. coli culture to be analyzed was sheared and mixed with a sample of labeled merogenote DNA. The unlabeled DNA was added to a final concentration of 100 μg/ml. The labeled merogenote DNA contained ^{3}H labeled DNA corresponding to the chromosomal segments of F' factors and ^{14}C labeled DNA corresponding to the entire E. coli chromosome. DNA in the mixtures was denatured by boiling and then renatured at 60°C. Samples (1 ml) were removed at intervals, chilled in an ice bath, and then frozen. Defrosted samples were fractionated on hydroxylapatite into single-stranded and double-stranded fractions which were assayed for both ^{3}H and ^{14}C. The inverse fraction of single-stranded ^{3}H labeled merogenote DNA was calculated as follows:

$$\frac{S_o}{S} = \frac{1}{1-[A/(A+B)C]}$$

where A = cpm in the double-stranded DNA fraction; B = cpm in the single-stranded DNA fraction; and C = fractional purity of the ^{3}H labeled merogenote DNA, $\frac{1}{100}$ × percent purity (Table 4). Similarly, the inverse fraction of single-stranded ^{14}C labeled chromosomal DNA was calculated to be:

$$\frac{S_o}{S} = \frac{A+B}{B}$$

Calculation of Frequencies of Chromosome Regions

For these calculations, the renaturation of ^{14}C labeled E. coli chromosomal DNA with unlabeled E. coli DNA was taken to represent the renaturation of the average of all the segments of the chromosomal DNA

in the unlabeled DNA preparation. If a ^{3}H labeled merogenote DNA preparation renatured at a more rapid rate than the ^{14}C labeled chromosomal DNA in a renaturation mixture containing unlabeled E. coli DNA, it would imply that the ^{3}H labeled segment was present in more copies than most segments of the chromosome (as represented by the renaturation kinetics of the ^{14}C labeled E. coli DNA with the unlabeled E. coli DNA preparation). The data has been plotted as (S_o/S) for ^{3}H labeled chromosomal segments $[(S_o/S)_{^{3}H}]$ versus (S_o/S) for ^{14}C labeled E. coli DNA $[(S_o/S)_{^{14}C}]$. The regression of $(S_o/S)_{^{3}H}$ on $(S_o/S)_{^{14}C}$ was calculated and used as a measure of frequency of the ^{3}H labeled chromosomal segment. The use of this method of plotting has distinct advantages over plotting S_o/S versus time as was done for Figure 5. In the kinetics experiment reported in Figure 5, the labeled and unlabeled DNA's were mixed together and sheared. Different DNA concentrations were achieved by dilution of sheared DNA with buffer. The ionic environments and sizes of DNA in the various DNA dilutions in that experiment were nearly identical. All conditions which affect DNA renaturation rate were also nearly identical for each DNA mixture so that the results obtained by plotting S_o/S versus time for each different DNA concentration were comparable. In contrast plotting (S_o/S) versus time, as in the experiment to show the dependence of renaturation rate on DNA concentration (Figure 5), would not be accurate enough for the experiments on renaturation kinetics as a measure of gene frequency. For example, in the experiments to be described below, unlabeled DNA was isolated from a variety of strains. It is not to be expected that the sheared DNA sizes would be the same in each unlabeled DNA preparation. Further, the ionic environment of

each DNA preparation will probably vary slightly. In order to compare renaturation kinetics among several DNA preparations, the DNA concentrations would have to be accurately determined if only ^3H labeled merogenote DNA were present in the renaturation mixtures. Similarly, each labeled merogenote DNA preparation is expected to vary somewhat in DNA size and in ionic environment.

Plotting $(S_o/S)_{^3H}$ versus $(S_o/S)_{^{14}C}$ allows us to measure the renaturation rate of the ^3H labeled DNA segments relative to an internal standard (^{14}C labeled E. coli DNA) which has undergone exactly the same treatments as the ^3H labeled merogenote DNA. It is obvious that the slight variations in salt concentration, temperature, sheared DNA piece sizes, and concentrations of unlabeled DNA would be internally compensated so that further corrections would not be necessary.

Marker Frequencies

Marker frequencies in rapidly growing E. coli

Initial experiments with renaturation kinetics utilized unlabeled DNA from E. coli strains B, C, 15, and K12 (Hfr, F^+, and F^- sublines) grown in L broth at 37°C. Generation times (G) of most E. coli strains in this medium were approximately 25 minutes. Using a value for chromosome replication time (R) of 40 minutes, "n" (average number of replicating forks per chromosome) would be expected to have a value of R/G = 1.6, (40 min./25 min.), under these conditions.

Sheared unlabeled DNA from E. coli growing exponentially in L broth was mixed with labeled DNA from merogenote DNA preparations, renatured as described previously, and analyzed for ^3H and ^{14}C in the single-stranded and double-stranded fractions. Several of the purified merogenote DNA's were used to assay the frequency of regions of the

chromosomes of each of the strains growing exponentially in L broth. The slope of the resultant line (that is, the value of the linear regression of $(S_o/S)_{3_H}$ on $(S_o/S)_{14_C}$ was taken as the frequency of the chromosome region identical to the merogenote segment). An example of the results of such an experiment, using labeled merogenote DNA from F104 and unlabeled DNA from cells (E. coli AT2243) growing exponentially in L broth, is shown in Figure 6. The renaturations and analyses were repeated for each of the combinations of labeled merogenote DNA and unlabeled E. coli DNA used. My earliest experiment, using unlabeled DNA from exponentially growing AT2243, gave region frequency data compatable with a frequency distribution curve having an origin for replication near 60 minutes on the E. coli genetic map, and a clockwise direction of replication (Figure 7). However, no regions between F103 clockwise to F14 were examined for frequency in AT2243; and the data do not rule out a bidirectional mode of replication. From this single experiment, it seems that an origin exists in the region near F14, and a replication fork travels clockwise from this region toward the region of F103 (approximately minute 40 on the E. coli genetic map). As region frequencies were determined for other E. coli strains, growing exponentially in L broth, it seemed that the region of the chromosome corresponding to F129 (not tested in the experiment involving AT2243) was usually present in a greater frequency than that of the region corresponding to F103 (Table 5, Figure 8), consistent with a model having a terminus in the region slightly counter-clockwise to the region of F103, with replicating forks traveling in both directions from the origin to the terminus. It is possible to fit a frequency distribution curve having an origin at minute 70 and a terminus at

Figure 6. Renaturation of labeled F104 merogenote DNA with unlabeled DNA from exponentially growing cells of E. coli AT2243. K' and its standard deviation are given. K', the measure of frequency of chromosomal regions, is the value of the regression of $(S_o/S)_{3H}$ on $(S_o/S)_{14C}$.

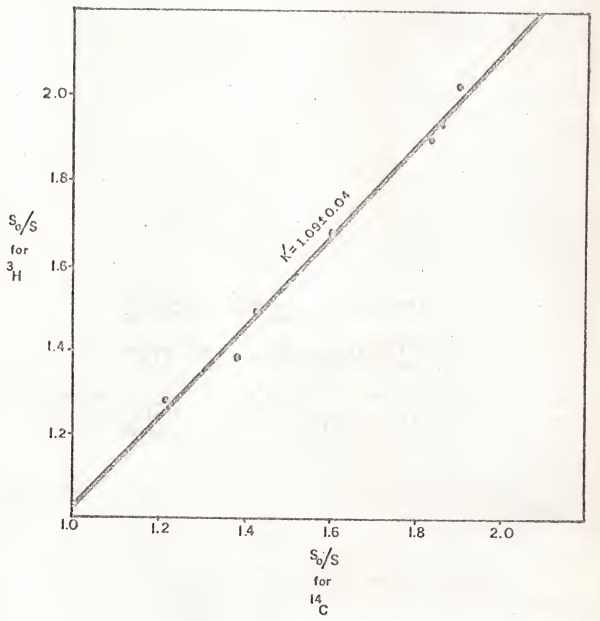

2.0
1.8
1.6
1.4
1.2
1.0
1.2
1.4
1.6
1.8
2.0
S_0/S for ^3H
S_0/S for ^{14}C
K = 1.03 ± 0.04

