SEGREGATION OF INDUCED FRAMESHIFT MUTATIONS AND THE SEQUENCE OF GENE REPLICATION IN *ESCHERICHIA COLI* K12

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ABSTRACT

We have constructed a strain of *E. coli* K12 carrying six mutations induced by the acridine half-mustard ICR-191. The mutations are widely spaced on the *E. coli* linkage map and are all easily reverted by ICR-191. Mapping of ten independent revertants for each of five markers indicated that the reversions induced by ICR-191 occurred near the original mutations. Exponentially and nonsynchronously growing cultures of this strain were exposed to ICR-191 for 0.85 generation, quickly washed free of mutagen, and resuspended in the original medium minus mutagen. Total viable cell number maintained its exponential increase both during and immediately after exposure to mutagen, whereas the number of revertants of any particular type remained constant for a characteristic period after removal of mutagen before finally assuming an exponential increase. Theoretically, the length of such a segregation lag should depend on the position of the particular reverted gene in the sequence of gene replication: the earlier a gene is replicated in the chromosome replication cycle, the longer its segregation lag should be. Our results are consistent with this prediction and fit a unidirectional, clockwise replication scheme with an origin between 55 and 74 min on the *E. coli* linkage map. The results also fit a very asymmetric bidirectional replication scheme.

IN 1968 VIELMETTER, MESSER and SCHÜTTE reported that virtually all mutants of *E. coli* freshly induced by N-methyl-N′-nitro-N-nitrosoguanidine (NG) produced sectored colonies (containing both mutant and nonmutant cells) when plated on suitable agar medium. When the mutants were permitted to grow and divide in liquid medium after exposure to NG and before plating out, the proportion producing sectored colonies gradually declined, until after a few generations of segregation they produced only pure mutant colonies. VIELMETTER, MESSER and SCHÜTTE (1968) pointed out that the kinetics of disappearance of such sectoring should, under certain assumptions, depend on the position of the particular mutated gene on the replicating chromosome: a gene that is replicated late in the chromosome replication cycle would be expected to show faster segregation kinetics than a gene that is replicated early. One of the assumptions in this analysis was that the mutagen induces mutations that are randomly distributed over the chromosome.

In this paper we have adapted this scheme to an analysis of the segregation

kinetics of mutants induced by ICR-191 (3-chloro-7-methoxy-9-[3-(chloroethyl) amino propylamino]acridine dihydrochloride), an acridine half-mustard which is known to cause frameshift mutations in bacteria (AMES and WHITFIELD 1966; MARTIN 1967; BERGER, BRAMMER and YANOFSKY 1968; NEWTON 1970). Rather than following the disappearance of sectored colony formers, however, we have chosen to follow the total number of mutant cells (heterozygous plus pure) as a function of time after termination of mutagenic treatment. We expect this number to increase only after a characteristic segregation lag, the length of which should depend on the relative position of the mutated gene in the chromosome replication cycle. We have derived theoretical expressions for the segregation patterns to be expected for either of two modes of action of mutagen: mutagen inducing mutations only near growing points or inducing mutations randomly distributed over the chromosome. The predictions based on these two modes are quite similar. The results from a set of segregation experiments involving six different genes have been fitted to the theoretical predictions and are consistent with a unidirectional, clockwise replication for our strain and conditions of growth with an origin between 55 and 74 min on the *E. coli* linkage map.

MATERIALS AND METHODS

Media: Liquid minimal medium was M63 (PARDEE, JACOB and MONOD 1959) with glycerol (0.2%) as the carbon source supplemented with 0.25% casamino acids and thiamine (5 μg/ml). Minimal agar (1.5%) was M63 with either glucose (0.5%) or lactose (0.5%) as the carbon source and supplemented with the necessary amino acids (each at a concentration of 20 μg/ml) and thiamine (5 μg/ml). Complete agar was lac-tet (HERMAN and DWORKIN 1971). Cells were plated by adding a sample to 2 ml soft agar (0.45% agar, 0.9% NaCl) at 45°C and then pouring onto agar medium.

Bacteria: The principal strain used in these experiments was RH491 (Table 1). Each of the mutations carried by RH491 was derived by mutagenizing with ICR-191 at 10 μg/ml overnight

TABLE 1

Bacterial strains

Strain	Sex	Description*	Derivation
A327	F′	F-*lac*+/*lacZS* Sm^S	†
CA7049	HfrH	*lacZU131* Sm^S	E. Signer
LH97	F⁻	*lacZL32*	†
LH133	F′	F-*lacZL32*/*lacZL32*	†
LH148	F⁻	*lacZL32 lys*⁻ Sm^R	†
RH9	F⁻	*lacZL32 lys*⁻ *ilv*⁻ *his*⁻ Sm^R	LH148 (see text)
RH12	HfrH	*lacZU131 leu*⁻ *trp*⁻ Sm^S	CA7049 (see text)
RH14	F⁻	*leu*⁻ *trp*⁻ *his*⁻ *lys*⁻ *ilv*⁻ Sm^R	RH12×RH9
RH491	F⁻	*leu*⁻ *lacZL32 trp*⁻ *his*⁻ *lys*⁻ *ilv*⁻ Sm^R	LH133×RH14 (see text)

* Sm^S and Sm^R refer, respectively, to sensitivity and resistance to streptomycin. Other abbreviations follow TAYLOR (1970).

† See HERMAN and DWORKIN (1971).

