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Replication Forks of *Escherichia coli* Are Not the Preferred Sites for Lysogenic Integration of Bacteriophage Mu

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The question of whether bacteriophage Mu prefers replication forks for lysogenic integration into *Escherichia coli* chromosomes was tested by using two different systems. In the first, inactivation of genes was scored in synchronized cultures infected by Mu at various times. No increase in the mutation frequency of a gene was found after infection at the time of its replication. In the second, the composition of colonies formed by bacteria lysogenized by Mu was determined; the newly formed lysogens should give rise to mixed colonies (containing lysogenized as well as nonlysogenized bacteria), uniform colonies, or both, depending on the mode of integration. Both types of colonies were found, and the fraction of uniform colonies was proportional to the relative length of the unreplicated segment of an average chromosome in the culture. The results in both systems clearly preclude the possibility that a lysogenizing Mu integrates with high preference at the chromosome replication forks.

Bacteriophage Mu integrates at many different sites upon lysogenization of *Escherichia coli* cells (5, 24), with a possible preference for certain "hot spots" (8, 23). Secondary transpositions to many different sites also occur, as is reflected by the variability of host sequences at both ends of the viral DNA (3, 4). The ability of Mu DNA to integrate into many sites led to the hypothesis that it is inserted preferentially at chromosome replication forks (9, 21, 22). The hypothesis of preference for replication forks for lysogenic integrations was supported by the frequencies of gene inactivation in nalidixic acid-treated cells (22) and by sequential inactivation of genes in synchronized cultures infected at intervals (21). Comparative kinetics of zygotic induction of synchronized, Mu-infected Hfr strains indicated that in a lytic pathway as well, at least the earlier transpositions occur preferentially at replication forks (9).

These results were satisfactory because they were in keeping with the prevailing ideas that Mu exploits nicks or gaps as well as special proteins found in the "replisome" (9) and that replication of Mu DNA is an obvious step for its integration (9). However, it is now generally accepted that each transposon participates in determining the specificity of the staggered cut made at the target (13), thus eliminating the need for existing nicks. Moreover, it has been demonstrated that the first integration of Mu is conservative and does not require previous replication (10, 15). It therefore seems worthwhile to reexamine the hypothesis that the first integration of Mu occurs preferentially at replication forks.

Using a sandwich hybridization assay, it has been demonstrated in recent years that chromosome replication forks are not preferred targets for secondary transpositions during lytic development of Mu (19). Moreover, the above-mentioned results of zygotic induction (9) were shown to have been misinterpreted and to stem from variation in Hfr transfer efficiency under the experimental conditions used (19). By considering transfer efficiency and by modifying the experimental conditions either to decrease (by chloramphenicol treatment) or to totally prevent (by using *Bam* 1066) secondary transpositions, Nakai and Taylor did not observe the pattern expected from preferred initial integrations at replication forks. Conclusions concerning lysogenic integrations cannot be drawn by using this system, because the vast majority of the infections would develop toward the lytic pathway; nevertheless, some doubts were raised in this respect (19). There still exists the possibility of a fundamental difference between the targets of Mu integrations in lysogenic and other transpositions.

In our study we used an independent method for monitoring the targets of lysogenic integrations and were able to show that Mu DNA does not integrate preferentially at replication forks of its host chromosome.

MATERIALS AND METHODS

Strains and growh conditions. The following *E. coli* K-12 strains were used throughout: CR34 (*leu thr thyA dra drm* [26]); A3 (*dnaA* T46 F$^-$ *leu thr thi lacY supE*), kindly provided by R. H. Pritchard (20); and CSH56(MupAp1), a lysogen of CSH56 (17) harboring a *c*ts62 ampicillin-resistant mutant of bacteriophage Mu, kindly provided by N. Symonds (14).

Cultures were grown in M9 minimal medium (17) supplemented with glucose (0.5%), $MgSO_4$ (1 mM), and the required nutrients (100 μg of each amino acid per ml and 10 μg each of thymine and thiamine per ml) at 37°C for CR34 (doubling time [τ] = 60 min) or at 31°C for A3 (τ = 90 min).

