Conservative integration of bacteriophage Mu DNA into pBR322 plasmid

(lysogenization/transposable elements/DNA transformation)

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ABSTRACT   In order to clarify the first step in Mu integrative recombination, we have infected a bacterial strain harboring the plasmid pBR322 and isolated Mu DNA in a supercoiled form associated with this plasmid. These structures show an association of Mu with pBR322 without any preliminary replication.

Integrative recombination is a basic step in the life cycle of the temperate and mutator bacteriophage Mu. This phage, which has developed a system permitting random insertion into the host genome, is also a potent transposable element (1).

Mu insertion is accomplished by means of the viral extremities (*att* sequences) and gives rise to linear and nonpermutated integration within a given gene (2). The host *rec* system does not take part in the process (3), but there is a preferential integration within the host replication fork (4–6) and an active replication of bacterial DNA is essential (7).

It is not clearly established whether, upon infection, Mu DNA replicates before or after its integration into the host chromosome. We have investigated the nature of the first step in Mu integrative recombination in order to elucidate whether the infecting Mu DNA is conserved or not during integration.

The basic idea of the experiment is schematized in Fig. 1.

We have studied the insertion of Mu in pBR322 upon infection of a bacterial strain harboring this plasmid and analyzed the supercoiled structure obtained in CsCl density gradients.

The system was chosen for the following reasons: (*i*) the association between Mu and pBR322 gives supercoiled structures easy to separate from bacterial DNA; and (*ii*) the density of the plasmid resulting from the insertion of Mu in pBR322 is virtually the density of Mu because pBR322 is small compared to Mu (4 vs. 37 kilobase pairs) and does not appreciably modify the plasmid density in density labeling experiments.

The experiments reported here show that a portion of recoverable Mu DNA is indeed in a supercoiled structure physically joined to pBR322, but still in a nonreplicated form.

To show that the Mu–pBR322 supercoiled structure isolated after CsCl/EtdBr and CsCl density gradients, has a biological activity, we have demonstrated a simultaneous transformation for plasmid and viral markers.

The results obtained are strongly indicative that a conservative event is the first step in Mu integrative recombination upon infection.

MATERIALS AND METHODS

Bacterial and Phage Strains. *E. coli* strains W5445 F⁻ and HUC166, which is W5445 harboring pBR322 (ampicillin and tetracycline resistant), were provided by B. Niaudet. *E. coli* strain RS54(Mu *cts62*) was from M. Howe. Mu *c⁺* was from the Bade collection.

Labeled Molecules. [*methyl*-^{3}H]Thymidine and carrier-free ^{32}P$_i$ were from Amersham. [^{13}C]Glucose (90% ^{13}C atoms) and ^{15}NH$_4$Cl (99% ^{15}N atoms) were from Merck, Sharp & Dohme.

Antibiotics. Tetracycline (Pfizer) was used for selection of transformants at a concentration of 15 μg/ml.

Media. Mu buffer is described in ref. 8; M9 and LB are described in ref. 9. M9.H is M9 medium in which [^{12}C]glucose and ^{14}NH$_4$Cl are replaced by the respective heavy (H) precursors. M9.HT is M9.H supplemented with [*methyl*-^{3}H]thymidine (10 μg/ml, 2 μCi/ml; 1 Ci = 3.7 × 10^{10} becquerels).

Preparation of ^{32}P-Labeled Mu $c⁺$ Stocks on Plates. The standard lysate procedure was followed except for preparation of all agar media: first phosphate was removed from LB according to Bukhari and Ljungquist (8), then the medium was sterilized and supplemented with ^{32}P$_i$ at 50 μCi/ml. The lysate was collected after 6 hr at 37°C, centrifuged 15 min at 7,500 rpm in a Sorvall SS34 rotor to eliminate bacterial debris and agar, and then concentrated by centrifugation in a Beckman 40 rotor at 23,000 rpm for 2 hr.

The pellet was resuspended in 0.3 ml of Mu buffer and purified on a CsCl step gradient (8). The phage band was collected with a syringe and then dialyzed against three changes of Mu buffer (buffer to phage solution, 1,000:1). Phage titer of the stock after dialysis was 10^{12} plaque-forming units/ml, specific activity was 2 × 10^{-6} cpm per phage.

