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Host DNA Replication Forks Are Not Preferred Targets for Bacteriophage Mu Transposition

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Bacteriophage Mu DNA integration in *Escherichia coli* strains infected after alignment of chromosomal replication was analyzed by a sandwich hybridization assay. The results indicated that Mu integrated into chromosomal segments at various distances from *oriC* with similar kinetics. In an extension of these studies, various Hfr strains were infected after alignment of chromosomal replication, and Mu transposition was shut down early after infection. The positions of integrated Mu copies were inferred from the transfer kinetics of Mu to an F^- strain. Our analysis indicated that the location of Mu DNA in the host chromosome was not dependent on the positions of host replication forks at the time of infection. However, the procedure for aligning chromosomal replication affected DNA transfer by various Hfr strains differently, and this effect could account for prior results suggesting preferential integration of Mu at host replication forks.

Bacteriophage Mu has been extensively studied in an effort to elucidate the molecular mechanism of transposition. The ability of Mu to transpose to random locations on the host chromosome is essential for both lysogenization and replication (3, 7, 28, 32). Its ability to transpose at high frequencies (as many as 100 times per cell during the course of lytic development, typically 45 to 60 min in duration) makes it amenable to many biochemical and genetic studies not possible with nonviral transposable elements, which transpose at frequencies of 10^{-4} to 10^{-7} per generation.

The hypothesis that Mu preferentially integrates at host replication forks (10, 26, 27) proposes a defined target for Mu integration. Replication forks may provide DNA structures, such as single-stranded and nicked duplex DNA, or functional complexes of host replication proteins that are necessary for the transposition reaction. Assays to determine the chromosomal positions of integrated Mu indicated that it is concentrated at sites of replication forks during infection (10, 26, 27). Paolozzi et al. (26, 27) assayed Mu integration by scoring inactivation of genetic activities among cells that survived infection. Fitts and Taylor (10) assayed Mu integration by monitoring transfer kinetics of Mu from infected Hfr cells.

One specific problem with the Fitts and Taylor procedure (10) is that Mu continues to transpose during mating of infected Hfr cells with F^- cells. Mu transfer kinetics reflect not only the positions of integrated Mu but also the increasing copies of Mu transposing during mating. Pato and Reich (28) have shown that Mu DNA synthesis requires ongoing synthesis of the Mu A gene product and that inhibition of protein synthesis shuts down Mu DNA synthesis with a half-life of 3 min. Therefore, we modified the experimental procedure of Fitts and Taylor (10) so that Mu transposition was shut down with chloramphenicol before mating. In addition, we used a more direct hybridization assay for Mu integration. We present evidence that host replication forks are not preferred targets for Mu integration.

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MATERIALS AND METHODS

Bacterial and bacteriophage strains. All bacterial strains used in this work were derivatives of *Escherichia coli* K-12 except *Proteus mirabilis* AT3557 (Table 1). Mu *c*ts62 p Apl (14; obtained from N. Symonds) was grown by heat induction of strain AB259 lysogenized with this phage. Mu *c*ts62 (*P. mirabilis*) (phage grown in *P. mirabilis*) and Mu *c*ts62 *B*1066 p Apl were grown by heat induction of strains AT3557 and AT3854, respectively.

Plasmids. Strain MG1043 containing pOX38, which consists of the largest *Hin*dIII fragment of F DNA, was obtained from M. S. Guyer (11). Bacterial strains (JA200) transformed with the plasmids pLC44-7, pLC20-30, pLC14-29, and pLC44-11 from the Clarke and Carbon (5) collection of hybrid plasmids were obtained from B. Bachmann (*E. coli* Genetic Stock Center, Yale University). The plasmids contained the following *E. coli* genes: pLC44-7, *ilvGEDAYC*, *hemD*, *rep*, *rho*, *gpp*, *bfm*, and *cya*; pLC20-30, *lacIYZ*; pLC14-29, *his* and *argS*; and pLC44-11, *gpt*, *phoE*, and *proAB* (24). pSPE*oriC* was obtained from P. Kuempel. It is a pBR322 plasmid with a 2-kilobase (kb) *E. coli* DNA insert (a *Pst*I fragment) containing *oriC* at the unique *Pst*I site (P. Kuempel, personal communication).

Media. Bacteria were cultured in L broth (LB) and M9 minimal medium (23). When required, M9 medium was supplemented with histidine, proline, and arginine at concentrations of 40, 167, and 145 μg/ml, respectively.

Amino acid starvation, nalidixic acid treatment, Mu infection, and sampling of host cells for DNA extraction. Auxotrophic bacterial cells, grown to 10^8 cells per ml in M9 medium supplemented with required amino acids, were collected by low-speed centrifugation or filtration through a membrane filter (Amicon Corp., 0.45-μm pore size), washed with and suspended at 10^8 cells per ml in M9 containing no amino acids, and starved by incubation at 37°C for 200 min with vigorous aeration. Cells were collected for immediate infection or nalidixic acid treatment, in which the culture at 10^8 cells per ml in LB containing 30 μg of nalidixic acid per ml was incubated at 37°C for 30 min with vigorous aeration. Nalidixic acid-treated cells were washed with 0.85% (wt/vol) NaCl before infection.