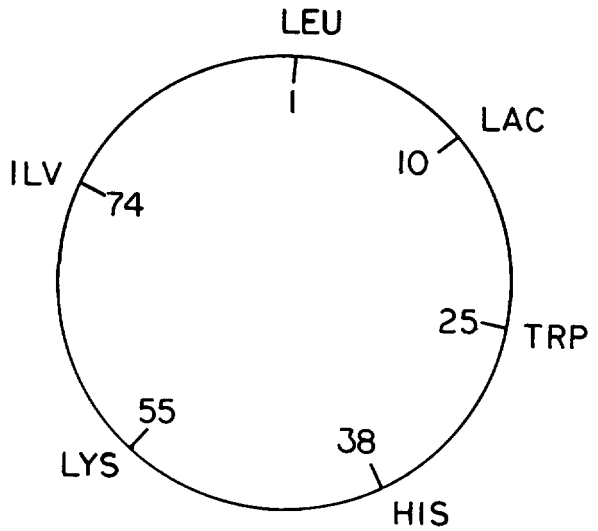

FIGURE 1.—*E. coli* linkage map showing positions of ICR-191-induced mutations carried by RH491.

followed by penicillin selection, as previously described (HERMAN and DWORKIN 1971). Generally, 25–100 mutants for each trait were tested, and one with a low spontaneous reversion rate and high reversion rate in response to ICR-191 was chosen. The *his*⁻ and *ilv*⁻ mutations were added in that order to LH148 to construct RH9. At the same time RH12 was constructed by mutagenizing CA7049 to add *leu*⁻ and *trp*⁻ sequentially. RH9 and RH12 were then mated, and a Lac+ SM^R recombinant was selected, purified, and tested for its amino acid requirements (RH14). To reintroduce *lacZL32*, RH14 was mated with LH133, and samples of the mating mixture were spread on lac-tet. Lac+ colonies were picked and tested for maleness by mating them with LH97 on minimal lactose agar. A strain that contained the episome (F-*lacZL32*) was restreaked on lac-tet. A Lac⁻ segregant was picked and cured of the episome by acridine orange treatment (HERMAN and DWORKIN 1971). It was tested by crossing with CA7049, LH97 and LH133, and then designated RH491. The presumed map positions of the ICR-191 induced markers carried by RH491 are shown in Figure 1. The map positions shown have been confirmed by crosses with CA7049 (data not shown). The mapping of revertants, which is described in the RESULTS and which is actually more important to our conclusions than the mapping of the original mutations, also supports the map given in Figure 1.

Segregation experiments: For each segregation experiment a single colony of RH491 from a lac-tet plate was picked, inoculated in 1 ml minimal medium and grown overnight at 37°C. The following morning the culture was diluted 1000-fold into minimal medium. When the culture reached about 7×10^7 cells/ml an aliquot was added to a flask containing ICR-191 to give 10 μg/ml of mutagen. All experiments with ICR-191 were done in dim light and used covered flasks. At the same time a parallel culture was incubated in the absence of mutagen for subsequent monitoring of spontaneous revertants. When the segregation of Lac+ revertants was to be tested, isopropylthiogalactoside (IPTG) was added as inducer at 5×10^{-4} M at the same time ICR-191 was added to increase the reversion rate (HERMAN and DWORKIN 1971). The cells were incubated with ICR-191 for 40 min, about 0.85 generation. Mutagen was then removed by Millipore filtration. Cells collected on the filter were washed with an equal volume of M63 medium and then resuspended in an equal volume of fresh minimal medium. The culture was again incubated at 37°C and samples were removed at 2-min intervals for 130 min and plated on appropriate selective media. The sample size varied from 0.1 ml to 0.4 ml according to the revertability of the marker being tested. In all cases, the addition of more liquid minimal medium to the pour agar

tubes did not increase the number of induced revertants able to make colonies. Thus it was concluded that the amount of medium included in the samples taken was sufficient to permit all potential mutants to make it through their phenotypic lags (if there were any phenotypic lags) before being challenged to initiate a colony. Each experiment followed the segregation of two markers. During the course of the experiment the optical density at 540 nm (OD_{540}) of the culture was monitored at 10-min intervals to estimate the doubling time. Samples were also taken periodically from the unmutagenized culture to determine numbers of spontaneous revertants. The OD_{540} of the unmutagenized cultures followed the mutagenized cultures closely. Spontaneous revertants for Lys+, His+, Lac+, Trp+ and Leu+ averaged about 1%, 1%, 2%, 5% and 10%, respectively, of the number of revertants found after mutagenesis, and the absolute numbers of revertants varied very little over the course of each experiment. Spontaneous Ilv+ revertants, on the other hand, varied from under 5% to nearly 50% (in different experiments) of the revertants found after mutagenesis, and here the number of spontaneous revertants did accumulate to some extent during segregation in liquid medium. Total numbers of colonies counted per marker per experiment ranged from 1,651 to 39,881. All data were corrected for spontaneous reversion.

Mating experiments: Parental cultures growing exponentially in Difco Penassay Broth were mixed to give about 0.9×10^8/ml donors and 1.5×10^8/ml recipients. Matings were allowed to proceed for 2 hr at 37°C without shaking. Samples of mated mixtures and unmated parents (as controls) were diluted appropriately and plated on various selective media.

Incorporation of ^{3}H-thymine: The labeling medium was liquid minimal medium supplemented with 4 μg/ml thymine-methyl-^{3}H (final specific activity: 78 mCi/mmole and 750 μg/ml of deoxyadenosine to promote uptake of thymine (Boyce and Setlow 1962). One-ml samples were taken at various times into 1 ml cold 20% trichloroacetic acid (TCA) containing 250 μg nonradioactive thymine. The TCA precipitates were subsequently collected and washed on Millipore filters. The filters were dried, added to a toluene scintillation fluid, and counted.