Mu Integration Experiments. A 5-ml culture of strain HUC166 was prelabeled in M9.HT medium for approximately 10 generations; thymine (200 μg/ml) was added to the medium to favor thymidine incorporation (7). The culture was then diluted 1:100 in 25 ml of fresh M9.HT medium supplemented with thymine at 200 μg/ml and grown to 2 × 10^8 cells per ml. Bacterial cells were centrifuged at 6,000 rpm for 10 min in a Sorvall SS34 rotor, washed twice, and resuspended in Mu buffer at the final concentration of 5 × 10^9 cells per ml.

This procedure was chosen to obtain a physiological "synchronization" of adsorption; in this condition bacterial replication, measured with 5-min pulses of [*methyl*-^{3}H]thymidine, slows down to about 10% of the initial rate of replication, but is not completely blocked (data not shown).

The bacterial culture at 5 × 10^9 cells per ml was then infected at a multiplicity of infection of 5 with 0.1 ml from a 2 × 10^{11} plaque-forming units/ml ^{32}P-labeled Mu stock. After 20 min at 37°C, the nonadsorbed phage was neutralized by anti-Mu serum (titer K = 3 per min). The infected bacteria were then centrifuged in a Sorvall, washed twice in Mu buffer, and re-

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Abbreviation: EtdBr, ethidium bromide.
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HUC166, an *Escherichia coli* K-12 strain harboring pBR322, is grown for 10 generations in heavy isotope-containing and tritiated medium. A culture at 2×10^8 cells per ml is concentrated to 5×10^9 cells per ml in buffer.

A ^{32}P-labeled Mu c^+ stock is prepared on plates of LB medium containing ^{32}P$_i$, concentrated by centrifugation, purified on a step gradient, and dialyzed against Mu buffer.

Cells are infected at 37°C in buffer at a multiplicity of infection of 5. After 20 min nonadsorbed phage is neutralized by anti-Mu serum and eliminated by centrifugation.

The infected culture is resuspended at 2×10^8 cells per ml in M9 heavy medium at 37°C (time 0) and sampled at 0 and 30 min. Samples are lysed artificially and centrifuged in CsCl/EtdBr dye buoyant density gradients (Fig. 2).

The upper DNA band (non-supercoiled fractions) is centrifuged in an CsCl equilibrium density gradient.

The lower DNA band (supercoiled fractions) is centrifuged again in CsCl/EtdBr to eliminate any nonsupercoiled DNA and then is centrifuged in a CsCl equilibrium density gradient (Fig. 4).

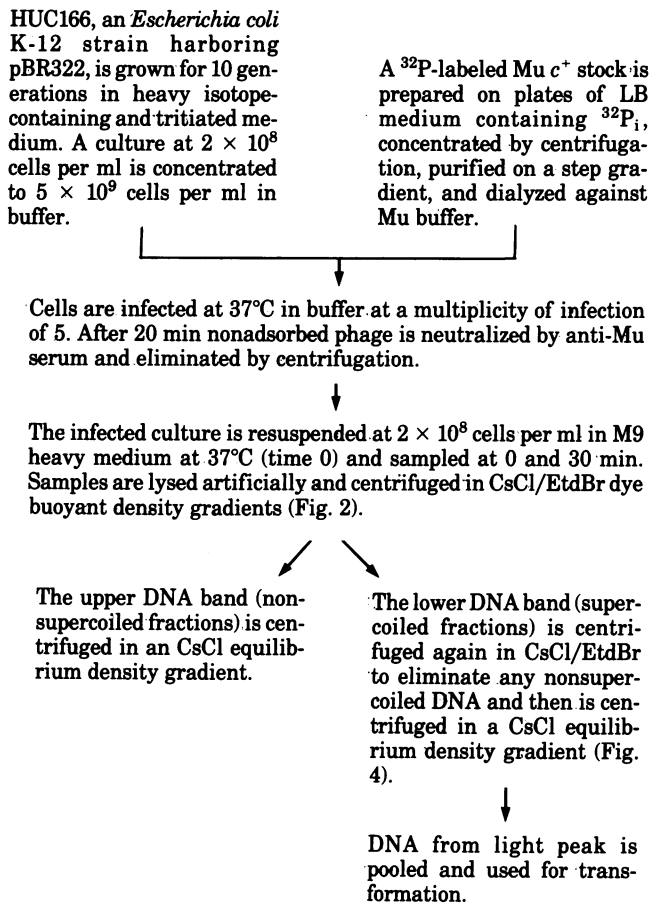DNA from light peak is pooled and used for transformation.