Cells were suspended at 10^8 cells per ml in LB containing

TABLE 1. Bacterial strains

Strain no.	Relevant characteristics[a]	Source
AB259	Hfr H *thi-1*	Laboratory strain
AT765	*lacI*::Mu *proC29 his rpsL metB1*	Laboratory strain
AT3557	(RP4::Mu cts62) *P. mirabilis thy*	Laboratory strain
AT3653	Hfr H Su⁻ *his-4 thi-1 relA1*	Laboratory strain (10, 16)
AT3751	Hfr KL16 Su⁻ *his-4 thi-1 relA1*	Laboratory strain (10, 16)
AT3752	Hfr KL228 Su⁻ *his-4*	Laboratory strain (10, 16)
AT3755	Hfr KL25 Su⁻ *his-4*	Laboratory strain (10, 16)
AT3757	Hfr H Su⁻ *his-4 thi-1 relA1 pdxA1*::Mu	Laboratory strain (10)
AT3848	Hfr KL16 (Mu p Ap1) *his-4 thi-1 relA1*	This work
AT3851	Hfr KL25 *his-4 zjj-205*::Tn*10 hsdR4*	This work
AT3854	*pro mel-1 supF* (Mu cts62 Bam1066 p Ap1)	This work
AT3855	*proA2 lacY1 tsx-29 gal-2 his-4 xyl-5 mtl-1 argE3 thi-1 hsdR4 zjj-205*::Tn*10*	This work
AT3965	Mu^r (pHN105) *thr-16 lysA36 purE41 his-53 metC65 ilv-277 rpsL97 xyl-14 lac-29 tsx-63 proC24 pyrF30 gyrA266*	This work
AT3973	Hfr KL16 Su⁻ *his-4 thi-1 relA1 hsdR4 zjj-205*::Tn*10*	This work
AT3989	Hfr KL25 *his-4* (Mu p Ap1)	This work
AT5009	Hfr KL228 Su⁻ *his-4 zhj-429*::Tn*10*	This work
AT5012	Hfr KL208 Su⁻ *his-4 zcf-117*::Tn*10*	This work
AT5013	Hfr OR21 Su⁻ *his-4 zbe-280*::Tn*10*	This work
JA200	*thr-1 leuB6 trpE63 recA56 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 supE44*	Clarke and Carbon (5)[b]
MG1043	*thyA747*(pOX38)	M. S. Guyer (11)

[a] Most genetic symbols are defined in reference 2. Genetic symbols for phage Mu are described in reference 34; *zhj*, *zcf*, *zjj*, and *zbe* are locations on the chromosome with no known genetic activity. Su⁻, Nonsuppressing; am, amber mutations.

[b] Obtained from Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University).

5 mM $CaCl_2$ for infection with Mu at a multiplicity of 3 PFU per cell. The infection was allowed to proceed with gentle aeration at 37°C. The cultures were sampled at various times by chilling portions of them on ice and adding 10 mM NaCN and 100 μg of nalidixic acid per ml.

DNA purification. Total DNA from whole *E. coli* cells was purified by the procedure of Kuempel (13). Whole cells were lysed by treatment with lysozyme followed by incubation for at least 1 h at 50°C in 1% (wt/vol) sodium dodecyl sulfate or sarcosyl (NL-30, ICN Pharmaceuticals Inc.) and 100 μg of proteinase K (E. Merck AG) per ml. The DNA was purified by digestion with RNase A and RNase T1 (Sigma Chemical Co.), extraction with phenol-chloroform (equal volumes of water-saturated, distilled phenol and chloroform), and repeated ethanol precipitations. The average single-strand length of DNA purified by this procedure was approximately 50 kb, as determined by alkaline agarose gel electrophoresis (22). For the two-probe Southern blot analysis described below, cells were lysed as described above, and DNA was purified from extracts by CsCl-ethidium bromide centrifugation (30).

All plasmids used in this work (except pOX38 DNA) were purified as follows. They were amplified by the addition of uridine and chloramphenicol to cultures of plasmid-bearing strains as described by Norgard et al. (25). Cleared lysates of these cultures were prepared by the procedure of Clewell and Helinski (6), and supercoiled DNA was purified twice by CsCl-ethidium bromide density centrifugation (30). pOX38 DNA was purified from strain MG1043 as described previously (35) by a modified procedure of Hirt (12); the supercoiled DNA was purified twice by CsCl-ethidium bromide density centrifugation (30).

Preparation of ^{32}P-labeled DNA probes. DNA probes were labeled with [α-^{32}P]dCTP (670 Ci/mmol, New England Nuclear Corp.) by the nick translation procedure of Rigby et al. (29) to a specific activity of 1×10^8 to 2×10^8 cpm/μg and purified by column chromatography on Sephadex G-50 (Pharmacia, Inc.).

Sandwich hybridization assay. The overall scheme for the sandwich hybridization procedure is depicted in Fig. 1. The assay was performed with Mu DNA (from phage grown in *P. mirabilis*) bound to disks of diazobenzyloxymethyl (DBM)-paper (7-mm diameter), which was prepared by the procedure of Alwine et al. (1). Each reaction (120 μl) of hybridization step 1 contained 100 μM triethanolamine hydrochloride (pH 7.5), 2× SSPE (1× SSPE is 10 mM sodium phosphate [pH 7.0], 0.18 M NaCl, 1 mM disodium EDTA), 2× Denhardt solution (1× Denhardt is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin [9]), 50% (vol/vol) formamide (MCB Manufacturing Chemists, Inc.; deionized by using Amberlite MB-1 ion-exchange resin), 200 μg of denatured salmon testes DNA (Sigma; sheared by passing DNA solution 10 times through a 26-gauge needle) per ml, 5 μg of Mu DNA (*P. mirabilis*) bound to a single DBM disk, and 1 or 2 μg of denatured sample DNA which was to be analyzed for integrated copies of Mu. DNA was denatured by being heated in a boiling-water bath

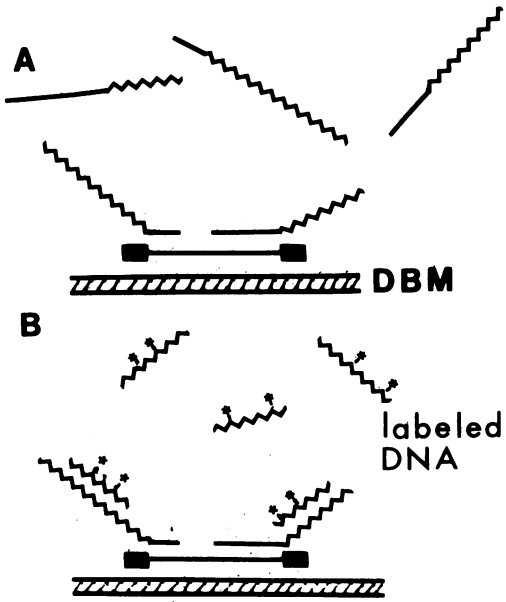

FIG. 1. Scheme for the sandwich hybridization assay. (A) Step 1: hybridization of chromosomal DNA containing integrated Mu DNA to Mu DNA (extracted from phage grown in *P. mirabilis*) bound to DBM-paper. (B) Step 2: hybridization to ^{32}P-labeled DNA probes to determine the amount of specific chromosomal sequences attached to Mu. Mu DNA, Straight line; *P. mirabilis* DNA, boldfaced line; *E. coli* DNA, jagged line.

for 5 min and quickly cooled in an ethanol-ice bath. The first hybridization reactions proceeded at 42°C for 16 h. The disks were washed three times with a 2× SSPE–0.1% sodium dodecyl sulfate solution at room temperature and three times with 0.1× SSPE–0.1% sodium dodecyl sulfate solution at 50°C. Each wash lasted 15 min.