THEORY

General theory for segregation of induced mutants: The formulation presented here will be general enough to include the possibility of bidirectional replication and will not assume at the outset that growing points move at constant rate. First, we shall assume that the chromosome replication cycle has a periodicity which is identical for all chromosomes in a culture and that the period is equal to one generation (in this paper one generation equals one doubling time). This periodicity is reflected by the parameter "chromosomal age", x, where $0 \leq x \leq 1$ (units are generations). The age of 1 is assigned to a chromosome just as it divides (or terminates) to give 2 chromosomes, which are simultaneously assigned the age of 0. (Under most growth conditions age 0 does *not* correspond to initiation of chromosome replication.)

We shall assume that a particular gene is duplicated once per generation when the chromosome reaches a particular age. Moreover, we shall assume that replication is symmetric (Caro 1970; Quinn and Sueoka 1970; Fritsch and Worcel 1971); that is, that the duplication of a particular gene can occur as the duplication of one copy per chromosome to two copies or as the simultaneous duplication of two copies per chromosome to four copies or, in general, as the simultaneous duplication of 2^{s-1} copies per chromosome to 2^s copies, where s must be an integer. Next we define a replication sequence parameter, r. Each gene is assigned a unique value of r according to the following recipe: a gene that goes from 2^{s-1} copies per chromosome to 2^s copies at chromosomal age $x = a$ is assigned $r = n -$

$s + a$, where n is defined by the condition that $r = 0$ for the chromosomal origin. It follows that the terminus corresponds to $r = n$, and one complete cycle of chromosome replication occupies n generations; that is, it takes n generations for one growing point in the case of unidirectional replication or a pair of growing points in the case of bidirectional replication to proceed from origin to terminus. The value of r for a given marker, then, is the time required from initiation of this cycle for a growing point (or one of a pair of growing points in the case of bidirectional replication) to encounter the particular marker. If growing points move at a constant rate, then the value of r for a marker is directly proportional to distance from the origin. As has been pointed out by others (YOSHIKAWA, O'SULLIVAN and SUEOKA 1964; HELMSTETTER and COOPER 1968), n is a function of the growth medium, it is not necessarily an integer, and it may be less than or greater than one.

Consider the following experiment: a mutagen is added to an exponentially and nonsynchronously growing population of cells. The mutagen does not significantly affect the rate of growth or division of the cells. After a period of exposure, T, the cells are quickly washed free of mutagen and resuspended in the original medium minus mutagen (time, $t = 0$). The cells maintain their exponential rate of growth and division. Samples are removed at various times and assayed for concentration of mutants, M, affected in a particular gene. The assay procedure allows for the possibility of phenotypic lag and thus assays for mutant progenitors or mutant yielders, although for simplicity we shall refer to mutant progenitors as mutants. Let the concentration of mutants at $t = 0$ be designated I. Assuming that the mutagen is effectively removed, as t increases from $t = 0$, the mutant titer, M, will at first remain constant (equal to I), owing to the segregation lag. Later, one generation after all mutant cells have become pure for the mutant gene, M will increase exponentially, presumably at the same rate as the culture as a whole. It is our purpose here to derive a relationship for the length of the segregation lag and in particular to show how it depends on the replication sequence parameter, r, of the mutant gene. We shall do this for two possible modes of action of the mutagen: a) the mutagen acts only near growing points (CERDÁ-OLMEDO, HANAWALT and GUEROLA 1968; NEWTON *et al.* 1972) and b) the mutagen does not act preferentially near growing points. We shall see that the predictions of these two modes of action are very similar.

We first consider the case of mutagen acting only on growing points. Consider a marker at r, which goes from 2^{s-1} copies to 2^s copies at chromosomal age $x = a$. Suppose that at $t = -(T - p)$, where $0 \leq p \leq T$, this marker is mutated while in the process of being replicated, such that one mutant (homoduplex) gene is produced in the wake of the growing point, leaving $2^s - 1$ nonmutant alleles in the same chromosome. A pure mutant chromosome will appear $s - a$ generations later, at $t = s - a - (T - p) = n - r - (T - p)$. Two mutant chromosomes will be produced one generation later still, at $t = n - r - (T - p) + 1$, and these will give rise to two mutant cells at $t = n - r - (T - \mathrm{p}) + 1 + d$, where d is the time from termination of chromosome duplication to segregation at cell division. In the simple case where the time of exposure to mutagen is very short ($T \ll 1$), a

marker at r would show a segregation lag of $n - r + 1 + d$, after which the mutants would divide synchronously. It clearly follows that a marker at the origin ($r = 0$) would show a segregation lag n generations longer than a marker at the terminus ($r = n$), with intermediately replicating markers showing intermediate segregation lags. For the case where T is not negligibly small (but still less than 1), the dm mutants produced in the interval $p - T$ to $p + dp - T$ will each generate dm new mutants by cell division in the interval t to $t + dt$, where $t = n - r - T + p + 1 + d$. To simplify our formulae, let $u \equiv n - r + 1 + d$. Thus the number of mutants, M, present from $t = 0$ to $t = u - T$ will be constant ($= I$) and for $(u - T) \leq t \leq u$:

$$M = I + \int_{p=0}^{p=t+T-u} dm(p) \quad . \tag{i}$$

Clearly, $\int_{p=0}^{p=T} dm(p) = I$. To clarify the meaning of equation (i), we point out that at the end of exposure to mutagen, I mutants are detected, and by definition, the function $m(p)$ represents the number of these mutants that were produced in the interval $t = -T$ to $t = -T + p$. We have estimated $m(p)$ for the reversion by ICR-191 of each of the six markers of RH491, and the results all normalized by I, are given in Figure 2. The measured functions, which show approximately linear increases in numbers of mutants after the first 7 min, are essentially identical for all six markers. This function of Figure 2 has been used to solve equation (i) graphically. The results are shown in Figure 3a. Slight heterogeneities in generation times will obviously tend to smooth out the wavy curve, so that the predicted experimental result would be a simple exponential increase in mutants following the segregation lag. The segregation lags for different markers will differ only according to their replication sequence parameters, r, since n, d, and T are the same for all markers. A marker at the terminus would be expected to have a segregation lag n generations shorter than a marker at the origin.

