Absence of Interparental Recombination in Multiplicity Reconstitution from Incomplete Bacteriophage T4 Genomes

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Interparental recombination between injected T4 DNA molecules is indetectable for incomplete petite phages (carrying a terminally deficient genome and therefore unable to circularize) as well as for genetically complete phages. The nonviable petite phages can individually replicate their DNA repeatedly, and they also undergo multiplicity reconstitution, producing complete phages, provided that a host bacterium is infected by several petite particles that carry genetically complementary segments of DNA. The formation of complete phages in multiplicity reconstitution must be due to recombination among incomplete progeny fragments, i.e., partial replicas of the T4 genomes. It evidently does not result from interparental recombination. To test for interparental recombination, light bacteria (containing no bromouracil) were simultaneously infected in light medium with light radioactive phage in minority (usually less than one per cell) and heavy (bromouracil-labeled) phage in majority (usually about nine per cell). Any interparental recombination should, under these circumstances of infection, lead to movement of the radioactive label of the minority light phage DNA to a position of higher density. That possibility was not observed.

It has not yet been conclusively established whether the DNA molecules of parental T-even bacteriophages that are injected into a host bacterium undergo recombination with one another under normal conditions of infection. The answer to this question is basic for the understanding of such phenomena as multiplicity reactivation (MRA) of UV-inactivated phages (2, 23) and, in general, the mechanisms of rescue of genetic material from lethally damaged or deficient genomes. Some data have been compiled on the genetic composition of single bursts issuing from bacteria that were infected with only one particle from each of two irradiated or deficient parental phages carrying distinguishing genetic markers (11, 25; C. Rayssiguier, Ph.D. thesis, Universite de Paris-Sud, Orsay, France, 1974). Those results, although they clearly implicate genetic recombination in the reconstitution (a more appropriate word than "reactivation" in the context of present experiments) of complete undamaged progeny, do not decide whether recombination occurs before or after the parental DNA has been replicated.

In 1963 a molecular experiment was designed to prove or disprove interparental recombination in T4 (19). Light (LL, no bromouracil [BU] substitution) bacteria, in a light medium, were infected with a minority of LL radioactive bacteriophage and a majority of heavy (HH, BU substituted) cold virus. At intervals, samples were analyzed in density gradients for distribution of the radioactive label. Since no displacement from its original LL location was observed, it was concluded that no recombination occurs among the input parental molecules in T4-infected bacteria (19). A year later the experiment was repeated in two laboratories, modified, however, by first inhibiting DNA replication through the addition of fluorodeoxyuridine (1, 16) and then in a different group of experiments by using DO amber mutants (15, 29). These later experiments indicated that interparental recombination does occur. The analyses were, however, made at very late times after infection (45 to 60 min) and under conditions in which parental molecules seem to deteriorate, i. e., during a long sojourn in cells where DNA replication is restricted. The results of Emanuel (Ph.D. thesis, Univ. of Pennsylvania, Philadelphia, 1972) indicate profound deterioration with many nicks and double-strand breaks found under those conditions. This leads us to suspect that such interparental recombination may play no significant part in recombination under normal conditions in which parent-progeny and interprogeny recombination probably play the primary role.

MRA for lethally damaged phages and multi-

plicity reconsitution (MRC) for phages such as petites with terminal genetic deficiencies (26) both clearly require recombination to reconstruct viable complete genomes. Two models have been suggested for their participation in those processes. The first model (6, 24) invokes breakage and rejoining (recombination) of the parental molecules, leading to the formation of a reconstituted viable parental molecule free from deficiencies or UV damages. That complete and undamaged molecule would from then forward carry out all the functions of the normal genome. The second model (2) would first require replicating the undamaged segments between UV lesions, or the incomplete genomes from the petites, leading to the formation of a pool of partial replicas. Those would subsequently recombine with the formation of integral or perhaps concatenated molecules, possible precursors of mature DNA.

Recently we have demonstrated that petite bacteriophages do replicate the entire length of their fractional genomes and that they do so repeatedly, even when used to singly infect host bacteria (14). This clearly shows that partial genomes (admittedly containing 85% of the total T4 genome) can and do replicate successfully and repeatedly without invoking circularization of the template. In addition, C. Rayssiguier (personal communication) has demonstrated that UV-inactivated phages, after infecting bacteria at a low multiplicity of infection (MOI), also replicate parts of their genomes repeatedly. Both of these discoveries suggest reconsideration of the idea that partial replicas may play a significant part in the recombination-dependent processes MRA, MRC, and marker rescue from UV-damaged genomes (cross-reactivation). An important role for partial replicas in such processes has long been advocated by Barricelli and Doermann on the basis of genetic evidence (2–4). Furthermore, Womack (30) has shown that efficiency of marker rescue from UV-inactivated T4 genomes varies according to location on the genetic map. Four clearly discernible peaks of rescue efficiency were noted, a number that corresponds quite well with the number of initiation sites for DNA replication observed by electron microscopy (7) and also determined by a physicochemical approach (12). The peaks could result from multiple replicas of the DNA restricted presumably to the undamaged genetic regions immediately to the right and the left of the principal initiation sites. The availability of numerous partial replicas from those particular segments of the genome would probably enhance the chance of rescue of genetic markers located in those regions (8; D. A.