Plates (containing 1.5% agar) were used for viability counting and for clonal selection, isolation, and characterization, as follows (17): LB, supplemented with thymine (10 μg/ml) and, where indicated, with ampicillin (50 μg/ml) or phosphonomycin (50 μg/ml); and VB, Vogel-Bonner (25) minimal medium, supplemented with thymine (10 μg/ml), thiamine (1 μg/ml), leucine and threonine (100 μg of each per ml), and, where indicated, ampicillin (50 μg/ml) or triornithine (100 μg/ml). A fresh lysate prepared by thermoinduction (42°C for 30 min) of strain CSH56(MupAp1), exponentially growing in LB, was used for infection.

Synchrony of chromosome replication. The method for synchrony was adapted from that of Cerdá-Olmedo and Hanawalt (6), as follows: exponentially growing cells of CR34 were collected by filtration (0.45-μm pore size),

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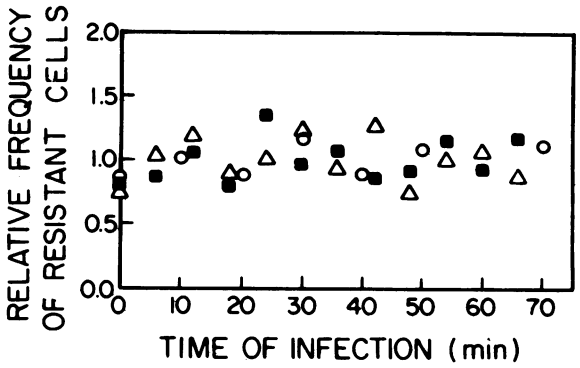

FIG. 1. Inactivation frequencies of *opp* and *glpT* in Mu-infected synchronous cultures of CR34. Synchrony was obtained by amino acid starvation followed by thymine starvation (see Materials and Methods). Cultures were infected at indicated times following thymine readdition. The frequencies of cells resistant to triornithine (○ and △, two experiments) and to phosphonomycin (■) are presented as ratios between their average frequencies in each experiment.

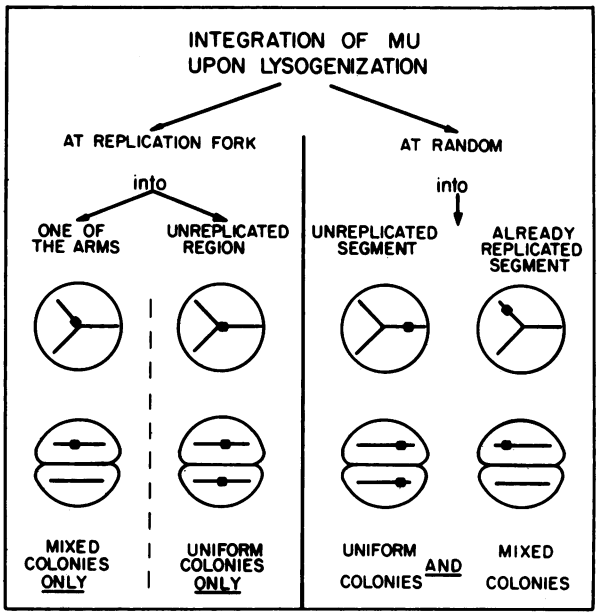

FIG. 2. Expected composition of colonies resulting from possible modes of Mu lysogenic integration. ■, Mu DNA.

washed, suspended in prewarmed M9 medium without amino acids (to allow completion of ongoing rounds of chromosome replication but inhibit new initiations), and incubated at 37°C for 120 min. The amino acid-starved culture was collected again, washed, and suspended in thymineless M9 medium (in the presence of the required amino acids). After 60 min at 37°C (conditions allowing the buildup of a maximal potential for initiation of chromosome replication), thymine was added.

Inactivation of genes by Mu infection. The following genes were selected as markers (1): *opp* (map position, 27 min), inactivation of which confers resistance to triornithine (2); and *glpT* (map position, 43 min), inactivation of which confers resistance to phosphonomycin (17).

Preliminary results indicated that infecting a batch culture with Mu increased the mutation frequency in these genes by about 2 orders of magnitude.