It should be clear that if the mutagen acted preferentially on a region replicated a certain prescribed time ahead of or behind the growing point, then the segregation pattern would be the same as we have already described except that all segregation lags would be shorter or longer by the same amount. Or if the mutagen acted on the growing point to produce a heteroduplex gene in the wake of the growing point and the heteroduplex was not repaired, then all segregation lags would be one generation longer.

We next consider the case of mutagen acting all over the chromosome irrespective of the growing points. Here we follow the treatment of VIELMETTER, MESSER and SCHÜTTE (1968). Again we concentrate on a marker at r, which goes from 2^{s-1} copies to 2^s copies at chromosomal age $x = a$. And again we consider dm mutations produced in the interval $p - T$ to $p + dp - T$. (As before, $T < 1$.) Mutated chromosomes of age $x < a$ will become pure at $t = s - x - T + p$ and will give rise to two mutant cells at $t = s - x - T + p + 1 + d = u + a - x - T + p$, where, as before, $u \equiv n - r + 1 + d$. Similarly, mutated chromosomes of

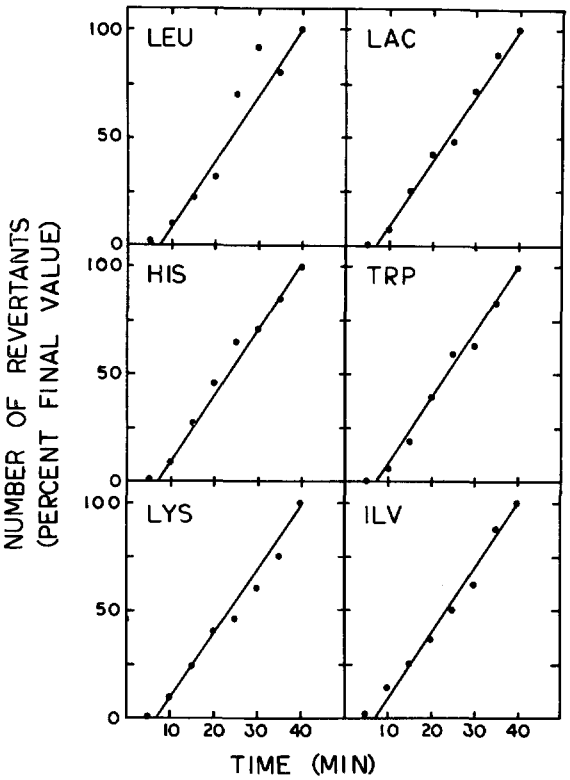

FIGURE 2.—Kinetics of ICR-191-induced reversion of mutations carried by RH491. When cultures growing exponentially in liquid minimal medium reached 3.5×10^8 cells/ml, ICR-191 was added to give 10 μg/ml ($t = 0$). At 5-min intervals 1 ml samples were removed, collected on a Millipore filter, washed twice with 2.5 ml of medium, resuspended in 1 ml of medium, and sampled for numbers of revertants and OD_{540}. The OD_{540} monitored recovery of cells through the washing procedure and was used to make corrections for slight variations in recovery. Corrections for spontaneous revertants were also made based on results from parallel, unmutagenized cultures. The reversion frequencies obtained in these experiments after the full 40-min exposures to ICR-191 were about 2×10^{-5}, 4×10^{-5}, 7×10^{-6}, 10^{-5}, 2×10^{-5}, and 7×10^{-5} for Lys+, His+, Trp+, Lac+, Leu+, and Ilv+ reversions, respectively.

age $x > a$ will give rise to two mutant cells at $t = u + a - x - T + p + 1$. A chromosome of age $x < a$ will have a probability of mutation equal to one-half that for a chromosome of age $x > a$ because it has half as many copies of the gene at r. Let this probability be c. Then, using the age distribution function for chromosomes of an exponentially and nonsynchronously growing population (POWELL 1956), $f(x) = (ln2)2^{1-x}$; we have: $dm(p) = c\int_0^a f(x)dx + 2c\int_a^1 f(x)dx$, which gives $c = (2^{a-1})(dm(p))$. It follows that for $t \geq u$, the number of mutants, M, is given by the following expression:

$$M = I + 2^{a-1}\int_{p=0}^{p=T} dm(p) \int_a^{u+a+p-T-t} f(x)(-dx) = 2^{t+T-u}\int_0^T 2^{-p}(dm/dp)dp. \quad \text{(ii)}$$

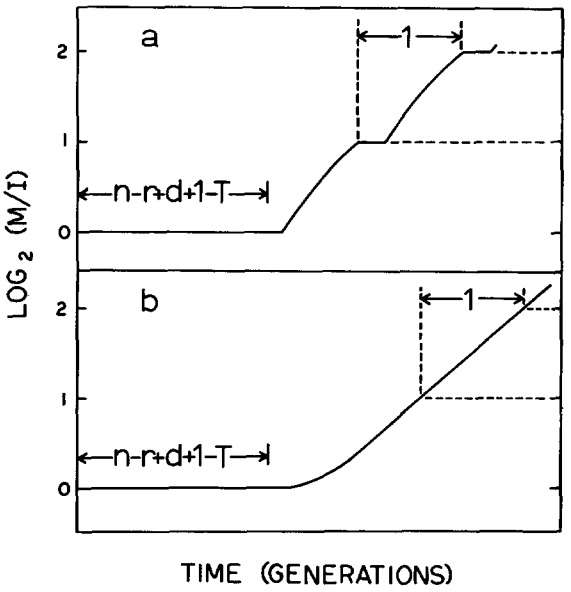

FIGURE 3.—Theoretical curves showing predicted kinetics of segregation of induced mutants. Curve (a) assumes that mutagen acts only on growing points. Curve (b) assumes that mutagen acts indiscriminately over the chromosome.