Conditions for hybridization step 2 were based on the procedure for rapid hybridization with dextran sulfate developed by Wahl et al. (36). DBM disks were incubated for 4 h at 45°C in a prehybridization solution (5 ml per 16 to 20 disks), which contained 5× SSPE, 5× Denhardt solution, 200 μg of salmon testes DNA (sheared and denatured) per ml, and 50% formamide. Sets of 16 to 20 DBM disks to be probed with the same ^{32}P-labeled DNA were incubated at 45°C for 36 h in a common hybridization solution (5 ml), which contained the ingredients of the prehybridization solution plus 10% (wt/vol) dextran sulfate (molecular weight, 5×10^5; Sigma) and a denatured DNA probe (5×10^5 cpm/ml) at a specific activity of 5×10^7 to 10×10^7 cpm/μg. Disks were washed as described above, dried, mounted, and exposed to X-ray film (Kodak X-Omat AR) at −70°C by using an intensifying screen (Dupont Cronex Lightning-Plus), and the amount of ^{32}P bound to each disk was quantitated from densitometer tracings.

Two-probe Southern blot analysis. An assay to compare the abundance of chromosomal sequences in two distinct segments of the *E. coli* chromosome was used to confirm alignment of chromosomal DNA replication of cultures after amino acid starvation and nalidixic acid treatment. Chromosomal DNA samples were digested with *Hin*dIII, resolved on neutral 0.7% agarose gels (22), and blotted onto nitrocellulose as described by Southern (31; 10× SSPE blotting buffer) from gels treated with 0.25 M HCl (36). Each blot was subjected to hybridization as described by Wahl et al. (36) with two ^{32}P-labeled DNA probes (3×10^5 cpm/ml of each probe in the hybridization solution).

Densitometer tracings. The grain absorbance of X-ray films was determined by using a densitometer (E-C Apparatus Corp.) and an integrating chart recorder (Cole-Palmer Instrument Co.).

Determination of the sites of integrated Mu DNA in Hfr strains. We monitored ampicillin resistance (Amp^r) transfer from Hfr strains infected with Mu *c*ts62 p Ap or Mu *c*ts62 *B*1066 p Ap to F$^-$ strain AT3965 (resistant to Mu, nalidixic acid, and chloramphenicol and immune to Mu). Infected cells were treated with chloramphenicol before mating. Tetracycline resistance (Tet^r) transfer was in some cases monitored simultaneously from host strains with Tn*10* insertions to measure the efficiency of Hfr transfer. Alternatively, Amp^r transfer was monitored from Hfr strains lysogenic for Mu p Ap to compare the transfer efficiencies of Hfr strains treated with chloramphenicol before mating.

Before infection or mating, cultures of Hfr cells subjected to amino acid starvation and nalidixic acid treatment were incubated in LB (10 ml at 10^8 cells per ml) at 37°C with vigorous aeration for various times. Infection proceeded at 37°C for 10 min with gentle aeration. (Typically, 67% of infected viable cells produced infective centers when plated with an indicator culture.) Chloramphenicol (100 μg/ml) was added, and incubation at 37°C continued for 5 min. Cells were collected by filtration, washed with 100 ml of Davis minimal medium salts (Difco Laboratories) solution containing 100 μg of chloramphenicol per ml, and resuspended in 10 ml of LB containing 100 μg of chloramphenicol per ml. A 5-ml portion of this culture was mixed with 5 ml of strain AT3965 culture (grown to 1.4×10^8 cells per ml in LB containing 12.5 μg of chloramphenicol per ml and adjusted to contain 100 μg of chloramphenicol per ml just before mating). Samples (0.5 ml) were taken from the mating mixtures at various times, and mating was interrupted as described by Low and Wood (17). Diluted and undiluted mating mixtures (diluent: Davis salts solution plus 30 μg of nalidixic acid per ml) were plated onto LB plates containing 30 μg of nalidixic acid, 12.5 μg of chloramphenicol, and either 25 μg of ampicillin or 15 μg of tetracycline per ml, and CFU were scored after 24 h of incubation at 37°C. These plates were prepared 1 day before use.

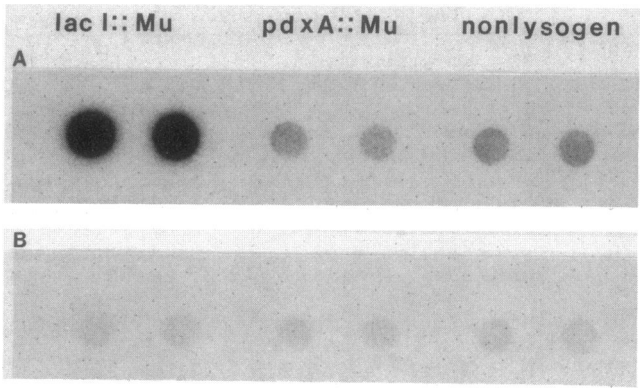

FIG. 2. Sandwich hybridization assay. DNA samples (1 μg per reaction) from strains AT765 (*lacI*::Mu), AT3757 (*pdxA*::Mu), and AB259 (nonlysogen) were incubated with Mu DNA (*P. mirabilis*) bound to DBM disks in hybridization step 1. ^{32}P-labeled pLC20-30 (*lac*) (A) or pLC44-11 (*proAB*) (B) probes were used in hybridization step 2.

Construction of pHN105. The recipient strain AT3965 used in the Hfr transfer analysis contained the plasmid pHN105, which conferred Mu immunity and resistance to chloramphenicol. It was constructed by inserting the left *Hin*dIII fragment of Mu DNA, which encodes the immunity repressor *c* (37), at the unique *Hin*dIII site of plasmid pACYC184 (4) by blunt-end ligation (19). The left *Hin*dIII fragment of Mu and *Hin*dIII-digested pACYC184 were treated with the Klenow fragment of *E. coli* DNA polymerase I (Bethesda Research Laboratories, Inc.) before ligation with T4 DNA ligase (Bethesda Research Laboratories).