FIG. 1. Basic scheme of the experiment. EtdBr, ethidium bromide.

suspended in 25 ml of M9.H medium at 2×10^8 cells per ml. A 10-ml sample was immediately withdrawn, time $t = 0$ of the experiment, and the rest of the culture was incubated at 37°C with aeration; after 30 min, $t = 30$, a second 10-ml sample was withdrawn. The two samples were lysed according to Katz *et al.* (10), a procedure that eliminates the bulk of the chromosomal DNA. The samples were then centrifuged for 60 hr at 44,000 rpm in CsCl/EtdBr dye buoyant density equilibrium gradients, according to Hudson *et al.* (11) in a Beckman SW 50.1 rotor.

Twelve-drop fractions were collected from the bottom of the tubes and the radioactivity of trichloroacetic acid-precipitable material was measured in aliquots of each fraction with an LKB model 1215 liquid scintillation spectrometer. Fractions corresponding to the supercoiled DNA peak were pooled and submitted to a further centrifugation in a CsCl/EtdBr gradient to eliminate completely any contamination by nonsupercoiled DNA; fractions corresponding to the nonsupercoiled DNA peak were also pooled independently. The EtdBr in each pool was eliminated by *n*-butanol extraction and the resulting DNAs were centrifuged in CsCl ($\rho = 1.71$ g/ml) at 33,000 rpm for 60 hr in a Beckman type 40 rotor. Twenty-drop fractions were collected from the bottom and trichloroacetic acid-precipitable radioactivity was measured in aliquots of each fraction.

The gradient density was determined by measuring the refractive index of each third fraction.

As a control, the same procedure was used to process the parental W5445 strain labeled with [^{3}H]thymidine and infected with ^{32}P-labeled Mu c^+, to check whether Mu DNA in a supercoiled form is detectable even in the absence of any plasmid.

Bacterial Transformation. Transformation experiments were performed at low temperature, as previously described (12),

with supercoiled DNA from the light peak of CsCl gradient. RS54(Mu *cts*62) was used as recipient in two independent experiments. Tetracycline resistant transformed clones were selected. Selection was made directly on LB plates supplemented with tetracycline, at 42°C in one experiment and at 32°C in the other. The transformed clones obtained were all tested and were found to be temperature resistant also.

All the clones obtained in the two experiments were purified on LB/tetracycline plates at 32°C. One colony per isolated clone was resuspended in 10 ml of LB, grown to 1×10^8 cells per ml, and then centrifuged after addition of CHCl$_3$. The supernatant phage production at 32°C was spot-tested at 42°C to distinguish between clear and turbid plaques.

RESULTS

CsCl/EtdBr Dye Buoyant Density Gradient Analysis. Two samples were withdrawn at chosen times after Mu infection of a bacterial strain harboring pBR322, lysed artificially, and run in a CsCl/EtdBr dye buoyant density gradient.

The pattern shown in Fig. 2 is representative for both samples. There is a comigration of ^{32}P-labeled viral and ^{3}H-labeled bacterial radioactivities in nonsupercoiled as well as in supercoiled fractions. This result is compatible with an association of Mu with pBR322 giving a supercoiled structure. The parental strain without plasmid, infected with Mu and processed in the same way, has not revealed any supercoiled structure (Fig. 3). In addition we found no evidence of trailing of phage particles at the level of the supercoiled band (data not shown).

^{32}P radioactivity recovered in supercoiled fractions corresponded to 3–4% of the input of radioactivity at the infection.