For $0 \leq t \leq (u - T)$, $M = I$. The expression for M in the interval $(u - T) \leq t \leq u$ can be derived readily and is similar to equation (ii). We have used the function shown in Figure 2 as $m(p)$ to solve our equations for M graphically. The results are given in Figure 3b. The general shape of the theoretical curve is very similar to the previous case. The absolute segregation lag is somewhat longer, but the relative lags of different markers depend on their relative values of r in exactly the same fashion as in the case of mutagen acting preferentially on growing points.

RESULTS

Effect of ICR-191 on growth and DNA replication: The foregoing theory assumes that the mutagen has a negligible effect on chromosome replication and cell division both during and immediately after the mutagenic treatment. We show here that this is approximately the case in our experiments with ICR-191. In the top curve of Figure 5 is plotted total viable cells assayed both during and following a 40-min exposure to 10 μg/ml of ICR-191. Little if any effect of mutagen can be discerned in experiments of this kind. We have also monitored the effect of ICR-191 on incorporation of ^{3}H-thymine into acid-insoluble material as a measure of DNA replication (Figure 4). The mutagen does appear to retard DNA replication, such that after 40 min the ICR-191-treated culture is about 10 min behind the control in amount of label incorporated. But the mutagen-treated culture certainly shows no signs of terminating DNA replication, and we shall assume that the conditions of the segregation theory are at least approximately satisfied.

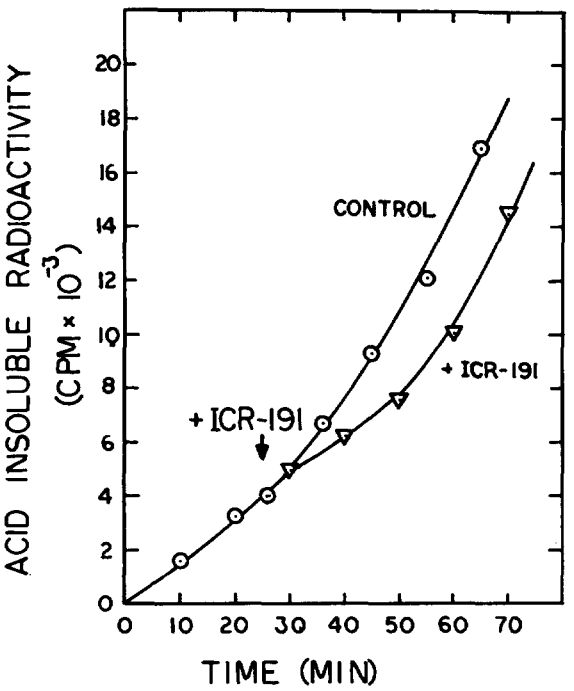

FIGURE 4.—Uptake of ^{3}H-thymine in the presence and absence of ICR-191. Radioactive label was added to exponentially growing cells at $t = 0$ (7×10^7 cells/ml). At $t = 25$ min a portion of the radioactive culture was added to a flask containing ICR-191 to give 10 μg/ml.

Segregation experiments: Segregation experiments were carried out as described in MATERIALS AND METHODS. Figure 5 shows two representative experiments for each marker. In order to treat our data as objectively as possible, each set of experimental points was fit to a theoretical curve by a Fortran computer program run on a Control Data Corporation 6600 computer. The program assumed that each set of experimental points was to be fitted to the following curve (a slightly simplified form of the theoretical curves of Figure 3): $\log_2 M = K_1$ for $0 \leq t \leq K_2$ and $\log_2 M = K_1 + t - K_2$ for $t \geq K_2$. The program searched for values of K_1 and K_2 which best fit the data. Its procedure for determining best fit was to take the logarithm of the colony counts at each time point (corrected for spontaneous revertants), subtract the theoretical value based on a particular K_1 and K_2, square the difference, and sum over all points. A $K_1$and K_2 were found which minimized this sum. The solid lines through the points in Figure 5 are the computer-derived curves. It is clear that the experimental points for each marker do follow the generally predicted segregation pattern. We shall refer to K_2 for any one experiment as the experimentally derived segregation lag for that experiment (although we shall hereafter use minutes as units rather than generations).