RESULTS

Sandwich hybridization assay. To test whether host replication forks are preferred targets for Mu integration during lytic development, we used a sandwich hybridization assay to measure the kinetics of Mu integration into discrete segments of the host chromosome (Fig. 1). The results show that this hybridization assay may distinguish Mu DNA at different chromosomal positions (Fig. 2). Chromosomal DNA extracted from lysogens with prophages at *lacI* and *pdxA* (AT765 and AT3757, respectively; Fig. 3) and from a nonlysogen (AB259) was subjected to hybridization step 1, and ^{32}P-labeled pLC20-30 (*lac*) and pCL44-11 (*proAB*) DNA probes were used in hybridization step 2. A strong signal was produced by the pLC20-30 probe with sample DNA from the *lacI*::Mu lysogen. Only background signals (less than 2% of the signal produced with DNA from the *lacI*::Mu lysogen) were produced by the pLC20-30 probe with DNA from the *pdxA*::Mu lysogen and the nonlysogen and by the pLC44-11 probe with DNA from both lysogens and the nonlysogen. This indicated that chromosomal DNA (average single-strand length, 50 kb) was sufficiently sheared by extraction

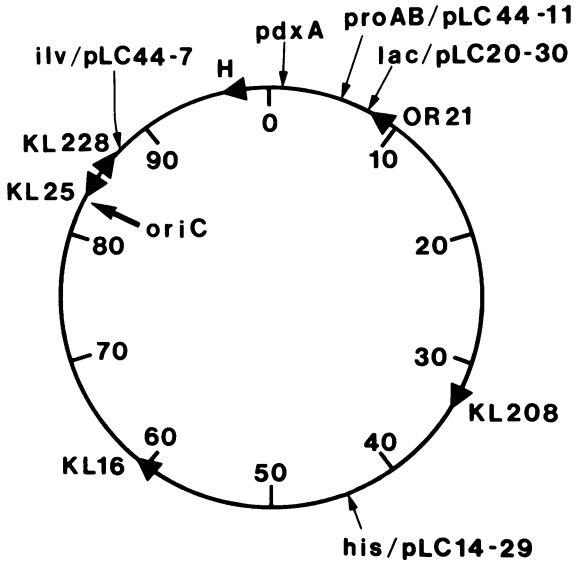

FIG. 3. Map of the *E. coli* chromosome. The positions of chromosomal segments represented by plasmids pLC44-7, pLC44-11, pLC20-30, and pLC14-29 and of Hfr transfer origins are shown (2, 16, 18).

and denaturation procedures to prevent any signal over background with *lacI*::Mu DNA and the pLC44-11 probe (*proAB* is approximately 2 min or 90 kb from *lac* on the *E. coli* map [2]).

Analysis of Mu transposition after infection of an exponentially growing cell population. AT3855 (a restrictionless strain), growing exponentially in LB, was infected with Mu cts62 (*P. mirabilis*). DNA sampled at various times postinfection was analyzed by the sandwich hybridization assay, with ^{32}P-labeled pLC44-7 (*ilv*), pLC20-30 (*lac*), and pLC14-29 (*his*) as probes (Fig. 4). Clearly, Mu transposed to all three parts of the chromosome (Fig. 3) during lytic development. The larger chromosomal DNA insert in pLC44-7 compared with pLC14-29 (15 versus 10 kb) or the higher abundance of *ilv* compared with *his* in exponentially growing cells may have contributed to differences in the slopes of the signals. The chromosomal DNA insert in pLC20-30 (25 kb) was approximately twice the length of inserts in pLC44-7 and pLC14-29, but as a probe it produced a maximum signal (at 36 min postinfection) that was 5 to 10 times the signals produced by pLC44-7 and pLC14-29. Possibly, pLC20-30 contains DNA sequences that are hot spots for Mu integration.

Analysis of Mu transposition in strain AT3855 infected after amino acid starvation. A culture of strain AT3855 was aligned for host chromosomal replication by amino acid starvation. Cells were released from starvation by being suspended in LB and immediately infected with Mu cts62 (*P. mirabilis*). DNA sampled at various times postinfection was analyzed by the sandwich hybridization assay. ^{32}P-labeled pLC44-7, pLC20-30, pLC14-29, pLC44-11 (*proAB*, 13-kb chromosomal DNA insert), and total *E. coli* DNA were used in hybridization step 2 (Fig. 5). The signals produced by pLC44-7, pLC14-29, and pLC44-11 were nearly identical for each DNA sample; however, the signals produced by pLC20-30 were again high (data not shown), as indicated above (Fig. 4). The results indicate that the kinetics of Mu integration into all four regions of the chromosome (Fig. 3) paralleled the kinetics of Mu integration into the entire chromosome. A host replication fork would require at least 1, 18, 19, and 32 min after initiation at *oriC* to replicate regions of the chromosome represented by the pLC44-7, pLC44-11, pLC20-30, and pLC14-29 probes, respectively. If replication forks provide preferred targets for integration, Mu integration into a particular segment of the chromosome should be stimulated after sufficient time has elapsed for it to be replicated by a replication fork. Instead, the results indicate Mu accumulated with similar kinetics into all parts of the chromosome.

Mu transposition in auxotrophic Hfr strains aligned for chromosomal replication by amino acid starvation and nalidixic acid treatment. It was possible that the results of Fitts and Taylor (10) reflected early Mu integration events, which took place at host replication forks, whereas analysis of the transposition events described above predominantly detected the bulk of Mu integration events which did not take place at replication forks. We therefore focused on early Mu transposition events after infection.