CsCl Density Gradient Analysis. Fractions 22–24, corresponding to the supercoiled DNA peak of the CsCl/EtdBr gradient, were pooled, centrifuged again in CsCl/EtdBr to eliminate any contamination from nonsupercoiled DNA and then, after extraction of the EtdBr, centrifuged in a CsCl density gradient ($\rho = 1.71$ g/ml). Fig. 4 shows the density distributions
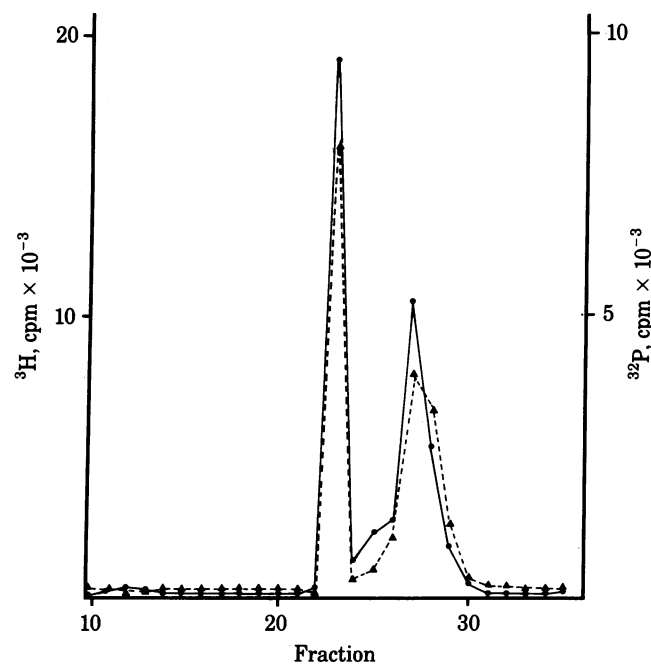

FIG. 2. CsCl/EtdBr gradient of DNA extracted from a bacterial strain harboring the pBR322 plasmid, after infection with Mu. The figure is representative for both time 0 and 30 min after the initial 20 min of infection in buffer. ▲, ^{32}P; ●, ^{3}H.
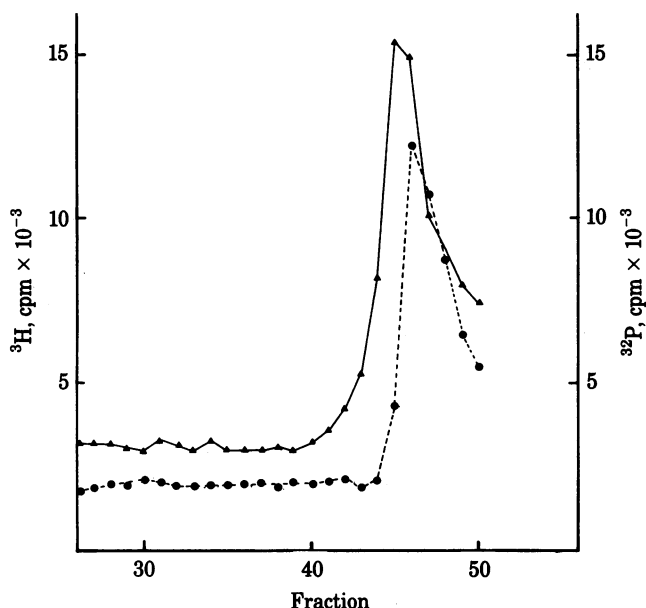
FIG. 3. CsCl/EtdBr gradient of DNA extracted from a bacterial strain without plasmid, after infection with Mu. ▲, ^{32}P; ●, ^{3}H.

obtained from this supercoiled DNA isolated at $t = 0$ (Fig. 4A) and at $t = 30$ min (Fig. 4B) of the experiment. In both cases, there was a separation of ^{3}H and ^{32}P peaks. At $t = 0$ all ^{32}P Mu radioactivity was found only in the light form, while 30 min later all ^{32}P had shifted to the hybrid form. ^{3}H radioactivity was always recovered in the heavy form, as expected after a long labeling with density markers.

From these data it is possible to conclude that: (i) Mu DNA is recovered in a supercoiled form due to its association to pBR322; (ii) the association at $t = 0$ between plasmid and parental viral DNA (light form of ^{32}P radioactivity from super-

coiled DNA) indicates that the first interaction between viral and host DNAs is of conservative type; (iii) at $t = 30$ min, viral DNA is all in the hybrid form, because of replication of the new replicon formed.

Unfortunately, under our experimental conditions it is impossible to determine any kinetics of replication.

The apparent lack of ^{3}H radioactivity associated with ^{32}P in Figs. 4 A and B is due to the fact that the bulk of pBR322 associated with Mu [^{32}P]DNA is too small to show a significant ^{3}H radioactivity above the ^{32}P "noise".

To overcome this difficulty, we repeated the experiment on a 20 times larger scale while reducing the ^{32}P specific activity correspondingly; the whole sample was processed at $t = 5$ min to give more information on viral replication kinetics. Fig. 5 shows the density gradient pattern for this last experiment. In this case it is possible to see evidence of a comigration of ^{3}H and ^{32}P radioactivities, corresponding to an association between pBR322 and Mu, in the light as well as in the hybrid form. The quantity of hybrid form would agree with a postintegrative replication of viral DNA, but the lack of a total block of bacterial replication does not permit us to confirm this suggestion.