A summary of all the segregation experiments is shown in Figure 6 with the mean segregation lag for each marker indicated. Figure 7 shows a plot of mean segregation lag for each marker as a function of map position. Lys$^+$ mutants segregated earliest, followed by His$^+$, Trp$^+$, Lac$^+$, Leu$^+$, and Ilv$^+$ in that order,

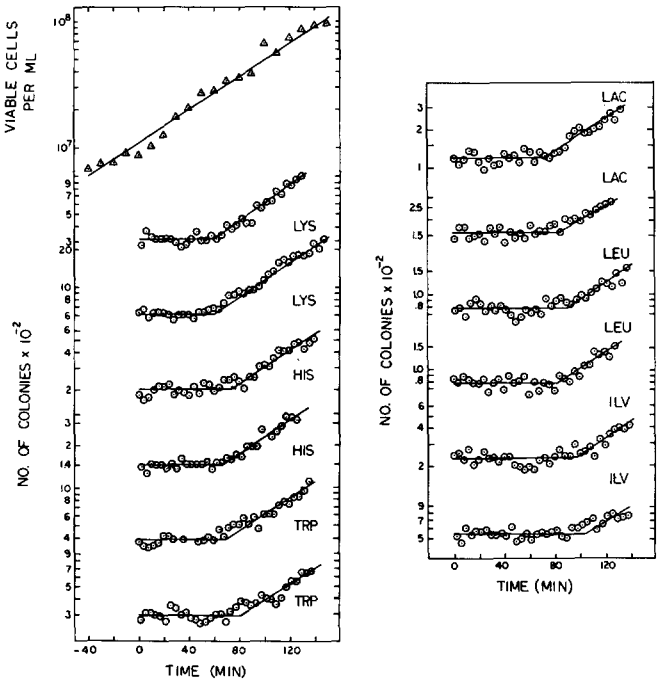

FIGURE 5.—Kinetics of segregation of revertants induced by ICR-191. Cells were exposed to ICR-191 from $t = -40$ min to $t = 0$. The top curve shows total viable cell number during and after mutagenesis.

although resolution of neighboring markers is in some cases not very good. According to theory, the markers are replicated in precisely the reverse order of their segregation, which implies that of these six markers, *ilv* is replicated first and *lys* is replicated last. This order is compatible with unidirectional replication proceeding in clockwise fashion around the map and beginning in the region bounded by *lys* (55 min) and *ilv* (74 min). The solid line drawn in Figure 7 corresponds to unidirectional replication at constant rate with a complete cycle of replication requiring 46 min ($n = 1$).

Mapping revertants: An important assumption in these segregation experiments is that the large majority of the "revertants" of a particular mutation, while probably not true revertants, are suppressor mutations in the same gene (or at least very close to it) as the original mutation. If reversion occurred by outside suppression the observed segregation lag would be that of the outside suppressor gene, not the original mutant gene. Intergenic frameshift suppression does occur (YOURNO, BARR and TANEMURA 1969), although it appears to be considerably less common than intragenic suppression (RIDDLE and ROTH 1970). The following experiments were carried out to determine if a significant fraction of the revertants in our experiments mapped away from the original mutations.

An *F-lac*$^+$ was transferred into RH491 by mating with A327 and selecting for a Lac$^+$ Sm^R recombinant. This F-*lac*$^+$ promotes transfer of chromosomal mark-

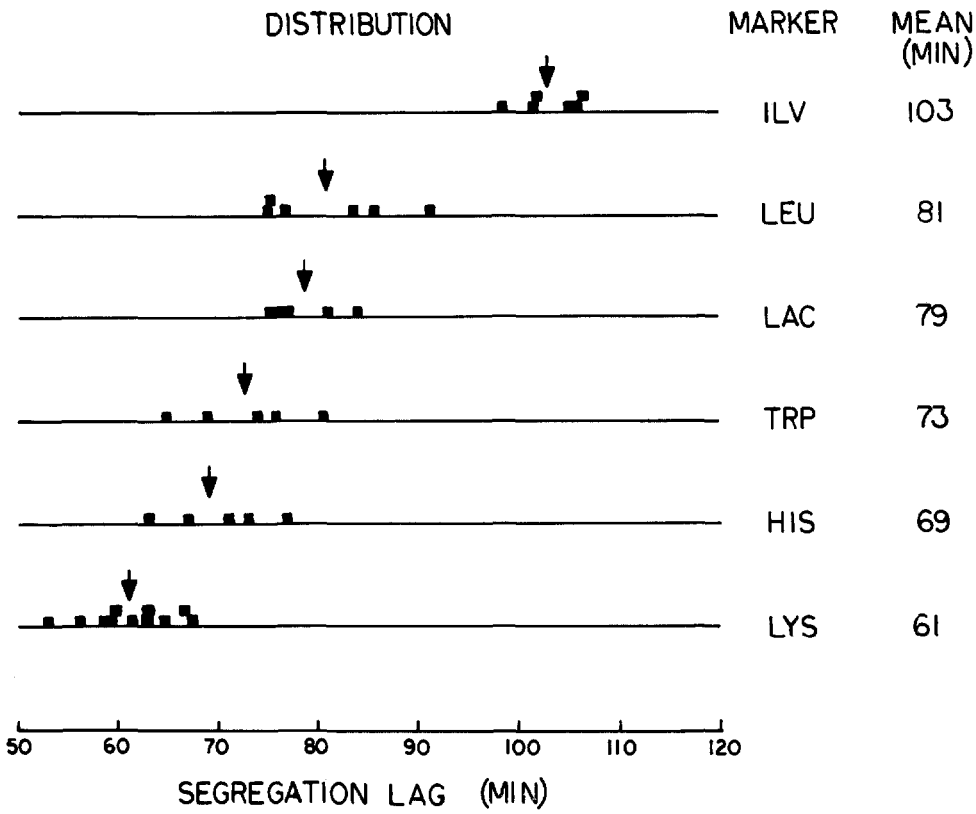

FIGURE 6.—Summary of all segregation data. Each square represents the segregation lag obtained in one experiment for one marker. Mean segregation lags for each marker are shown by arrows.