A culture grown in glucose minimal medium was centrifuged, and the cells were suspended in LB containing 5 mM $CaCl_2$, 10 mM $MgSO_4$, and 2 mM KCN and infected at 30°C for 10 min (at a multiplicity of infection [MOI] of about 1.3). Infected samples were washed, diluted in LB, and incubated at 30°C for 40 min, when ampicillin (50 μg/ml) was added. After overnight growth, each culture was diluted (in M9 medium for Opp^-; in LB for $GlpT^-$) and allowed to grow for an additional 3 h, and samples were spread either on triornithine-containing VB plates or on phosphonomycin-containing LB plates, as appropriate, and on ampicillin-containing plates for viability counting of lysogens. The number of mutants for each point presented (Fig. 1) was more than eight.

Composition of colonies (mixed or uniform). A culture grown in glucose minimal medium was infected at 31°C for 6 min in LB containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$, cooled to prevent further cell divisions, washed twice, and diluted at 4°C to give about 100 colonies per plate when spread on VB plates. Plates were incubated for 36 h at 31°C, and colonies were replicated on ampicillin-containing LB plates and incubated at 31°C. Ampicillin-resistant colonies were then identified on the original plates. Each such colony was diluted to obtain 60 to 200 daughter colonies when spread on a VB plate. At least 50 daughter colonies from each original colony were replicated on LB and on ampicillin-containing LB plates. A colony was defined as uniform if all its tested daughter colonies were found to be ampicillin resistant. Otherwise it was defined as mixed.

Pretreatment of strain A3. A culture of A3 was grown at 31°C to an A_{540} of 0.25, transferred to 42°C for 65 min, and then shifted back to 31°C, after which infection of samples was carried out at intervals.

Determination of effective MOI. The effective MOI (*m*) was calculated from the relative fraction of uninfected cells in an infected culture [P(0)], according to the zero term of the Poisson distribution, $P(0) = e^{-m}$. To this end, the number of survivors (closely resembling the number of uninfected cells) and the number of infective centers (closely resembling the number of infected cells) were determined, and P(0) was calculated as [number of survivors/(number of survivors + number of infective centers)].

RESULTS

Distribution of mutations in synchronous cultures. A culture of strain CR34 was infected with MupAp1 at intervals along a synchronous chromosome replication cycle, and inactivation frequencies of two genes (*opp* and *glpT*) among lysogens were determined (Fig. 1). No increase in mutation frequency of each gene was evident around the time of its duplication, as would be the case if lysogenic integration of bacteriophage Mu occurred preferentially at replication forks. Similar results were obtained for a third gene (*cycC*), situated closer to *oriC* (data not shown). Since these results contradict those of Paolozzi et al. (21), we set up another, entirely different system.

Composition of colonies. The system was based on the composition of colonies formed by bacteria lysogenized with Mu (Fig. 2); lysogens formed upon infection would give rise exclusively to mixed colonies (containing lysogenic as well as nonlysogenic bacteria) if integration occurred at one of the arms of a replication fork and solely to uniform colonies if it occurred at an unreplicated region near the fork. Random integration, on the other hand, would result in both

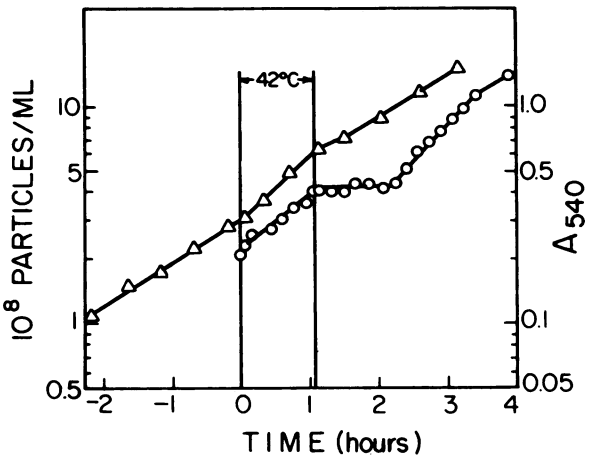

FIG. 3. Growth parameters of an A3 culture shifted from 31 to 42°C and back. Symbols: △, A_{540}; ○, particle counts.

mixed and uniform colonies; the fraction of the latter was expected to be proportional to the relative length of the unreplicated segment of an average chromosome in the culture. The term random, as opposed to preference for forks, is used throughout, without excluding possible specificity for hot spots (8) or for a consensus sequence, as was suggested for the transposition of mini-Mu (18).