In addition, fractions from the nonsupercoiled DNA peak obtained after CsCl/EtdBr were pooled and run in a CsCl density gradient. The results obtained (not shown) are comparable to those obtained by Ljungquist *et al.* (7).

Bacterial Transformation. Fractions 31–40 from the light peak (see Fig. 4A) were pooled, dialyzed, and utilized in transformation experiments to test whether the supercoiled Mu DNA found after infection of strain HUC166 derives from the association of viral and plasmid DNA and whether it has biological activity or not.

The quantity of DNA, estimated from ^{32}P cpm recovered after the CsCl density gradient, was roughly 5 ng expressed as concentration of viral DNA; the quantity of pBR322 alone corresponding to fraction 31 was less than 0.1 ng.

This "light supercoiled DNA" was used to transform strain RS54 (Mu *cts*62) in two independent experiments, at low tem-
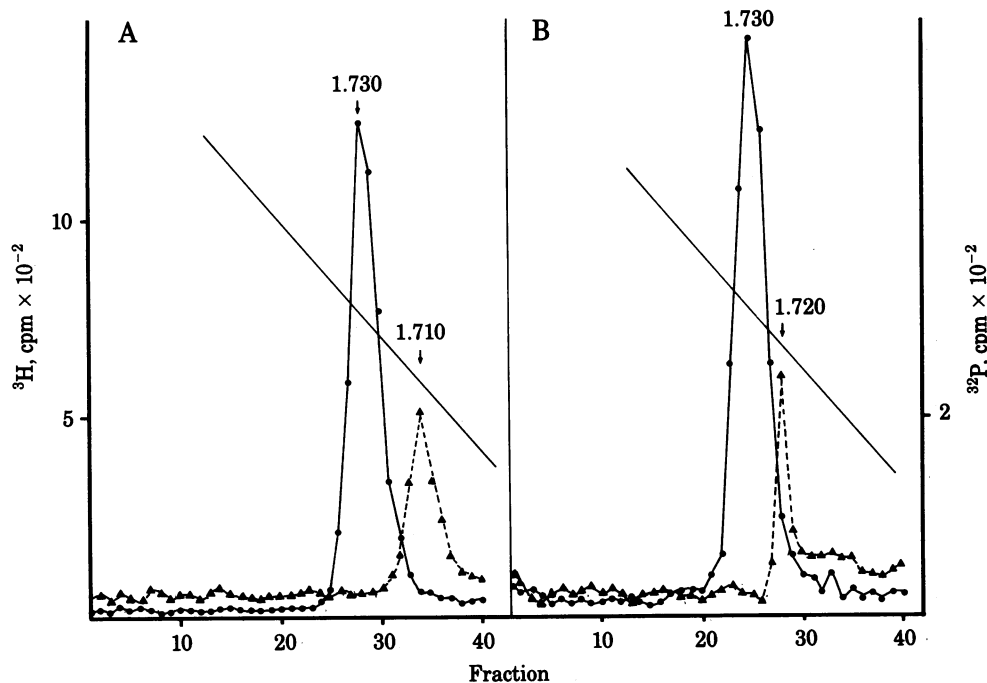

FIG. 4. CsCl density gradient of supercoiled DNA fractions collected at time 0 (A) and 30 min (B) of the experiment. Measurements of refractive index were obtained every third fraction. The values reported on figures correspond respectively to heavy (1.730), hybrid (1.720), and light (1.710) densities, all in g/ml. ▲, ^{32}P; ●, ^{3}H.
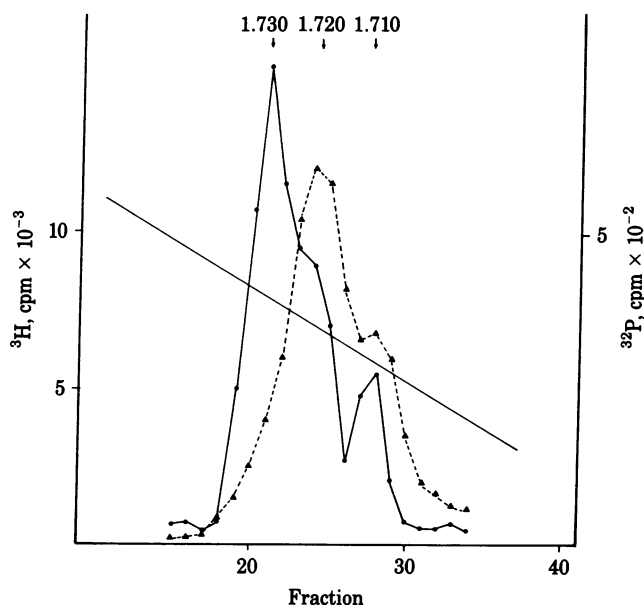
1.730 1.720 1.710

perature; tetracycline resistant transformed clones were selected.