ers in merodiploids in the following sequence: *lac–leu–ilv–lys–his–trp*. Ten independent ICR-191 induced His$^+$ revertants of the F-*lac*$^+$/RH491 strain were picked and restreaked. One ICR-191-induced Ilv$^+$ revertant of each of these ten strains was picked and restreaked. Lys$^+$ and Leu$^+$ reversions were introduced in similar fashion in that order, so finally we had 10 F′ strains, each carrying independent His$^+$, Ilv$^+$, Lys$^+$, and Leu$^+$ reversions. These ten strains are designated 4R1, 4R2, etc. The four reversions were mapped with respect to each other in each of the ten strains by crossing them individually with a Trp$^+$ revertant of RH491. After 120-min mating periods, selection was exerted for four types of recombinants: Leu$^+$ Trp$^+$, Ilv$^+$ Trp$^+$, Lys$^+$ Trp$^+$, and His$^+$ Trp$^+$. If the reversions map very close to the original mutations, we expect that the relative numbers of these four recombinant types will decrease in the order just given, owing to spontaneous rupture of the chromosome during transfer. For a breakage probability of 0.5 per 10 min of map the expected ratios would be 1.0:0.33:0.09:0.03 for Leu$^+$ Trp$^+$:Ilv$^+$ Trp$^+$:Lys$^+$ Trp$^+$:His$^+$ Trp$^+$. This expectation was realized by the data shown in Table 2 except for the extremely low frequency of occur-

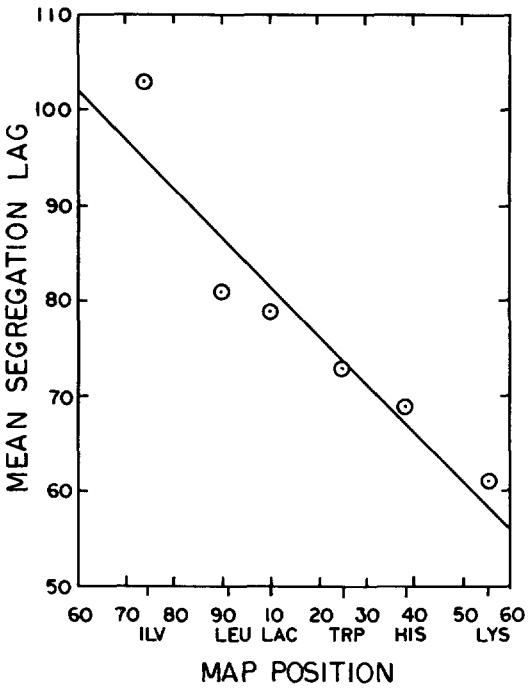

FIGURE 7.—Mean segregation lag for each marker as a function of map position.

rence of Lys$^+$ Trp$^+$ recombinants in crosses involving 4R2, 4R3, and 4R4 as donors of the Lys$^+$ markers. Precise frequencies of Lys$^+$ Trp$^+$ recombinants were difficult to determine, owing to the poor growth of this particular recombinant type issuing from crosses involving 4R2, 4R3, and 4R4. We have entertained two possible explanations for the low numbers of Lys$^+$ Trp$^+$ recombinants in these three crosses. One was that the Lys$^+$ reversions were suppressor mutations

TABLE 2

Relative recombinant frequencies in crosses between His+ Ilv+ Lys+ Leu+ revertants as donors and a Trp+ revertant as recipient

Donor	Recombinant ratio		
	Ilv$^+$ Trp$^+$/Leu$^+$ Trp$^+$	Lys$^+$ Trp$^+$/Leu$^+$ Trp$^+$	His$^+$ Trp$^+$/Leu$^+$ Trp$^+$
4R1	.42	.09	.05
4R2	.24	<.01	.02
4R3	.40	<.01	.07
4R4	.22	<.01	.01
4R5	.24	.10	.06
4R6	.23	.05	.02
4R7	.21	.04	.02
4R8	.31	.08	.03
4R9	.28	.06	.02
4R10	.24	.08	.03

mapping near *trp* or between *trp* and *lac*. The second was that the reversions did occur near the original *lys*$^-$ mutation but that there was something aberrant about their integration in crosses. We shall try to distinguish between these alternatives after first mapping 10 independent Trp$^+$ revertants.

Consider the immediate progenitors of the donors listed in Table 2. Recall that these strains were His$^+$ Ilv$^+$ Lys$^+$ Leu$^-$ Trp$^-$/F-*lac*$^+$. Ten independent ICR-191-induced Trp$^+$ revertants of the progenitor 4R1 were picked, purified and mated with a Leu$^+$ revertant of RH491. The ten Trp$^+$ donors were designated 3R1T1, 3R1T2, etc. Ninety-six Lys$^+$ Leu$^+$ recombinants from each mating were picked and tested for inheritance of two unselected markers His$^+$ and Trp$^+$. For all ten strains the number of recombinants that were His$^+$ exceeded the number that were Trp$^+$ (although in three cases the difference was small), indicating that the Trp$^+$ revertants map on the counterclockwise side of His$^+$.