A culture of strain CR34 was infected with MupAp1 at 30°C (MOI of about 1). Several colonies in which Mu entered a lysogenic state were identified by replicating them on ampicillin-containing plates, and the compositions of 5 colonies were determined by scoring 50 daughter colonies of each for resistance (see Materials and Methods). All five were found to be mixed, containing both ampicillin-resistant and ampicillin-sensitive bacteria. A one-to-one correspondence was found between resistance to ampicillin and immunity to Mu in all daughter colonies. Mixed colonies were unlikely to be a consequence of the loss of Mu during cell divisions, since resistant daughter colonies contained only lysogenic bacteria.

The existence of mixed colonies under our experimental conditions, where there is no more than one ongoing replication cycle per cell at a time (τ is equal to the time between initiation of replication and cell division [C + D] [11]), precludes the possibility that Mu integrates exclusively at the unwound prereplicated region of the fork.

To differentiate between the two other possibilities, integration at one arm of the replication fork and integration at random, it was necessary to establish conditions under which the relative portion of the replicated chromosome in the culture differed significantly. For this purpose, we used strain A3 (*dnaA46*). During 65 min at the restrictive temperature, cells in which chromosome replication had been completed divided (Fig. 3). Upon being shifted back to the permissive temperature, each cell starts a new round of replication of its single chromosome. The relative length of the unreplicated segment decreases with time at the permissive temperature. The frequency of uniform colonies thus is inversely proportional to the time of infection after temperature is restored only if Mu integrates at random.

A culture of strain A3 was grown at 31°C, shifted to 42°C for 65 min (to enable completion of ongoing replication cycles and subsequent divisions), and then shifted back to 31°C. Samples were infected at intervals after being shifted back, and the frequency of uniform colonies in each was determined (Table 1). This frequency decreased as chromosome replication proceeded, as was expected for random integration.

TABLE 1. Fraction of uniform colonies obtained upon infection of A3 samples at various times after shift back to 31°C

Infection time (min) after shift to 31°C	No. of colonies		Ratio of uniform colonies/ total colonies
	Uniform	Mixed	
1	28	3	0.90
25	26	12	0.68
50	1	27	0.04
70	3	27	0.10

Uniform colonies could be formed by double lysogenization. Simultaneous lysogenization occurs when a high MOI is used (12). The effective MOI was determined (as described in Materials and Methods) in a similar experiment in which a culture was infected at 1 and 50 min after being returned to 31°C. The effective MOI was found to be low (0.4) and equal in both cases, while the proportions of uniform colonies differed consistently. In our experiments, multiple simultaneous lysogenizations should therefore have had very little chance to be formed and by no means can be held accountable for the observed differences (Table 1).

DISCUSSION

The lack of differences in the inactivation frequencies of three tested genes in cells infected with Mu at different times along a synchronous replication cycle (Fig. 1) indicates that Mu does not integrate preferentially at replication forks upon lysogenization. Previous results in a similar biological system (21) had supported preferred lysogenic integration at replication forks, because each marker gene had been inactivated at the highest rate upon infection around the time of its replication. Such experimental systems depend on the exact time at which lysogenic integrations occur: the pattern expected from preferred integration at replication forks would be achieved only if these integrations occurred synchronously close to the time of infection. We used KCN to synchronize Mu adsorption but were unable to determine the exact time at which lysogenic integrations occurred. In the above-mentioned investigation (21), Paolozzi et al. tried to limit the integration time by transferring to 32°C a culture of a lysogen harboring an X mutant of Mu *c*ts62 grown and infected at 42°C. This synchrony of integrations is, however, not very efficient; only 70% of the cells recover immunity within 15 min after the temperature shift to 32°C (21), and since the duration of infection is 8 min, integration is limited in 70% of the cells to 23 min, which constitutes a substantial portion of the chromosome replication cycle.