Figure 7. Frequencies of regions on the chromosome of E. coli AT2243 growing exponentially with a generation time of 25 minutes. Frequencies of regions on the chromosome, measured by renaturation kinetics as described in the text, are plotted against the positions of the regions on the E. coli genetic map (opened to a linear map at minute 60). Errors are the standard deviations of the regressions used in the determinations of frequencies. The curve is a plot of the frequency distribution function, $f_n(X) = 2^{n(1-X)} \; n \; \ln 2/(2^n-1)$, assuming an origin for chromosome replication at minute 60, a clockwise direction of replication, and an average of 1.6 rounds of replication proceeding on each chromosome.

region frequency
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
60
70
80
90/0
10
20
30
40
50
60
70
map position
ilv
mel
thr
lac
gal
trp
his
tyr
lys
argC str
F14
F118
F104
F'lac-prod
F8
F103

Table 5. Frequencies of chromosomal regions in rapidly growing E. coli

E. coli strain	Generation time[a]	F14	F118
AT2243	25 min	1.42 ± 0.08[b]	1.30 ± 0.08
AB312	32 min	-	1.12 ± 0.06
$2{,}000_{X111}$	28 min	1.41 ± 0.05	1.18 ± 0.11
AB264	25 min	1.56 ± 0.10	1.33 ± 0.04
AB259	30 min	1.31 ± 0.07	1.16 ± 0.05
E.coli B	25 min	1.46 ± 0.02	1.25 ± 0.05
E. coli C	27 min	1.51 ± 0.05	1.30 ± 0.04
E. coli 15T (555-7)	24 min	1.56 ± 0.03	1.46 ± 0.05
E. coli B/r	28 min	-	1.29 ± 0.08

[a] Generation time as determined by turbidity increase; generation times determined by increases in DNA, protein, and cell numbers were determined in most cases and found to be the same.

[b] Standard deviation of the regression of a plot of $(S_o/S)_{3_H}$ against $(S_o/S)_{14_C}$ used in determination of frequencies of labeled DNA regions by the kinetics of DNA renaturation (see text).

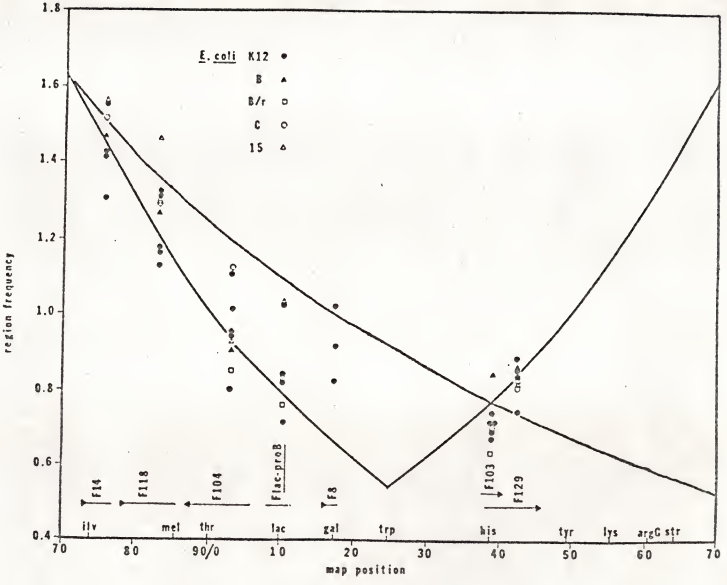
[c] Frequency not determined for this region in this strain.

Frequencies of chromosomal regions in E. coli growing exponentially in L broth were determined by renaturation kinetics as described in the text.

Table 5. Continued

Chromosome Region Frequency Corresponding to:				
F104	Flac-proB	F8	F103	F129
1.09 ± 0.04	1.03 ± 0.18	1.02 ± 0.09	0.71 ± 0.06	-[c]
0.95 ± 0.06	0.82 ± 0.06	0.92 ± 0.08	0.67 ± 0.02	0.64 ± 0.09
0.80 ± 0.05	-	-	0.69 ± 0.04	0.84 ± 0.04
1.02 ± 0.03	0.84 ± 0.08	0.82 ± 0.08	0.73 ± 0.03	0.89 ± 0.05
0.95 ± 0.04	0.66 ± 0.03	-	0.72 ± 0.06	0.85 ± 0.05
0.90 ± 0.07	1.01 ± 0.13	-	0.84 ± 0.05	0.85 ± 0.07
1.12 ± 0.10	0.82 ± 0.15	-	0.70 ± 0.04	0.80 ± 0.10
0.92 ± 0.08	1.04 ± 0.13	-	-	0.80 ± 0.06
0.84 ± 0.03	0.76 ± 0.04	-	0.64 ± 0.06	0.83 ± 0.04

Figure 8. Frequencies of regions on the chromosome in rapidly growing E. coli. Unlabeled DNA isolated from cultures growing exponentially in L broth was measured for chromosome frequencies by renaturations with labeled merogenote DNA, as described in the text. Frequencies of regions are plotted against their positions on the genetic map, for E. coli strains K12, B, B/r, C, and 15. The theoretical curves, plotted for comparison, represent the frequency distribution function, $f_n(X) = 2^{n(1-X)} \, n \ln 2/(2^n-1)$, for a population of cells whose chromosomes replicate either (a) in a bidirectional fashion with an origin at minute 70 and a terminus at minute 25, or (b) in a clockwise fashion with an origin at minute 70, with an average of 1.6 rounds of replication on each chromosome.


E. coli K12
B
B/r
C
15
region frequency
map position
F14
F118
F104
F1ac-pro8
F8
F103
F129
ilv
mel
thr
lac
gal
trp
his
tyr
lys
argG
str

minute 25 on the E. coli genetic map to data obtained from the E. coli strains grown in L broth (Figure 8). The curve in Figure 8 is a plot of the frequency distribution function:

$$f_n(X) = 2^{n(1-X)}{}_n \ln 2/(2^n-1)$$

for the model of chromosome replication having bidirectional replication in each cell (see Mathematical Appendix for a discussion of various models for chromosome replication). The value used for "n" was 1.6 (the number of replicating forks expected per chromosome in a cell population growing exponentially with a generation time of 25 minutes). If one disregards, for the moment, the three points for frequencies obtained with F8 DNA, the data obtained in these experiments fit the curve for bidirectional replication quite well. The F8 DNA was the most impure of the labeled merogenote DNA preparations (see Table 4), having only 43% of the ^3H labeled DNA present as DNA which reanneals with E. coli DNA, and therefore, the values obtained with F8 DNA are suspected of having the greatest error.

The data (Table 5, Figure 8) show that chromosomal DNA corresponding to the region on F14 is present in the highest frequency, and, according to current theory, is nearest the origin. Likewise, the chromosomal DNA corresponding to the region on F103 is present in the lowest frequency, and, therefore, is nearest the terminus. Chromosomal regions decrease in frequency as these regions are located clockwise from the region of F14 to the region of F103. Bidirectional replication is suggested by the frequency of F129 region being higher than the frequency of the F103 region.

An important observation, derived from Table 5 and Figure 8, is that there are no significant differences in region frequencies (and,

hence, mode of replication) among E. coli strains B, B/r, C, 15, and K12. Further, there are no differences among Hfr, F^+, and F^- derivatives of E. coli K12, an observation in agreement with previous data obtained by quantitative DNA reannealing techniques (Tables 2 and 3, Figure 2).

The data accumulated in the above experiments do not measure the frequencies of a sufficient number of regions of the chromosome to support strongly the theory of bidirectional chromosome replication. Such a pattern of replication has been indicated by the data of other workers (Masters, 1970; Masters and Broda, 1971; Bird, R. E., Louarn, J., Martuscelli, J., and Caro, L. G., The Origin and Sequence of Chromosome Replication in Escherichia coli, submitted for publication, J. Mol. Biol., 1972). To provide support for this model, the relative frequencies of regions lying between minute 40 and minute 70 on the E. coli genetic map must be measured. Two merogenotes appropriate for this purpose, F108 and F122, unfortunately have a thy^+ allele on the merogenote; and attempts to isolate thy derivatives of the diploid hybrids harboring either of these merogenotes were unsuccessful. Therefore, these hybrids could not be labeled properly for the isolation of labeled merogenote DNA. Also, no merogenote in the area between gal and his (minute 17 and minute 39) was available in a Proteus hybrid. Thus, it was necessary to use an alternate method of determining relative frequencies of regions in this area of the chromosome. The method selected involved the use of certain transposition Hfr strains which have a chromosomal region translocated (together with the sex factor) from its normal position (near minute 10 on the E. coli map) to one of three positions in the region between minute 40 and minute 70

on the E. coli map, and one position between minute 17 and minute 39 on the E. coli map.