We now return to the problem with Lys$^+$ in 4R2, 4R3, and 4R4. The immediate (leu$^-$) progenitors of these strains were reverted to Trp$^+$ (now designated 3R2T1, 3R3T1, and 3R4T1) and mated with the Leu$^+$ revertant of RH491. Lys$^+$ Leu$^+$ and Trp$^+$ Leu$^+$ recombinants were selected. Just as in the crosses of Table 2, these three Lys$^+$ reversions were transmitted to recombinants at an aberrantly low frequency: Lys$^+$ Leu$^+$ recombinants were less than one-third as frequent as Trp$^+$ Leu$^+$ recombinants (whereas in the crosses of Table 3, Lys$^+$ Leu$^+$ recombinants, as expected, were more frequent than Trp$^+$ Leu$^+$ recombinants). But when Lys$^+$ Leu$^+$ recombinants were picked, purified and checked for inheritance of the unselected Trp$^+$ marker, in all three crosses Trp$^+$ was inherited infrequently (Table 4). We conclude from these results that the low frequency of Lys$^+$ transmission in crosses involving 4R2, 4R3, and 4R4 as donors (as well as 3R2T1, 3R3T1, and 3R4T1) is not indicative of the map positions of the Lys$^+$ revertants in these strains but appears to be inherently low. Table 4 also indicates that the inheritance of His$^+$ by Lys$^+$ Leu$^+$ recombinants in these crosses is abnormally high (compare with Table 3): apparently those recombinants that

TABLE 3

*Mapping Trp$^+$ revertants: inheritance of unselected traits by Lys$^+$ Leu$^+$ recombinants**

Donor	Unselected traits His$^+$	Trp$^+$
3R1T1	9	7
3R1T2	13	7
3R1T3	19	15
3R1T4	17	7
3R1T5	18	6
3R1T6	22	7
3R1T7	12	8
3R1T8	9	6
3R1T9	24	10
3R1T10	27	9

* 96 Lys$^+$ Leu$^+$ recombinants were tested in each case.

TABLE 4

Inheritance of unselected traits by Lys+ Leu+ recombinants

Donor	Number of Lys+ Leu+ recombinants tested	Unselected traits His+	Trp+
3R2T1	29	21	9
3R3T1	104	71	16
3R4T1	101	47	7

propagate the Lys+ trait tend to be those that inherit rather more of the donor chromosome than would normally be the case.

In summary, our mapping data, while not definitive, give no indication of outside suppression but, on the contrary, indicate that the large majority of the Leu+, Ilv+, Lys+, His+, and Trp+ revertants induced by ICR-191 in RH491 map near the original mutations.

We have not attempted any mapping of Lac+ revertants. We expect, however, that at least half of the Lac+ revertants *lacZL32* map in the *lac* operon since IPTG increases the frequency of Lac+ reversion by a factor of two, and this effect has been shown to depend specifically on expression of the *lac* operon (HERMAN and DWORKIN 1971).

DISCUSSION

In the experiments reported here we have measured the segregation lags of six different mutant classes induced by ICR-191. Theory predicts that the length of a segregation lag depends upon the relative position of the reverted gene in the chromosome replication cycle: the earlier a gene is replicated in the cycle, the longer its segregation lag should be. When we relate our observed segregation lags to the positions of the six markers on the genetic map, we find that the results are consistent with an origin for RH491 between 55 min and 74 min and a single growing point moving clockwise. This interpretation fits the chromosome replication pattern proposed earlier by numerous workers (see KLEIN and BONHOEFFER 1972 and PATO 1972 for reviews) but is inconsistent with recent evidence favoring bidirectional chromosome replication (MASTERS and BRODA 1971; BIRD *et al.* 1972; PRESCOTT and KUEMPEL 1972; YAHARA 1972). We should point out, however, that an extremely asymmetric bidirectional scheme which would be consistent with our results would be one in which *ilv, leu, lac, trp* and *his* are replicated by one growing point moving clockwise and *lys* is replicated very late in the chromosome replication cycle by a second growing point moving counterclockwise. The possibilities also remain that different strains of *E. coli* utilize different modes of replication and that growth conditions affect the mode of replication.

The interpretation of our experiments is not free of possible sources of error. The first of these is that the revertants we have followed are not mutations very close to the original mutations, as we have supposed, but are outside suppressor mutations. We feel that we have covered this problem by our mapping studies,

which showed that ten independent revertants for each of five markers in RH491 mapped in the relative positions expected if reversion occurred primarily by way of intragenic suppression. Perhaps we have decreased the likelihood of external suppression by restricting ourselves to highly revertible mutations.

Two other possible sources of error concern the mode of ICR-191 mutagenesis. The first of these depends on whether ICR-191 acts only on growing points or all over the chromosome. If ICR-191 acts only one way or the other or if both processes occur in the same proportion for each marker, then our conclusions are unchanged (see THEORY). Our conclusion would be affected, however, if some markers were reverted principally by one mechanism and other markers were reverted principally by the other mechanism. We consider this possibility unlikely. The results of NEWTON *et al.* (1972) favor a mechanism of ICR-191 mutagenesis in which only growing point regions are mutagenized.

Another possible source of error concerns repair of heteroduplexes. If the primary product of ICR-191 mutagenesis were a heteroduplex and if the probability or kinetics of repair of heteroduplexes differed for different markers, then their segregation lags would be affected to differing extents. We cannot eliminate this possibility, and we consider it the most serious objection to the interpretation we have presented.

One of the advantages of the method we have used for estimating the sequence of marker replication is that it does not require a synchronized culture. A second advantage is that a unidirectional origin should in principle be determinable to a very precise degree. Two markers closely bracketing a unidirectional origin-terminus should give segregation lags differing by a period corresponding to almost a full cycle of chromosome replication.

A potentially interesting application of this technique would be to strains of *E. coli* where the origin of replication is believed to have been moved by virtue of integrative suppression (LINDAHL, HIROTA and JACOB 1971; NISHIMURA *et al.* 1971).

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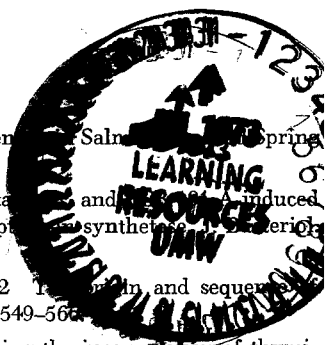

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