Cultures of strains AT3851 (Hfr KL25) and AT3973 (Hfr KL16) (Fig. 3) were subjected to amino acid starvation and nalidixic acid treatment (the method used by Fitts and

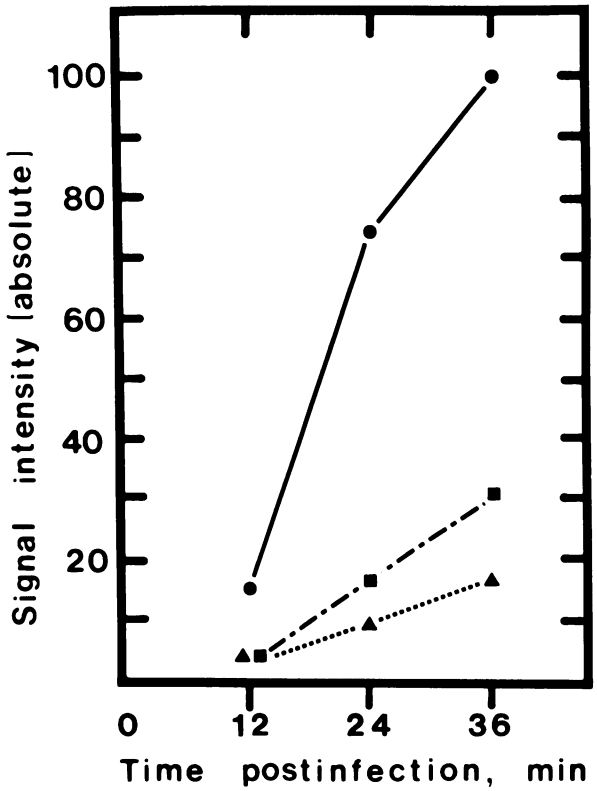

FIG. 4. Mu transposition after infection of exponentially growing AT3855 cells. DNA samples (1 μg per reaction) from AT3855 cells were analyzed by the sandwich hybridization assay. ^{32}P-labeled pLC20-30 (*lac*) (●), pLC44-7 (*ilv*) (■), or pLC14-29 (*his*) (▲) probes were used in hybridization step 2. The background signal produced by each probe with the DNA sample at 0 min postinfection was subtracted from all signals produced by that probe. The background signals for the pLC14-29, pLC44-7, and pLC20-30 probes were approximately 8, 8, and 1%, respectively, of the corresponding signals produced at 36 min postinfection. The signal produced by the pLC20-30 probe at 36 min postinfection was set at a value of 100, and signals produced by all probes were set relative to this value.

Taylor [10] to align chromosomal replication) and infected with Mu cts62 (*P. mirabilis*) immediately after nalidixic acid treatment. The sandwich hybridization analysis (Fig. 6) indicated that Mu has integrated into F DNA in Hfr KL16 at 10 and 15 min after release from nalidixic acid treatment, although a replication fork initiating at *oriC* would require at least 18 min to reach the site of F insertion. The greater amount of Mu transposing onto F in Hfr KL25 compared with Hfr KL16 was proportionate to the greater amount of Mu transposing onto the entire chromosome. The results indicate that the proximity of F to *oriC* in Hfr KL25 compared with Hfr KL16 did not make F any more of a preferred target for Mu integration.

Amp^r transfer from Hfr strains infected with Mu cts62 p Ap after amino acid starvation and nalidixic acid treatment. We modified the Fitts and Taylor procedure (10) so that Hfr cells were infected for 10 min and then treated with chloramphenicol before mating. We determined that Hfr cells treated for 5 min with 100 μg of chloramphenicol per ml were still able to transfer chromosomal sequences; however, Hfr cells infected with Mu in the presence of chloramphenicol (cells which formed infective centers when released from chloramphenicol) were unable to transfer Mu DNA (data not shown).

Strains AT3755 (Hfr KL25), AT3752 (Hfr KL228), AT3653 (Hfr H), and AT3751 (Hfr KL16) were subjected to amino acid starvation and nalidixic acid treatment and immediately infected with Mu cts62 p Ap. The infection was allowed to proceed for 10 min before chloramphenicol treatment. Mating was with F$^-$ strain AT3965 to monitor Amp^r transfer. The results (Fig. 7) indicated that the closer F was inserted to *oriC* in the Hfr strain (Fig. 3), the more efficient was the transfer of Amp^r. When the Hfr KL16 strain was incubated for 20 min in LB before infection with Mu

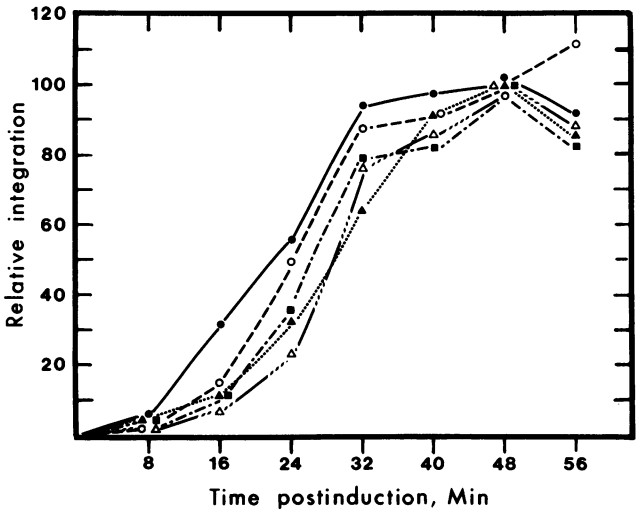

FIG. 5. Mu transposition in AT3855 cells infected after amino acid starvation. DNA samples (1 μg per reaction) from infected AT3855 cells were analyzed by the sandwich hybridization assay. ^{32}P-labeled pLC44-7 (▲), pLC20-30 (○), pLC14-29 (■), pLC44-11 (△), and total *E. coli* DNA (●) probes were used in hybridization step 2. The absolute signal intensities on autoradiograms were normalized to the signal produced by each probe at 48 min postinfection. The signals at 48 min produced by pLC44-7, pLC14-29, and pLC44-11 were virtually identical. The signals produced by pLC20-30 and the *E. coli* DNA probe were approximately fivefold greater.