The location of these translocated Flac-proB DNA's permitted the measurement of relative frequencies of these regions by the reannealing kinetics method used previously. For this quantitation, labeled lac-proB DNA isolated from PF80 was used. Each of the isogenic transposition Hfr strains and the lac-proB deletion parent of the Hfr's (X7026) were grown separately in L broth at 37°C and harvested during exponential growth (with generation times of between 30 and 35 minutes). DNA was extracted and used for frequency determinations as described previously. Since an integrated F factor has no effect on chromosome replication (Tables 2,3 and 5, Figures 2 and 8), the frequency of the integrated lac-proB region in each of the strains would be dependent only on its position on the replicating chromosome. Frequencies of the regions carried by F14 and by F103 were determined for each strain, as was the frequency of the transposed lac-proB region. In addition, frequencies of F14, F103, F104, and F129 were determined for the purpose of comparison with the frequencies obtained previously for these regions in other strains of E. coli.

It is important to note that Hfr cells in which the temperature-sensitive Flac-proB has reverted to the autonomous state of replication would tend to segregate Lac$^-$ cells during growth at 37°C. The extent of this segregation was measured. Before harvesting cells of the transposition Hfr strains, samples of the cultures were plated at 37°C and at room temperature on MacConkey's Agar containing lactose. This procedure would determine the fraction of Hfr, F$^-$, and autonomous Flac-proB cells in the population. Cells in which the temperature-

sensitive Flac-proB is integrated into the chromosome give Lac^+ colonies on plates incubated at 37°C and on plates incubated at room temperature. Further, the Lac^+ colonies on the plates incubated at room temperature give rise to Lac^+ colonies on replica plates incubated at 42°C. Lac^- cells give rise to Lac^- colonies on plates incubated at 37°C and on plates incubated at room temperature. Lac^- colonies give rise to Lac^- colonies an replica plates incubated at 42°C. Cells harboring an autonomous Flac-proB, give rise to Lac^+ colonies at both 37°C and room temperature. Lac^+ colonies at room temperature, which are composed of cells harboring an autonomous Flac-proB, give rise to Lac^- colonies on replica plates incubated at 42°C. Similar proportions of Lac^- colonies arose on dilutions of the exponentially growing populations plated at 37°C and at room temperature. Further, on replicating colonies from the plates incubated at room temperature to plates subsequently incubated at 42°C, no Lac^- colonies were found at 42°C which corresponded to colonies which were Lac^+ at room temperature. Therefore, Lac^+ colonies were the result of cells containing an integrated Flac-proB, and very few cells harboring an autonomous Flac-proB were present in cell populations growing at 37°C. The proportion of Hfr cells in the populations varied with each strain between 80% and 100%. Frequencies of the lac-proB region, in each population, were corrected by dividing the measured frequency by the fraction of stable Lac^+ colonies on the replica plates incubated at 42°C.

The results obtained (Table 6, Figure 9) indicate that, in these transposition Hfr strains, the relative frequencies of the regions corresponding to merogenotes F14, F103, F104, and F129 were similar

Table 6. Frequencies of chromosomal regions in rapidly growing transposition Hfr strains of isogenic *E. coli*

E. coli Transposition Hfr strain	Generation time[a]	Chromosome Region Frequency Corresponding to:				
		F14	F104	F103	F129	Flac-proB
X7026 (F^-)	35 min	1.26 ± 0.04[b]	-	0.83 ± 0.01	0.84 ± 0.06	-[c]
EC2	31 min	1.32 ± 0.03	0.89 ± 0.02	0.85 ± 0.05	0.90 ± 0.06	1.45 ± 0.31
EC6	30 min	1.20 ± 0.05	-	0.89 ± 0.07	-	1.19 ± 0.18
EC9	35 min	1.34 ± 0.09	-	0.80 ± 0.06	-	1.19 ± 0.03
EC28	34 min	1.28 ± 0.05	-	0.80 ± 0.05	-	0.87 ± 0.07

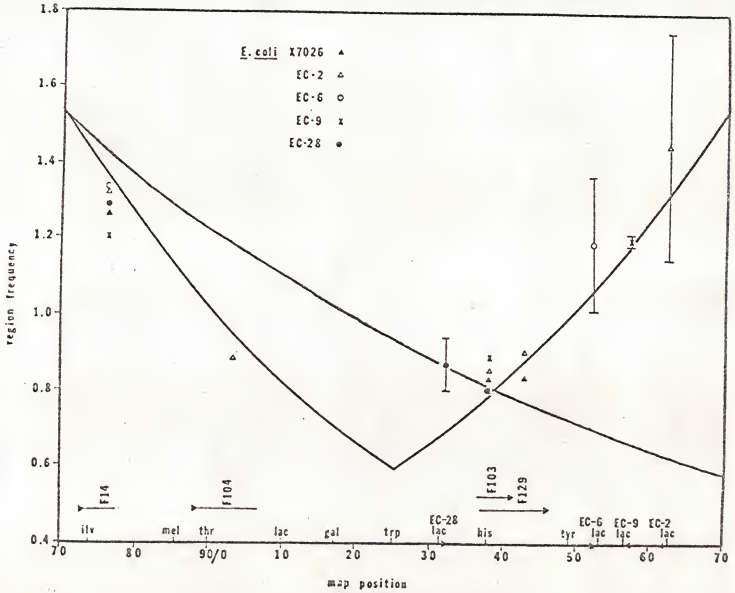
[a] Generation time as determined by turbidity increase; generation times determined by increases in DNA, protein, and cell numbers were determined and found to be the same.

[b] Standard deviation of the regression of a plot of $(S_o/S)_{3_H}$ against $(S_o/S)_{14_C}$ used in determination of frequencies of labeled DNA regions by the kinetics of DNA renaturation (see text).

[c] Frequency not determined for this region in this strain.

Frequencies of chromosomal regions in *E. coli* transposition Hfr strains growing exponentially in L broth were determined by renaturation kinetics as described in the text.

Figure 9. Frequencies of chromosomal regions in transposition Hfr strains of *E. coli*. Unlabeled DNA isolated from cultures growing exponentially in L broth was quantitated by renaturations with labeled merogenote DNA. Frequencies of regions are plotted versus their positions on the genetic map, for *E. coli* strains X7026 (F^-), EC-2, EC-6, EC-9, and EC-28. The theoretical curves, plotted for comparison, represent the frequency distribution function, $f_n(X) = 2^{n(1-X)}\ n\ \ln 2/(2^n-1)$, for a population of cells whose chromosomes replicate either (a) in a bidirectional with an origin at minute 70 and a terminus at minute 25, or (b) in a clockwise fashion with an origin at minute 70, with an average of 1.6 rounds of replication on each chromosome.


E. coli X7026
EC-2
EC-6
EC-9
EC-28
region frequency
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
F14
F104
F103
F129
ilv
mel
thr
lac
gal
trp
EC-28 lac
his
tyr
EC-6 lac
EC-9 lac
EC-2 lac
70
80
90/0
10
20
30
40
50
60
70
map position

to their frequencies in other E. coli strains. Further, the frequencies of the integrated lac-proB regions increased as they are located clockwise from the site of F129 to the site of F14. These results would be expected of chromosomes which replicated in two directions from a single point of origin. If chromosome replication would have been unidirectional, the relative frequencies of the various regions between minute 40 and minute 70 would have been expected to continue to decrease in frequency, then to increase in frequency, with a sharp break between the region with minimum frequency (terminus) and the region with maximum frequency (origin).

All the above data were compared to theoretical curves for a variety of plausable models for chromosome replication (derived precisely for this purpose, see Mathematical Appendix). The data best fit a model for chromosome replication having an origin for replication near minute 70 on the E. coli genetic map, with bidirectional replication on each chromosome to a terminus near minute 25.