The contradiction between our results and those reported by Paolozzi et al. (21) required a reexamination of the question in an entirely different system. The examination of the composition of colonies obtained from Mu-infected cells which we have devised is such an independent method (Fig. 2). It was expected that integration to one of the arms of a replication fork or to an already replicated segment would give rise to mixed colonies containing lysogenic as well as nonlysogenic bacteria. After infection of *E. coli* CR34, we did find such mixed colonies. This precludes the possibility that Mu integrates exclusively at the not-yet-opened area of the replication fork, because under the growth conditions

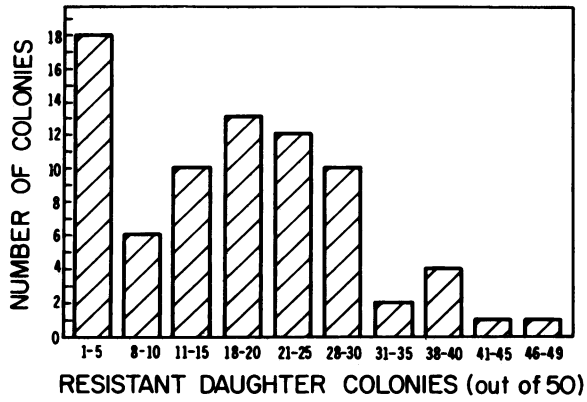

FIG. A1. Composition of mixed colonies resulting from Mu infection at 50 min after return to 31°C (compliation of three experiments).

used (doubling time of 60 min in M9 with 10 μg of thymine per ml), there is no more than one ongoing replication fork per cell at a time (11, 26).

Infection of strain A3 at different times after the shift back to the permissive temperature (Fig. 3) was aimed at discriminating between the two other possibilities (Fig. 2). The fraction of uniform colonies was found to decrease with the time of infection (Table 1), as was expected if integration is random. Cell divisions in the culture began about 75 min after the shift back to 31°C (Fig. 3). Some of the cells that had been infected at 70 min might have dividied before spreading, and this factor can account for the slightly higher portion of uniform colonies relative to that in the 50-min-infected cells (Table 1). These results are predicted by random integration and cannot be explained by preferred integration at replication forks for the following reasons. (i) Integration at one of the arms at the replication fork would result almost exclusively in the appearance of mixed colonies. (ii) Integration at both prewound and wound areas of a replication fork would also give rise to both mixed and uniform colonies, but in this case a constant ratio would be expected between the two types of colonies, independent of infection time. (iii) Dependence on the time of infection, as found here, could also result from integration at a prewound area near the replication fork if there were additional initiations, but this possibility was eliminated when mixed colonies were found upon infection of CR34 under conditions in which no more than one replication cycle per cell existed at a time.

The results in both our systems were found to be concordant and in our opinion preclude the hypothesis that lysogenic integrations of Mu occur with high preference at the replication fork. This is an extension of a similar conclusion (19) for secondary Mu transpositions during lytic development and for first integration of an infecting Mu, which develops mostly toward the lytic pathway.

APPENDIX

The distribution of the relative fraction of ampicillin-resistant bacteria in 77 mixed colonies developed after infection at 50 min in three experiments (including the one whose results are presented in Table 1) is shown in Fig. A1. In the absence of any other influence, a mixed colony should contain, on the average, 50% ampicillin-resistant bacteria. A close-to-normal distribution was observed (Fig. A1), with a mean slightly lower than 50%, along with an additional peak at a very low fraction of ampicillin-resistant bacteria.

The following explanations can account for these observations: (i) Growth rate may have decreased in some lysogens. The presence of a prophage has been reported to alter the growth rate of lysogenic bacteria in chemostat cultures (7, 16), the change being dependent on the growth conditions (16). (ii) Late lysogenic integrations of Mu after one or more cell divisions would have reduced the fraction of lysogens in mixed colonies. (iii) Additional initiations of chromosome replication may have occurred. After pretreatment, some A3 cells will begin a second round of chromosome replication before termination of the first synchronous cycle. Integration to a segment which had replicated more than once would also reduce the fraction of lysogens. The phenomenon described here is only a by-product of the present investigation.

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