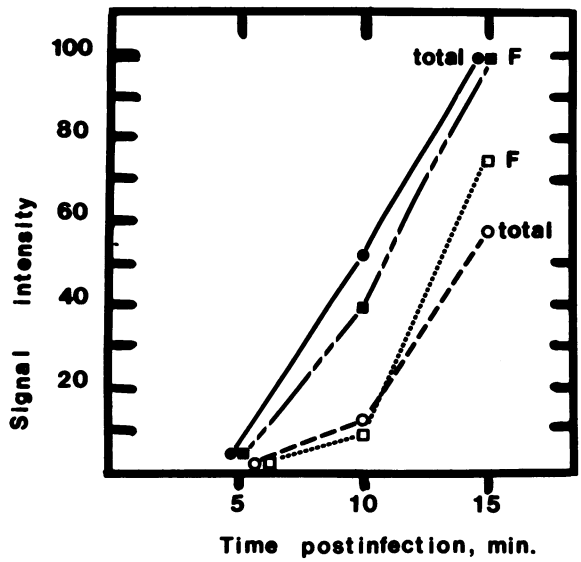

FIG. 6. Mu transposition in strain AT3851 (Hfr KL25) and AT3973 (Hfr KL16) cells infected after amino acid starvation and nalidixic acid treatment. DNA samples (2 μg per reaction) from infected AT3851 (●, ■) and AT3973 (○, □) cells were analyzed by the sandwich hybridization assay. ^{32}P-labeled pOX38 DNA (F specific; ■, □) and total *E. coli* DNA (AB259 DNA; ●, ○) probes were used in hybridization step 2. The signals at 15 min with DNA from infected AT3851 were set at 100 for each probe. The background signals (at 0 min postinfection), which were substracted, were approximately 15% of these signals.

(incubation time that would allow host replication forks to reach the transfer origin), Amp^r transfer was stimulated 10-fold to a level similar to that of Hfr KL228 and Hfr KL25. These results were consistent with those of Fitts and Taylor (10).

As controls, cultures of AT3989 and AT3848 (Hfr strains KL25 and KL16, respectively, bearing Mu p Ap prophages which are transferred early) were subjected to amino acid starvation and nalidixic acid treatment and incubated in LB for 10 or 30 min before chloramphenicol treatment and mating with AT3965 cells. Exponentially growing cultures of AT3989 and AT3848 (10^8 cells per ml in LB) were also treated with chloramphenicol and mated with AT3965 cells. Amp^r transfer from Hfr KL16 was stimulated 10-fold with an additional 20 min of incubation after nalidixic acid treatment (Fig. 8). This indicates that the 10-fold increase in Amp^r transfer by the KL16 strain (Fig. 7) was the result not of an increased number of Mu being integrated near the transfer origin but of an increased efficiency of Hfr DNA transfer.

Although Hfr KL16 cells incubated 10 min after alignment of chromosomal replication transferred Amp^r with slightly less efficiency than the nonaligned cells, a 10-min incubation of aligned KL25 cells was sufficient to increase Amp^r transfer efficiency 2- to 5-fold over nonaligned cells; a 10-fold increase was attained with 30 min of incubation. These results suggest that the closer F is inserted to *oriC*, the shorter the incubation period necessary to stimulate DNA transfer by aligned cells. Such a phenomenon could account for different efficiencies of Amp^r transfer by the various Hfr strains shown in Fig. 7.

To account for the different efficiencies of Hfr transfer, the analysis was repeated with strains with Tn*10* insertions (4 to 7 map units away from the transfer origins) that are transferred early. Strains AT5009 (Hfr KL228, Tn*10* at 80 min on

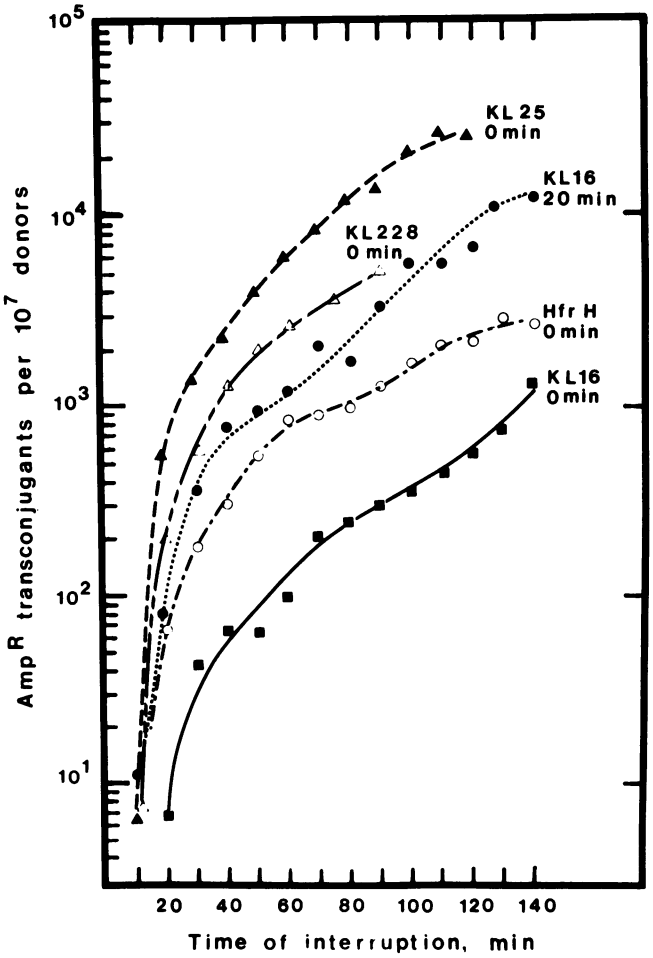

FIG. 7. Kinetics of Mu transfer from Hfr strains infected after amino acid starvation and nalidixic acid treatment. Hfr cells were infected with Mu cts62 p Ap after alignment of chromosomal replication, treated with chloramphenicol, and mated with strain AT3965 cells to monitor Amp^r transfer. 0 min, Hfr cells infected immediately after nalidixic acid treatment; 20 min, Hfr KL16 incubated for 20 min in LB before infection. Host strains: Hfr KL25 (▲), AT3755; Hfr KL228 (△), AT3752; Hfr H (○), AT3653; and Hfr KL16 (■, ●), AT3751.

the *E. coli* map), AT5013 (Hfr OR21, Tn*10* at 14 min), and AT5012 (Hfr KL208, Tn*10* at 25 min) (B. Bachmann, personal communication) were infected after alignment of DNA replication, and Amp^r and Tet^r transfer to AT3965 was simultaneously monitored after chloramphenicol treatment (Fig. 9). No delay of Amp^r transfer with respect to Tet^r transfer was observed even with strain AT5012, which has a transfer origin close to the terminus of chromosomal replication. Moreover, incubation of strain AT5013 for 20 min to allow replication forks to proceed near the transfer origin before infection stimulated both Tet^r and Amp^r transfer approximately 10-fold, confirming that the increased amount of Mu transfer is a simple consequence of increased Hfr transfer efficiency. The results were virtually identical when aligned Hfr strains were infected with Mu cts62 *B*1066 p Ap (data not shown). A second round of transposition by this phage is highly improbable because of the low frequency of transposition in the absence of the *B* gene product (34). Although the reduced efficiency of transposition was reflected by a 10-fold reduced level of Amp^r transfer, we observed no delay of Amp^r transfer from strains AT5013 and AT5012 infected immediately after nalidixic acid treatment. These results indicated that the sites for Mu integration, including the first integration event, were not dependent on the position of host replication forks at the time of infection.