Marker frequencies in E. coli subjected to synchronizing procedures

Experiments were performed to determine the effect of various chromosome synchronizing procedures on the replication state of the chromosome as determined by relative frequencies of selected chromosomal regions. Cell populations of E. coli K12 $2{,}000_{X111}$, growing exponentially in minimal medium, were treated by the addition of either 150 µg/ml chloramphenicol (CAM), 0.25% phenethyl alcohol (PEA) (the effects of chloramphenicol and phenethyl alcohol have been described in the Literature Review), or 10^{-3}M L-valine. A widely used method for aligning chromosomes in a non-replicating state, amino acid starvation, was not feasable for our analyses of frequencies since the large

number of cells needed for the preparation of unlabeled DNA could not be filtered, washed, and resuspended in fresh medium in a reasonable amount of time. The alternative method for starving cells for an amino acid, the addition of valine, is based on the ability of valine to completely inhibit the synthesis of isoleucine in E. coli K12 (Tatum, 1946). The addition of valine, then, was used to starve cells for isoleucine.

The effects of CAM, PEA, and valine on the growth of treated cultures, as measured by increase in turbidity and by the synthesis of DNA and protein, are shown in Figures 10, 11 and 12, respectively. The residual DNA synthesis amounted to 40-50% during inhibition, an amount consistent with the completion of a round of chromosome replication in cells having one replication fork. Unlabeled DNA was extracted from cells harvested 2 hours after the inhibition of macromolecular synthesis.

Two other methods, known to give cell division synchrony, were used for attempts to align chromosomes in a completed state. In the first method (Cutler and Evans, 1966), cells of strain $2{,}000_{X111}$ were grown to stationary phase in 2.5 liters of minimal medium and harvested one hour after entry into stationary phase. The latter was defined as the period of constant turbidity readings. Harvested cells were used for the preparation of unlabeled DNA. An aliquot of cells diluted into fresh medium 60 minutes after entry of the culture into stationary phase showed division synchrony as determined by cell counts with a Petroff-Hauser counter. Growth to stationary phase and subsequent cell counts showing division synchrony are shown in Figure 13. Stationary phase cells prepared as above have been reported

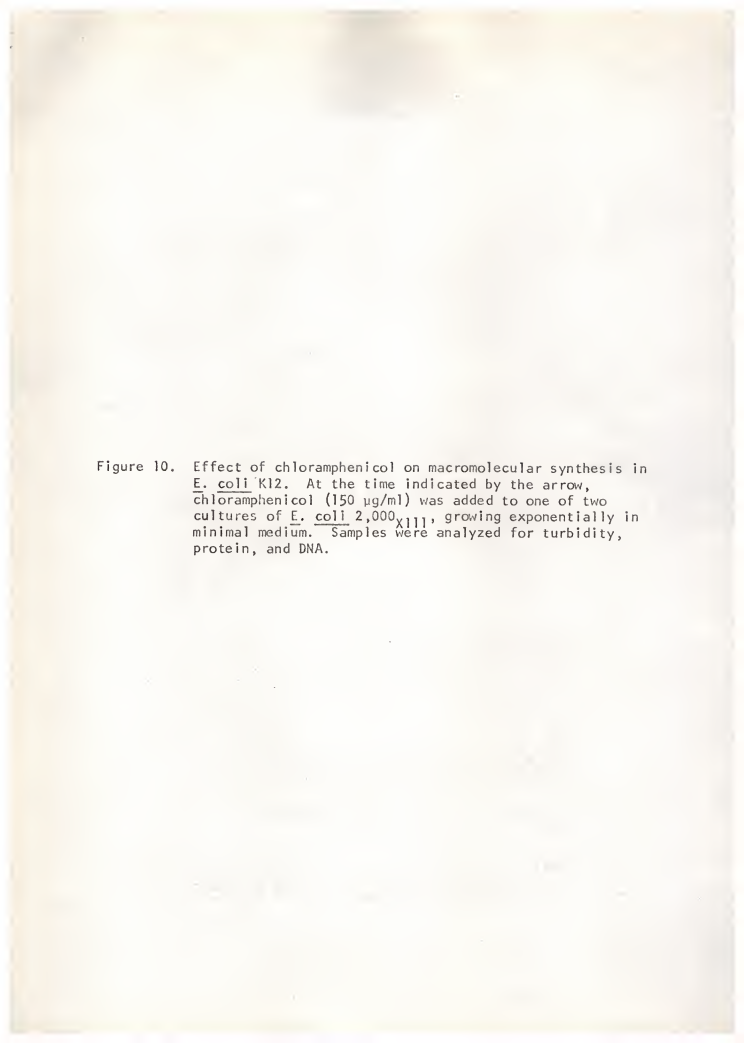


Figure 10. Effect of chloramphenicol on macromolecular synthesis in E. coli K12. At the time indicated by the arrow, chloramphenicol (150 µg/ml) was added to one of two cultures of E. coli $2{,}000_{X111}$, growing exponentially in minimal medium. Samples were analyzed for turbidity, protein, and DNA.

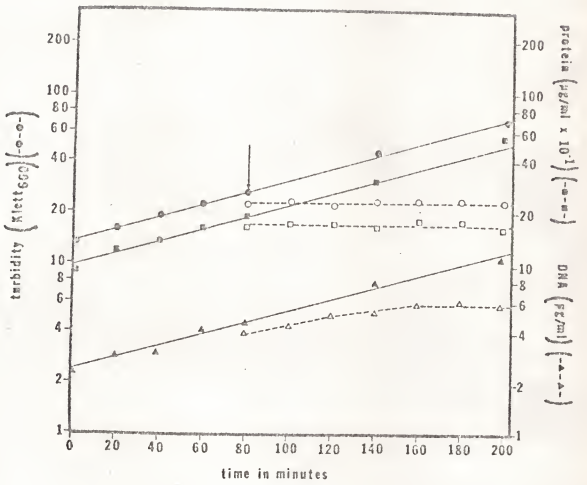

turbidity (Klett 660) (-o-o-)
protein (µg/ml x 10⁻¹) (-o-o-)
DNA (µg/ml) (-▲-▲-)
time in minutes
0
20
40
60
80
100
120
140
160
180
200
1
2
4
6
8
10
20
40
60
80
100
200

Figure 11. Effect of phenethyl alcohol on macromolecular synthesis in E. coli K12. At the time indicated by the arrow, phenethyl alcohol (0.25%) was added to one of two cultures of E. coli $2{,}000_{X111}$, growing exponentially in minimal medium. Samples were analyzed for turbidity, protein, and DNA.

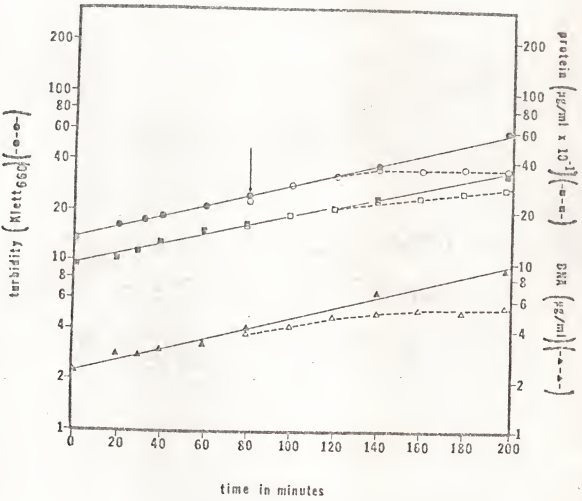

turbidity (Klett 660) (-o-o-)
protein (µg/ml x 10⁻¹) (-o-o-)
DNA (µg/ml) (-•-•-)
time in minutes
200
100
80
60
40
20
10
8
6
4
2
1
0
20
40
60
80
100
120
140
160
180
200

Figure 12. Effect of L-valine on macromolecular synthesis in E. coli K12. At the time indicated by the arrow, valine (10^{-3} M) was added to one of two cultures of E. coli $2{,}000_{X111}$, growing exponentially in minimal medium. Samples were analyzed for turbidity, protein, and DNA.