In the first experiment the selection was done directly at 42°C, and 20 transformants were obtained. In the second experiment selection was done at 32°C. The 24 clones obtained were checked and all were found to be temperature resistant and immune to Mu superinfection at 42°C. The supernatants from transformed clone cultures grown at 32°C were examined for spontaneous phage production. The phage produced, plated on *E. coli* strain C600 at 42°C, gave rise to turbid and clear plaques corresponding, respectively, to Mu c^+ and Mu *cts* phenotypes, with the first type prevailing 6:1. Moreover, as expected for the two phages, the turbid phenotype is always associated with larger plaques as in Mu c^+, whereas the clear one is associated with smaller plaques as in Mu *cts*. Ten plaques per phenotype were checked for stability by replating of resuspended phage, and all were found to maintain their phenotypes.

DISCUSSION

Integrative recombination is a basic step in the life cycle of temperate bacteriophage Mu. In addition, the phage displays the ability to transpose its DNA during the lytic cycle.

Many models have been proposed to explain the mechanism of Mu-mediated transposition, but, until now, the question of whether Mu replicates before or after its integration has not been answered. In this work we have investigated Mu integration, mainly the first step of the recombinative event that follows infection, to determine whether the first integrated viral DNA molecule is conserved or not.

The basic idea of the experiment was to infect a bacterial strain harboring pBR322 with Mu and to isolate Mu DNA in a supercoiled form associated with this small plasmid. The analysis in a CsCl density gradient of the Mu–pBR322 structure isolated with a CsCl/EtdBr gradient allowed determination of whether Mu DNA associated with pBR322 had replicated or not, assuming the change in density due to pBR322 to be negligible because of its small size compared to Mu.

Our results show that, upon infection by ^{32}P-labeled Mu of a strain harboring pBR322 labeled with ^{3}H and heavy carbon and nitrogen markers, part of the parental viral DNA is found in the supercoiled band.

The analysis in a CsCl density gradient of the supercoiled DNA, isolated at $t = 0$ with a CsCl/EtdBr gradient, shows that ^{32}P radioactivity is recovered only in the light form. Therefore Mu DNA is conserved during the first integration. This conclusion was confirmed by the large-scale and the transformation experiments.

The large-scale experiment showed a definite comigration of ^{3}H-labeled pBR322 and ^{32}P-labeled Mu in the light band. The only way to observe pBR322 DNA in a light form after a long density labeling is in a condition in which this DNA is associated with a much longer light DNA molecule as, in our case, with Mu DNA.

To demonstrate that this DNA complex had a biological activity, we showed that it harbored transforming activity for both viral and plasmid markers. We transformed a strain lysogenic for Mu *cts*62 to eliminate zygotic induction. Transformation was carried out at low temperature for antibiotic resistance and all the transformed clones obtained were tested and found to be able to produce two kinds of plaques, corresponding to Mu c^+ and Mu *cts*, with the first type prevailing, and to be immune to Mu superinfection at 42°C. In addition there is evidence of segregation of antibiotic-sensitive clones that also results in loss of immunity to Mu at 42°C.

An interesting result is given by the abundance of hybrid form shown in Fig. 5. Even though there was a partial block of bacterial replication under the experimental conditions described, there was a large quantity of the hybrid form found after only 5 min at 37°C. This hybrid material may represent postintegrative replication of Mu DNA.

In conclusion, our data show that the first interaction of Mu with the target chromosome is of conservative type and is followed by an immediate replication of viral DNA.

When all the various models proposed for Mu-mediated transposition are divided into two groups according to whether the transposable element replicates before or after its recombination with the target site [for review see Bukhari (13)], our result fits more easily with the second class. However, it should be taken into account that the mechanism of Mu integration during lysogenization and the mechanism of transposition during lytic cycle could differ in various aspects, as suggested also by other authors (14–16).

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