Verification that amino acid starvation and nalidixic acid treatment aligns chromosomal DNA replication in Hfr cells. We used a Southern blot analysis to verify that chromosomal replication initiates at *oriC* and not at the site of F insertion. *Hin*dIII-digested DNA samples from strain AT5012 (Hfr KL208) cells sampled after amino acid starvation and at various times after nalidixic acid treatment were resolved by neutral agarose electrophoresis and blotted on nitrocellulose. Each of two duplicate blots was incubated with two probes: ^{32}P-labeled pSPE*oriC* or pOX38 (F DNA) and pLC24-29 (*his*). Densitometer tracings were used to determine the ratio of signals of *oriC*- and F-specific bands versus *his*-specific bands. At 10 and 30 min after nalidixic acid treatment, the *oriC*/*his* ratio increased to 1.85, indicating that *oriC* sequences were being amplified (Table 2). On the other hand, the F/*his* ratio did not go above 1.00. These results indicated that host chromosomal replication preferentially if not exclusively initiates at *oriC* after amino acid starvation and nalidixic acid treatment.

DISCUSSION

The sandwich hybridization assay was used to monitor the relative kinetics of Mu integration into various parts of the host chromosome during lytic development. When host cells were infected with Mu immediately after alignment of chromosomal replication, integrated copies of Mu DNA ac-

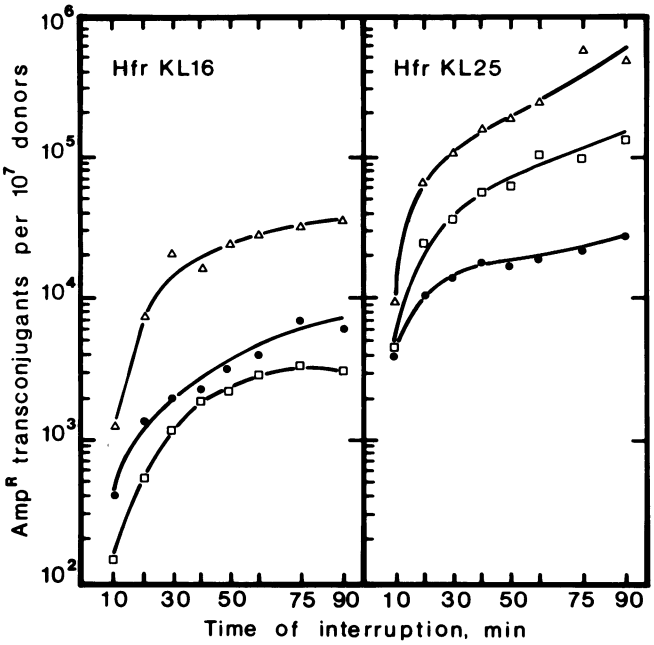

FIG. 8. Kinetics of Mu transfer from lysogenic Hfr strains. Strain AT3848 (Hfr KL16) and AT3989 (Hfr KL25) cells growing exponentially in LB were treated with chloramphenicol and mated with AT3965 cells (●). AT3848 and AT3989 cells subjected to amino acid starvation and nalidixic acid treatment were incubated for 10 (□) or 30 (△) min before treatment with chloramphenicol and mating with AT3965 cells. Amp^r transfer was monitored.