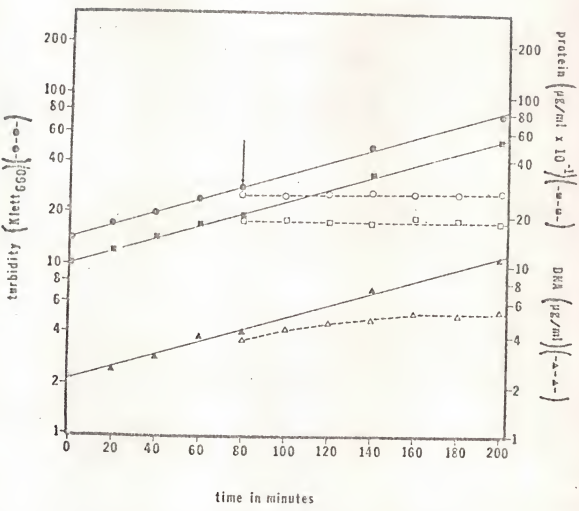
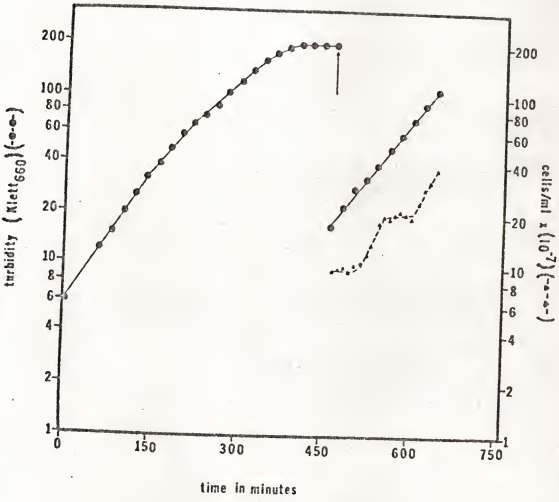

200
100
80
60
40
20
10
8
6
4
2
1
0
20
40
60
80
100
120
140
160
180
200
time in minutes
turbidity (Klett 660) (-o-o-)
protein (µg/ml x 10) (-o-o-)
DNA (µg/ml) (-•-•-)

Figure 13. Growth and division in stationary phase cells of *E. coli* K12 following dilution into fresh medium. Cells of *E. coli* $2{,}000_{X111}$, grown two hours into stationary growth phase were diluted into fresh minimal medium, and subsequent growth and cell divisions were followed by increases in turbidity and cell numbers.

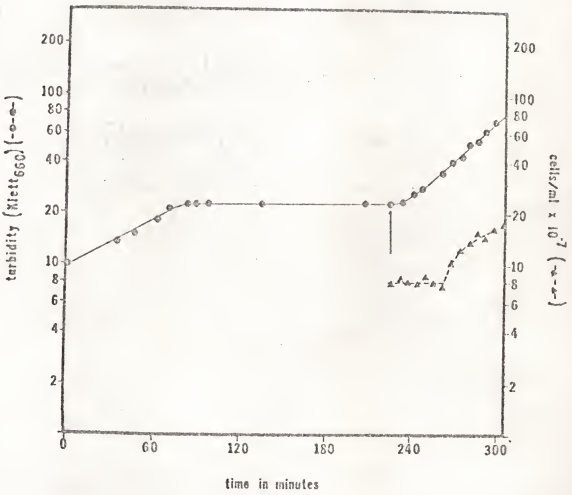

turbidity (Klett660) (-e-e-)
cells/ml (10^7) (-•-•-)
time in minutes
200
100
80
60
40
20
10
8
6
4
2
1
0
150
300
450
600
750

to show division synchrony (Cutler and Evans, 1966) and synchronous replication of the chromosome from a single replication origin (Cutler and Evans, 1967a and b).

In the second method (Matney and Suit, 1966), cells of strain $2{,}000_{X111}$ were grown to stationary phase in the presence of a growth limiting concentration (8 μg/ml) of proline, a required amino acid. Cells entered into stationary phase at a low cell density (Klett 23) and were harvested 4 hours after entry into stationary phase, showing synchronized division as determined by cell counts with a Petroff-Hausser counter. Growth of cells to stationary phase and subsequent cell counts are shown in Figure 14. Cells entering stationary phase under conditions of amino acid depletion have been shown to undergo synchronous divisions after the addition of normal concentrations of the amino acid exhausted by growth of the cells (Matney and Suit, 1966).

In cells subjected to the above synchronizing procedures, the frequencies of regions located near the origin and near the terminus were determined by renaturation kinetics as a measure of the completion of chromosome replication. Unlabeled DNA isolated from cells having undergone one of the above procedures was reannealed with labeled F14 merogenote DNA (a region near the replication origin) and with labeled F103 merogenote DNA (a region near the replication terminus). The frequencies of F14 region and of F103 region were determined for chromosomes aligned by the above procedures and also for the same E. coli K12 strain ($2{,}000_{X111}$) growing exponentially in minimal medium. If all chromosomes in the cell population complete the replication of the chromosome and do not initiate new rounds of replication during the

Figure 14. Growth and division in E. coli K12 cells after amino acid depletion. Proline (50 μg/ml) was added to a culture of E. coli $2{,}000_{X111}$ previously grown to stationary phase in a limiting concentration (8 μg/ml) of proline. Subsequent growth and cell divisions were followed by increases in turbidity and cell numbers.


turbidity (Klett$_{660}$) (-o-o-)
cells/ml x 10^{-7} (-x-x-)
time in minutes
200
100
80
60
40
20
10
8
6
4
2
0
60
120
180
240
300

inhibition procedures used above, then all regions of the chromosome will be present in the same frequency, one copy per chromosome. As alignment of the chromosomes in the cell populations occurs in a greater proportion of cells, the frequencies of a region near the origin for replication (F14) and for a region near the replication terminus (F103) will approach one. Origin and terminus regions were chosen for analysis since, in exponentially growing cultures, their frequencies deviate the most from a value of one. The frequencies of the F14 and F103 regions, in cells whose chromosomes were aligned by one of the above procedures, are shown in Table 7 together with frequencies of the two regions in a population of cells growing exponentially in minimal medium. It may be seen that frequencies of F14 and F103 approach one in cells whose chromosomes have been aligned presumably in a completed state by each of the above techniques, with the exception of cells aligned by the amino acid depletion technique. Dr. J. Suit has made a similar observation (personal communication). Techniques, other than amino acid depletion, used above to align chromosomes appear to result in populations of cells whose chromosome replication is completed to the terminus. From my experiments, I cannot predict the subsequent replication pattern of the chromosomes if cells are released from inhibition. Experiments to answer this question are planned.

Marker frequencies in cells grown at increasing generation times

In cells growing at increasingly slower growth rates it is generally accepted that the initiation of new rounds of chromosome replication occurs less frequently, and that the rate of chromosome replication, although also decreasing, is decreasing at a slower rate than the growth

Table 7. Effects of synchrony procedures on frequencies of chromosomal regions in E. coli

Treatment or Growth Condition	Reference Figure[a]	Chromosome Region Frequency Corresponding to: F14	F103
Minimal Medium, Exponentially Growing Cells	-	1.32 ± 0.08[b]	0.84 ± 0.09
Chloramphenicol	Figure 10	1.08 ± 0.03	1.02 ± 0.10
Phenethyl Alcohol	Figure 11	0.96 ± 0.03	0.91 ± 0.06
Valine	Figure 12	1.08 ± 0.04	0.89 ± 0.01
Stationary Phase	Figure 13	1.02 ± 0.04	0.95 ± 0.03
Amino Acid Depletion	Figure 14	1.13 ± 0.03	0.82 ± 0.04

[a] Text figure showing growth of strain $2{,}000_{X111}$ during the treatment or growth condition of the experiment.

[b] Standard deviation of the regression of a plot of $(S_o/S)_{3_H}$ against $(S_o/S)_{14_C}$ in determination of frequencies of labeled DNA regions by the kinetics of DNA renaturation (see text).

E. coli $2{,}000_{X111}$ was treated with inhibitors or subjected to physiological conditions as described in the text. Frequencies of an origin region of the chromosome (F14) or of a terminus region of the chromosome (F103) were determined for chromosomes in cells of a culture by renaturation kinetics as described in the text.

rate. Therefore, in cells growing with increasingly longer generation times, the fraction of the generation time during which chromosome replication is taking place is decreasing. The average number of replicating rounds occurring on each chromosome, "n", is expected to decrease with increasing generation times.

We have tested the above hypothesis as follows: Unlabeled DNA prepared from several different cell populations of strain AB259 growing exponentially with different generation times was renatured with labeled F14 merogenote DNA and with labeled F103 merogenote DNA. As "n" decreases, the frequencies of both origin and terminus regions should approach unity. The prediction that "n" (the average number of replicating rounds per chromosome) decreases with increasing generation times was confirmed with cells of E. coli K12 AB259 growing with generation times between 30 and 115 minutes at 37°C (Table 8), since the ratio of the origin region (F14) to the terminus region (F103) decreases with increasing generation times.

Marker frequencies in additional strains of rapidly growing E. coli K12

In my final experiments, I have measured the frequencies of an origin region (F14) and a terminus region (F103) in two additional strains of E. coli K12, Hfr B7 and Hfr P4X, growing exponentially in L broth. Further, the frequencies of a number of regions were measured in two E. coli K12 strains of interest, Hfr χ574 and Hfr DG163, growing exponentially in L broth. χ574 was chosen since the integrated F factor showed the lowest frequency of the isogenic Hfr strains examined in quantitative experiments (Table 3). Based on the data obtained for frequencies in renaturation kinetics experiments (Figures 7 and 8), an F factor integrated at the Hfr site of χ574 would be expected to have

Table 8. Frequencies of chromosomal regions in E. coli AB259 growing at various growth rates

Medium[a]	Generation time[b]	Region Frequency	
		F14	F103
L broth	30 min	1.31 ± 0.07[c]	0.72 ± 0.06
Glucose	54 min	1.21 ± 0.07	0.86 ± 0.04
Glycerol	81 min	1.22 ± 0.03	0.89 ± 0.02
Lactate	100 min	1.13 ± 0.11	0.94 ± 0.05
Acetate	115 min	1.08 ± 0.05	1.01 ± 0.02

[a] Medium was either L broth or minimal medium containing the carbon and energy source listed as sole source of carbon and energy.

[b] Generation time as determined by turbidity increase; generation times determined by increases in DNA, protein, and cell numbers were determined in most cases and found to be the same.

[c] Standard deviation of the regression of a plot of $(S_o/S)_{^3H}$ against $(S_o/S)_{^{14}C}$ used in determination of frequencies of labeled ^3H DNA regions by the kinetics of DNA renaturation (see text).

a higher frequency than that observed in the quantitative experiment. I felt that it would be of interest to investigate marker frequencies in strain χ574 by the renaturation kinetics technique in order to see if any marked differences were apparent between frequencies in χ574 and other E. coli strains. The second strain, DG163 (Hfr B4) has been reported to have two replication origins, one near gal on the genetic map and the other near the normal replication origin for E. coli (Wolf, Newman, and Glaser, 1968; Eberle, 1970). If this strain had a second functional origin near gal, an increase in frequency of Flac-proB (counter-clockwise direction of replication from gal) or of F103 (clockwise direction of replication from gal) would be expected as well as a decrease in the frequency of the F14 region. It may be seen in Table 9 that the frequencies of origin and terminus regions in strains Hfr P4X and Hfr B7 and the frequencies of several regions in χ574 and DG163 show no significant differences from region frequencies in other E. coli strains, reported in Tables 5 and 6. I have no explanation for the different results obtained with χ574 region frequencies obtained in the quantitative experiments and region frequencies obtained in experiments using renaturation kinetics as an assay system. The region frequencies obtained with DG163, are not in agreement with the interpretations of Wolf, Newman, and Glaser (1968) and of Eberle (1970), but confirm the results of Masters and Broda (1971) who found no differences in chromosome replication in Hfr B4.

Table 9. Frequencies of chromosomal regions in additional strains of E. coli

E. coli strain	Generation time[a]	Chromosome Region Frequency Corresponding to: F14	F104	Flac-proB	F103
Hfr B7	26 min	1.47 ± 0.09[b]	-[c]	-	0.72 ± 0.06
Hfr P4X	28 min	1.35 ± 0.04	-	-	0.77 ± 0.07
x574	43 min	1.25 ± 0.09	1.05 ± 0.08	1.00 ± 0.11	0.83 ± 0.05
DG163	29 min	1.52 ± 0.07	0.93 ± 0.06	0.89 ± 0.09	0.69 ± 0.09

[a] Generation time as determined by turbidity increase; generation times determined by increases in DNA, protein, and cell numbers were determined in most cases and found to be the same.

[b] Standard deviation of the regression of a plot of $(S_o/S)_{3_H}$ against $(S_o/S)_{14_C}$ used in determination of frequencies of labeled DNA regions by kinetics of DNA renaturation (see text).

[c] Frequency not determined for this region in this strain.

Frequencies of chromosomal regions in E. coli strains growing exponentially in L broth were determined by renaturation kinetics as described in the text.

CONCLUSION

A method has been developed for measuring directly the relative frequencies of the various regions of the chromosome in exponentially growing E. coli. This study has used these techniques to answer a number of questions concerning chromosome replication in several strains of E. coli. The primary study was to determine the point of origin and direction of replication of the E. coli chromosome.

The technique has the following advantages: This measure of frequency allows me to examine the chromosomes of exponentially growing populations of cells without having to resort to such normalizing procedures as comparing gene frequencies, or to examine the increases in frequencies during the growth of synchronized cell populations. Objections to the use of cells whose growth is physiologically unbalanced are circumvented by the methods chosen for this study.

Region frequencies were measured by a quantitative nucleic acid reannealing procedure, which utilized unlabeled DNA from Proteus mirabilis F' hybrid strains and labeled DNA from a series of isogenic strains of E. coli K12. The results have led me to conclude that F^-, F^+, and Hfr strains replicate their chromosomes in the same fashion. Thus, the F factor, when present in the integrated state, does not alter the origin for chromosome replication, or the direction of replication. A measurement of F factor frequencies in labeled DNA prepared from three Hfr strains provided evidence suggesting that chromosome replication is polarized in E. coli K12, with an origin

near minute 60 on the E. coli genetic map. A clockwise direction of replication was indicated by the frequencies of the F factor in the limited number of Hfr strains examined.

Frequencies of regions of the E. coli chromosome were also measured in experiments using renaturation kinetics as an assay. The renaturation kinetics assay is based on the observation that the rate of reannealing is a function of DNA concentration, and hence, gene frequency. For this purpose, unlabeled DNA from exponentially growing E. coli was reannealed with labeled segments of the chromosome purified from P. mirabilis F' hybrid strains. This technique allowed measurements of frequencies of regions on the chromosomes of exponentially growing cells. The results indicated that in E. coli strains K12 (F^-, F^+, and Hfr sublines), B, B/r, C, and 15, there is an origin for chromosome replication located near minute 70 on the E. coli map. Also, at least one replicating fork proceeds from the origin, replicating the chromosome in a clockwise direction. My interpretation of the data suggested that perhaps a second replicating fork proceeds from the origin in a counter-clockwise direction as well. This second possibility was confirmed in experiments measuring the frequency of the lac-proB region in a series of transposition Hfr strains of E. coli K12. Thus I have concluded from these data that all the strains of E. coli examined replicate their chromosomes in both directions from an origin near minute 70.

Frequencies of chromosomal regions in E. coli growing at various growth rates were also measured by renaturation kinetics. These studies indicated that initiation of chromosome replication becomes less frequent in cells growing with increasing generation times.

Frequencies of a chromosomal region near the origin for chromosome replication and a region near the terminus for chromosomal replication were measured in cells which had been subjected to various synchronizing procedures. Some of these procedures have been used in the past to determine the origin and direction of chromosome replication. My data indicated that most of these procedures aligned chromosomes in a completed state since the relative frequencies of the origin and the terminus were near to a value of one. I have not yet answered the question as to what effect release from synchronizing procedures will have on subsequent chromosome replication. Amino acid depletion by growing cells led to a population of cells which underwent synchronized divisions upon the re-addition of the required amino acid. However, chromosomes in such populations did not have region frequencies compatible with the completion of rounds of chromosome replication. This same result has been obtained by Dr. J. Suit (personal communication).

In separate experiments, the frequencies of origin and terminus regions of two Hfr strains of E. coli, not investigated previously, were measured.

In final experiments, the frequencies of several regions were determined for two Hfr strains of special interest.