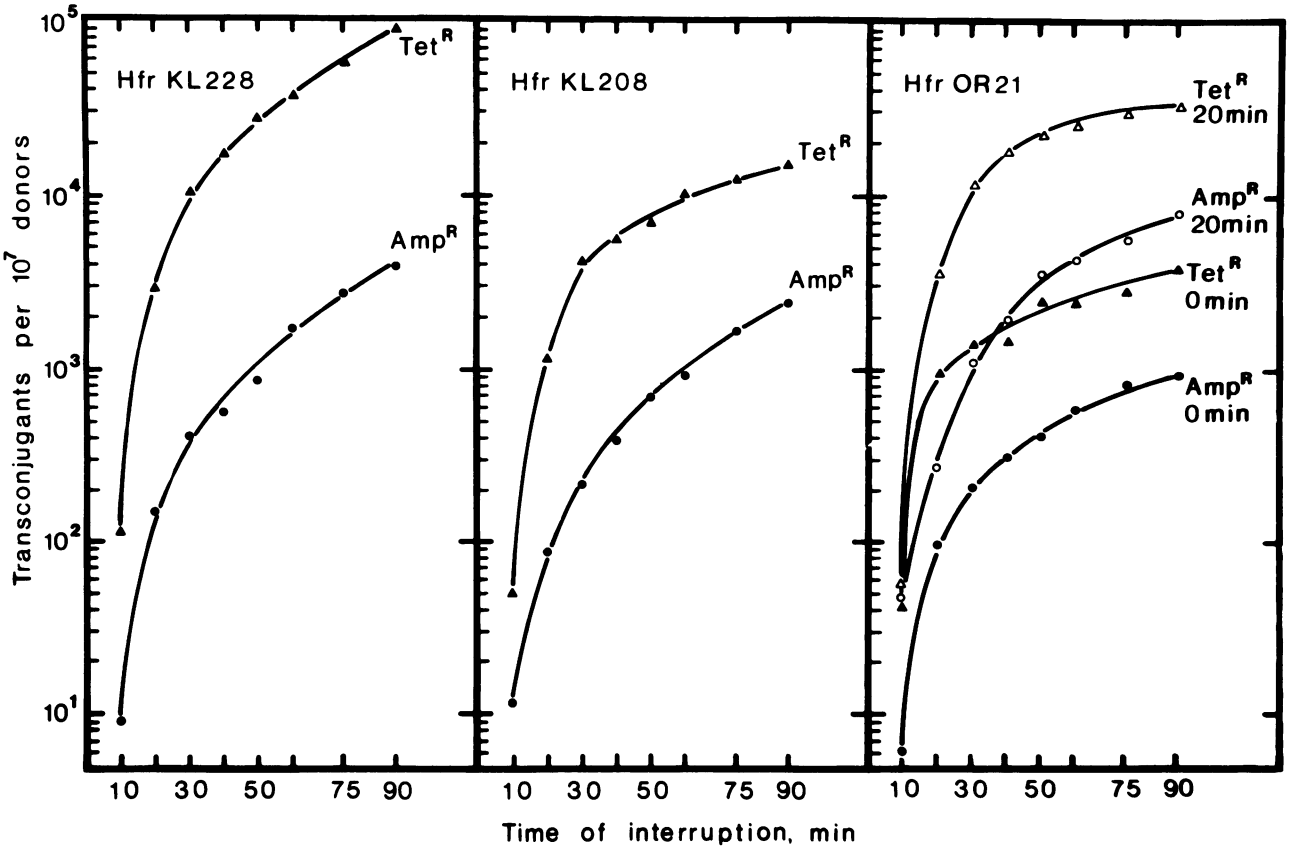

FIG. 9. Transfer kinetics of Mu and a chromosomal marker from Hfr cells infected after amino acid starvation and nalidixic acid treatment. Strain AT5009 (Hfr KL228), AT5012 (Hfr KL208), and AT5013 (Hfr OR21) cells were infected with Mu *c*ts62 p Ap after alignment of chromosomal replication, treated with chloramphenicol, and mated with AT3965 cells to simultaneously monitor Amp^r (●, ○) and Tet^r (▲, △) transfer. Aligned cells were infected after 0 (▲, ●) or 20 (△, ○) min of incubation in LB.

cumulated into all parts of the host chromosome with similar kinetics.

Our revised version of the Fitts and Taylor (10) Hfr transfer analysis indicated that the transfer efficiencies of Hfr cells were increased during recovery after amino acid starvation and nalidixic acid treatment. Our results indicated that the recovery time necessary for stimulation may vary among Hfr strains and suggested that strains with F inserted near *oriC* may in general require a shorter time for stimulation than do strains with F inserted farther away. Any variation of Mu transfer by Hfr strains infected after alignment of chromosomal replication was shown to be the consequence of variation in Hfr transfer efficiency. Because of current thinking that the first Mu transposition event after infection may be distinct from the ensuing transposition events during lytic development (34), we extended our analysis with Mu *c*ts62 *B*1066, which is unlikely to transpose more than once after infection. The results were analogous to those obtained with B^+ phage.

When early Mu transposition events to F in Hfr strains KL25 and KL16 were analyzed by the sandwich hybridization assay (Fig. 6), the rate of transposition into F was apparently higher in KL25, in which F is inserted very close to *oriC*, but the corresponding control experiment showed that this higher level of transposition into F simply reflected the higher level of transposition into the entire chromosome. The greater efficiency of Mu transposition in Hfr KL25 compared with KL16 may reflect differences such as the efficiency of phage absorption and other parameters that may affect the efficiency of Mu replication and transposition. This experiment, as well as the Hfr transfer analysis, exemplified the necessity for controls to monitor the vagaries of physiological parameters that may profoundly affect the quantitative results. Fitts and Taylor (10) erroneously assumed in the interpretation of their results equal efficiency of DNA transfer by various Hfr strains after alignment of chromosomal replication.

Paolozzi et al. (26, 27) have also presented evidence in support of the hypothesis that Mu preferentially integrates at

TABLE 2. Effect of amino acid starvation and nalidixic acid treatment on the abundance of *oriC*- and F-specific sequences in strain AT5012

Source of DNA sample	*oriC*/*his* ratio[a]	F/*his* ratio[a]
Amino acid starved[b]	1.00	1.00
Exponential[c]	3.05	0.833
Synchronous[d] (min)		
0	1.54	0.811
10	1.86	0.817
30	1.85	0.881

[a] The results were normalized by assigning the ratio of *oriC* and F versus the *his* signals a value of 1.00 for DNA from amino acid-starved cells, in which all chromosomal segments should be present in equal abundance (20).

[b] Cells subjected to amino acid starvation for 200 min.

[c] Cells growing exponentially in LB.

[d] Cells subjected to amino acid starvation and nalidixic acid treatment and incubated for the given amount of time in LB.

host replication forks. Because they monitored Mu integration by scoring mutations at various genetic loci, their results could reflect only Mu integration events that result in lysogenization, and these events may take place preferentially at host replication forks. However, it is unlikely that integration events that result in lysogenization evaded detection by our Hfr transfer analysis, for we were able to monitor integration of Mu *c*ts62 *B*1066 p Ap. Like Fitts and Taylor (10), Paolozzi et al. (26, 27) made assumptions in interpreting their quantitative results. For example, they estimated the extent to which gene dosage effects would quantitatively influence the incidence of Mu-induced mutations near *oriC* when cells were infected after nalidixic acid treatment (27). Moreover, they interpreted the mutations they scored to be the result of Mu insertions at the genetic loci of interest, but they did not report whether Mu prophages were actually linked to the mutations (26, 27). Daniell et al. (8) have demonstrated that Mu infection leads to a 100-fold increase in the frequency of *lac* mutations, of which 13% were deletions of the gene unlinked to the Mu prophage. Perhaps Mu deletes a genetic locus without directing transposition at that site, and the results of Paolozzi et al. (26, 27) may reflect mutations that are preferentially generated at host replication forks by this mechanism.

Other studies (15, 21, 33) have suggested that optimal Mu transposition is dependent on host-directed chromosomal replication. Some effects of host replication forks other than to provide targets for integration may optimize Mu growth. A cell with a replicating chromosome may have the optimal amount of enzymes necessary for Mu transposition. Moreover, replication of integrated Mu copies by host replication forks may be necessary for optimal Mu development. Understanding the physiological parameters that influence the rate of Mu DNA replication should provide insights into the characteristics of Mu transposition in vivo.

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