The first Hfr strain, χ574, had been used previously in quantitative renaturation experiments. Data obtained in the quantitative experiments, using this strain and strains isogenic to it, had indicated unidirectional replication. Data obtained by renaturation kinetics contradicted the first study, since the frequencies in χ574 were not significantly different from frequencies of the same regions

in other E. coli strains. The data from other strains of E. coli indicates that chromosome replication is bidirectional. However, the data in renaturation kinetics experiments using unlabeled DNA from χ574 is not accurate enough to determine whether chromosome replication is unidirectional or bidirectional in this strain. The contradiction between the data obtained with χ574, in quantitative renaturation experiments, and with other E. coli strains, in renaturation kinetics experiments, will remain only until labeled chromosome segments in the region from minute 40 to minute 70 can be prepared.

Region frequencies were also determined for chromosomes of exponentially growing E. coli DG163. In agreement with the data of Masters and Broda (1971), obtained with this strain, I have found DG163 to have one origin at or near the position of the origin in other E. coli strains. Both of these findings fail to confirm those of Wolf, Newman, and Glaser (1968) and Eberle (1970). These authors investigated chromosome replication in Hfr DG163 after release from amino acid starvation and have concluded that this strain contained two origins for chromosome replication, one at or near the origin for other strains of E. coli and a second near gal on the E. coli map. The differences in interpretation may be a reflection of the treatment (amino acid starvation) used to align chromosomes in the experiments of Wolf, Newman, and Glaser (1968) and of Eberle (1970).

In conclusion, marker frequency analysis suggests, by comparison with the theoretical curves presented in the Mathematical Appendix, that chromosomes initiate replication from an origin located near minute 70 on the E. coli map; and replication forks proceed in both

directions from the origin in most, if not all, cells in the population. At increasing generation times, chromosome replication is initiated less frequently. Evidence has been presented indicating that most of the synchronizing procedures examined do, in fact, align chromosomes in a completed or non-replicating state, with one important exception, that of amino acid depletion.

APPENDIX

APPENDIX

The Age and Frequency Distributions

In an exponentially growing population of bacterial cells, the ages of cells in the population are random. Similarly, chromosomes are found in various stages of replication. The distribution of cell ages and of chromosome ages in the population are identical for a population growing exponentially in "balanced growth". Balanced growth, by definition, is an equilibrium state in which all parameters of growth are increasing with the same growth rate constant, (μ).

The precise distribution of cell ages in an exponentially growing population may be determined, and from it the distribution of the chromosome ages is identical by analogy.

Consider a population of exponentially growing cells dividing by binary fission. Since each "old" cell divides to give two "young" cells, it may be seen that the age distribution has the curious property of having twice as many young cells, recently formed by division, as old cells, which are about to divide.

For the derivation of age and frequency distribution functions, I will use the following symbols:

n = number of replicating rounds per chromosome

N = cell number

t = time

τ = generation time

μ = exponential growth rate constant

x = age of a cell or chromosome (from 0 to 1)

X = map position of a genetic locus on a replicating chromosome (from an origin,0, to a terminus, 1)

$f(x)$ = age distribution of cells or chromosomes

For a population of cells dividing by binary fission, the rate of increase in cell number is a function of the cell number already present:

$$\frac{dN}{dt} = \mu N$$

$$\int_{N_o}^{N} \frac{1}{N}\, dN = \mu \int_{t_o}^{t} dt$$

$$\ln N]_{N_o}^{N} = \mu t]_{t_o}^{t}$$

$$\ln \frac{N}{N_o} = \mu(t-t_o)$$

$$N = N_o e^{\mu(t-t_o)}$$

If I let exactly one generation pass, so that $N = 2N_o$ and $(t-t_o) = \tau$, then:

$$2N_o = N_o e^{\mu\tau}$$

$$2 = e^{\mu\tau}$$

$$\ln 2 = \mu\tau$$

$$\mu = \frac{\ln 2}{\tau}$$

Also, μ is the slope of a plot of lnN against t:

$$\frac{d\ln N}{dt} = \mu$$

If it is assumed that each cell divides upon reaching an age of one generation and that each old cell divides to give two cells of age zero, then at any time the instantaneous increase in cell numbers will be the result of the division of those cells which are one generation in age at that instant of time.

As an example, if one examines a culture of exponentially growing cells, the rate of increase in cell numbers at time zero is the result of the division of those cells which which are one generation in age at the beginning of the experiment. After 1/2 generation has passed, the rate of increase in cell numbers at that time will be the result of the division of those cells which were 1/2 generation in age at the beginning of the experiment (and hence, one generation in age after the passing of 1/2 generation). Similarly, the increase in cell numbers after one generation has passed will be the result of the division of those cells which were of age zero at the start of the experiment ($t = 0$).

Therefore, at any time t:

$$N = N_o e^{\mu t}$$

$$\frac{dN}{dt} = \mu N_o e^{\mu t}$$

However dN/dt will be numerically equal, at any time t, to the number of cells in the population which are exactly one generation in age at time t. In the interval $0 \leq t \leq \tau$, the number of cells which will be of division age at time t is that fraction of cells whose age is

x at time zero,

where: $x = \tau - t$.

The number of cells of division age at time t is given by the expression: $N_o f(x)$.

Hence:

$$N_o f(x) = \mu N_o e^{\mu t}$$

$$f(x) = \mu e^{\mu t}$$

$$f(x) = \frac{\ln 2}{\tau} e^{\frac{\ln 2}{\tau}(\tau - x)}$$

$$f(x) = \frac{\ln 2}{\tau} 2^{(1-\frac{x}{\tau})}.$$

If time and age of cells are expressed in generations, then:

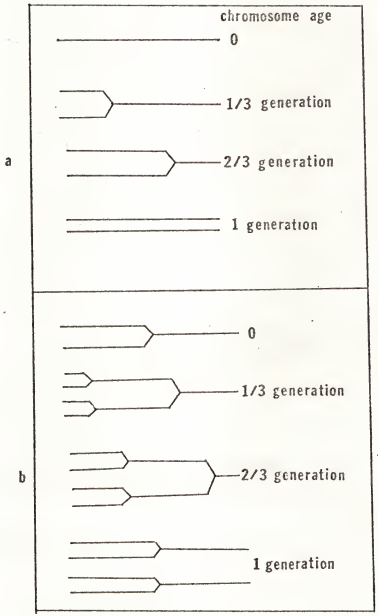
$$f(x) = 2^{1-x} (\ln 2)$$

In further discussion, time and age are expressed in generations.

Likewise, chromosomes have an age distribution analogous to that described for cells. Figure 15 is a series of line drawings representing chromosomes in stages of replication. For my derivation of the frequency distribution function, I have assumed that a replication fork is initiated at a specific locus on the circular bacterial chromosome and travels at a constant speed to the terminus. In this model the origin and the terminus occupy the same locus (or very close loci). Figure 15a presents the case in which new rounds of replication are initiated at the same time as the completion of old rounds. The average number of rounds of replication (n) proceeding on each chromosome is equal to one.

On any replicating chromosome, those gene loci lying between the

Figure 15. Pictorial representation of replicating chromosomes. The stylized chromosomes have been opened into linear representations at the presumed origin for chromosome replication. The movement of replicating forks along the chromosome during the cell cycle is shown for chromosomes having (a) an average of one replicating round per chromosome or (b) an average of two replicating rounds per chromosome.


chromosome age
0
1/3 generation
a
2/3 generation
1 generation
0
1/3 generation
b
2/3 generation
1 generation

origin and the replicating fork will have been replicated (and hence, present in two copies) while those lying between the replicating fork and the terminus will be present in only one copy (not having been replicated). On a chromosome of age x, genetic loci lying between zero and X on the replication map are present in two copies, assuming a constant velocity for replicating forks, while those lying between X and one are present in one copy. Another way of viewing this is that two copies of a gene occupying position X on the chromosome will be present on chromosomes where x>X , and one copy where x<X. Thus, the frequency of genetic loci, g(X), may be represented as:

$$g(X) = \int_0^X f(x)\ dx + 2\int_X^1 f(x)\ dx$$

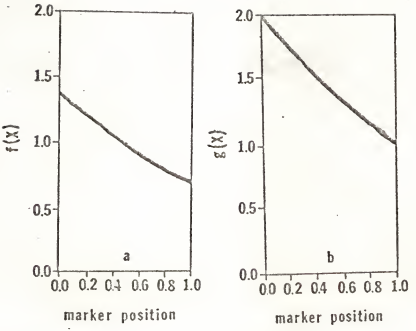
$$g(X) = \int_0^X (\ln 2)\ 2^{(1-x)}\ dx + 2\int_X^1 (\ln 2)\ 2^{(1-x)}\ dx = 2^{1-X}$$

Similarly, if more than one round of replication is traversing the chromosome, for example, n such rounds (Figure 15b shows two rounds), each separated from the next round by a distance 1/n of the chromosome, then the frequency of genetic loci on a chromosome having n rounds of replication, $g_n(X)$, may be derived by integrating each 1/n portion of the chromosome separately and summing:

$$g_n(X) = 2^{n(1-X)}$$

Since $g_n(X)$ is the frequency of loci per replicating chromosome and the work to be described utilizes purified DNA, it is more convenient for me to use a function normalized for DNA concentration. The relative amount of DNA per chromosome is given as:

Figure 16. Graphic representation of (a) normalized, $f_n(X)$, and (b) unnormalized, $g_n(X)$, frequencies of chromosome regions plotted against X, the distance of the region from the origin for chromosome replication. The number of rounds of replication per chromosome was taken to be one.


2.0
1.5
1.0
0.5
0.0
f(x)
a
0.0 0.2 0.4 0.6 0.8 1.0
marker position
2.0
1.5
1.0
0.5
0.0
g(x)
b
0.0 0.2 0.4 0.6 0.8 1.0
marker position

$$\int_0^1 g_n(X)\ dX = \int_0^1 2^{n(1-X)}\ dX = \frac{2^n-1}{n\ln 2}$$

The frequency of loci may be normalized to the DNA content of a non-replicating chromosome as follows:

$$f_n(X) = \frac{(n\ln 2)\ 2^{n(1-X)}}{2^n-1}$$

The normalized and unnormalized frequency distributions (for $n = 1$) are shown in Figure 16.

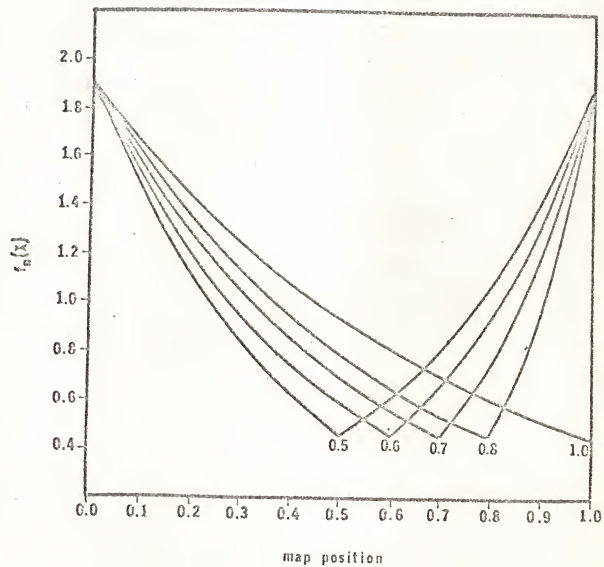
One can also calculate the increase in DNA following a specific block in initiating new rounds of replication as:

$$\% \text{ increase} = \frac{2^n - \int_0^1 2^{n(1-X)}\ dX}{\int_0^1 2^{n(1-X)}\ dX}\ (100) = \frac{n\ln 2\ 2^n}{2^n-1} - 1\ (100)$$

In view of the possibility of bidirectional replication, I have plotted in Figure 17 the results expected for chromosome replication beginning at a fixed origin and proceeding in both directions in the same cell.

If the terminus is not fixed (but instead, varies within a small portion of the chromosome), the curves will deviate from those drawn. If the terminus varies, no gene locus will be exactly at the terminus in more than a fraction of the chromosomes in the population. The frequencies of gene loci will not reach the minimums shown for the curves in Figure 17 if the terminus is not fixed. A similar argument applies for the case where the origin varies within a small portion of the chromosome. In this case, no gene will be at the origin in more than a small fraction of the chromosomes; and hence, the

Figure 17. Marker frequencies in populations of cells replicating each chromosome in two directions. The graph shows marker frequencies for two rounds of replication per chromosome, n=2, in cells having an origin for replication at a map position of zero and termini at either 0.5, 0.6, 0.7, or 0.8 of the distance of the chromosome from the origin. A curve of marker frequencies for unidirectional replication (terminus at 1.0) is included for comparison.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
$f_n(x)$
0.5
0.6
0.7
0.8
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
map position

frequencies of chromosomal regions will not reach the maximums in the curves shown in figure 17.

In figure 18, frequencies of chromosomal regions are plotted for the case where replication begins at a fixed origin; but replication forks may proceed in either direction from this origin. It may be seen that the theoretical results differ significantly from those of figure 17.

Kinetics of Renaturation

The renaturation of single-stranded DNA to form double-stranded DNA is a second order kinetic reaction. The concentration of single-strands decreases with time as follows:

$$\frac{dS}{dt} = -KS^2$$

$$\int \frac{1}{S^2}\, dS = -K \int dt$$

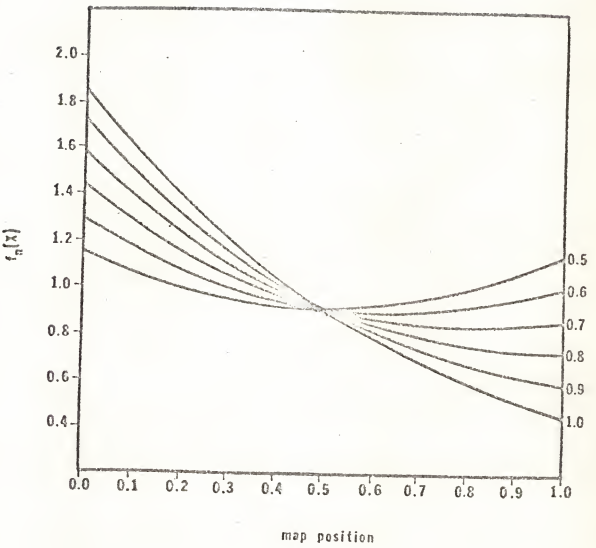
$$\frac{1}{S} = Kt + \frac{1}{S_o}$$

$$\frac{S_o}{S} = KtS_o + 1$$

where "K" is the second order rate constant and "S_o" is the concentration of single-stranded DNA at time zero.

If I plot S_o/S against time, the observed second order rate constant, "K'", is proportional to the DNA concentration, ($K' = KS_o$). In my experiments I have purified segments of the chromosome from Proteus mirabilis - Escherichia coli F' hybrids. Regions of the chromosome corresponding to the purified segments will be present at different concentrations in DNA prepared from exponentially growing cells. The rate at which segments renature with corresponding E. coli

Figure 18. Marker frequencies in populations of cells replicating each chromosome in either one direction or the opposite direction from a single origin. The graph shows marker frequencies for two rounds of replication per chromosome, n=2, in cells replicating their chromosomes from a fixed origin at map position zero and either a clockwise or a counter-clockwise direction of replication. Curves are presented for populations replicating their chromosomes in one particular direction in fractions of either 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 of the cells.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
$f_n(x)$
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
map position
0.5
0.6
0.7
0.8
0.9
1.0

DNA regions will be proportional to the frequency of the regions on the chromosome.

or:

$$K' = KS_o = Kf_n(X).$$

In my experiments I do not measure K' directly, but include a ^{14}C labeled tracer of bulk E. coli DNA mixed with the ^{3}H labeled segments (see Results and Discussion, p 70). The ^{14}C labeled DNA renatures with an observed rate constant dependent on a host of variables and is a measure of the renaturation of all the segments on the chromosome. The ^{3}H labeled DNA renatures with the unlabeled DNA in the same mixture but is dependent on the concentration of only that region on the replicating chromosome, relative to the concentration of the average segment represented here by the ^{14}C labeled DNA. The ratio of the rate constant for the renaturation of ^{3}H labeled DNA with unlabeled DNA to the rate constant for the renaturation of ^{14}C labeled DNA with unlabeled DNA is taken as a measure of f(X) in the cell population from which the unlabeled DNA was harvested:

$$K'_{3H}/K'_{14C} = f(X).$$

It should be pointed out that the treatment of data as described would allow the distinction among replication from a single origin, random replication, bidirectional replication, and replication from an integrated sex factor.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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