Campbell, Ph.D. thesis, Univ. of Washington, Seattle, 1969).

Because multiple infection with incomplete genomes of petite phages leads to the formation of productive infectious centers, these mutants provide an ideal means of testing whether interparental recombination is an essential step in MRC. This paper will demonstrate that interparental recombination is not detected at any time after multiple infection with petite phages. This leads to the conclusion that the process of MRC of petite phage genomes is accomplished by recombination between partial replicas (incomplete progeny molecules). MRA of UV-inactivated phages may perhaps be accomplished in a similar manner.

MATERIALS AND METHODS

Bacterial and phage strains. *Escherichia coli* strain B23 and T4D bacteriophage mutant *ptg*191 (9) were used in all experiments. This phage mutant produces normal-size phages (plaque formers) as well as petite particles of the intermediate type (9). The latter contain about 86% of the full T4 genetic complement (14), the deficient segment being terminal and circularly permuted over a population of petite particles (26). In addition, lysates contain giant phages that contain multiple genomes in concatenated form.

Growth medium. The growth medium was TCG (22). BU substitution was achieved by supplementing TCG with 200 μg of bromodeoxyuridine, 5 μg of flourodeoxyuridine, and 20 μg of uracil per ml of medium. This will be referred to as "heavy" medium and the phage produced in it as "heavy" (HH) phage.

Purification of petite and complete phages. Purification of petite and complete phages was performed as follows. Phages produced in a TCG lysate of B23 cells infected with T4D*ptg*191 were purified by two alternating cycles of low- and high-speed (15 min at 16,000 rpm in a Beckman model 40 rotor) centrifugation. The resulting purified phage were resuspended in Tris-salt buffer (0.01 M Tris-0.15 M NaCl, pH 7.4) and layered on the surface of a 15 to 40% linear sucrose gradient (sucrose solutions prepared in 0.1 M NaCl-0.032 M KH$_2$PO$_4$-0.001 M MgCl$_2$, pH 5.2). Centrifugation was carried out at 19,500 rpm for 70 min at 20 C in a Beckman swinging-bucket titanium rotor (SW41-Ti). At the conclusion of the run, giant phage present in the *ptg*191 lysate were located at or near the bottom of the gradient, while complete and petite bacteriophages formed two distinct, well-separated visible bands that could be separately siphoned off using a peristaltic pump. The petite sample used in the experiments contained less than one complete phage per 2,000 petite particles. The sample of complete phages presumably contained very few petite particles, but no precise method is avalable for detecting a small number of petite particles in a suspension containing predominantly complete phages. After separation, the moieties were dialyzed against Tris-salt medium to remove sucrose and thereafter used

for the experiments described below. They are referred to as petites and completes, respectively.

DNA extraction from infected cells. Infected-cell suspensions were taken at any time during the experiment and diluted 10-fold in ice-cold LTL-EDTA (0.05 M NaCl-0.05 M Tris-0.015 M EDTA, pH 7.4). Cells were sedimented, resuspended in LTL-EDTA to a concentration of 6×10^8 cells/ml, and extracted by the sodium dodecyl sulfate-Pronase-phenol method. Efficiency of recovery upon extraction is 100% (20).

CsCl equilibrium density fractionation. CsCl equilibrium density fractionation was performed by combining 2.6 ml of saturated CsCl with 0.7 ml of sample in a polyallomer tube that was centrifuged in an SW50.1 Beckman rotor at 29,600 rpm for 72 h. Fractions were collected from the bottom of the tube onto fiber-glass filters in scintillation vials and dried at 80 C. Recovery of input DNA from CsCl gradients averages 100%. Isotopes were measured in a Packard scintillation counter, using 2 ml of toluene-based scintillator fluid per vial.

RESULTS

A. Phage DNA synthesis and kinetics of DNA replication in cells infected with petite bacteriophage at a high MOI. This group of experiments was carried out to make two comparisons between cells multiply infected with petites and those similarly infected with completes. First, the kinetics of net synthesis of DNA and, second, the timing of replication of parental molecules were compared.

(1) **Net DNA synthesis in bacteria infected at a high MOI of petite compared with complete bacteriophages.** *E. coli* B23 was grown in TCG medium to a density of 3×10^8 cells/ml (confirmed by colony counts). At that moment the bacterial suspension was divided, and one aliquot was infected with an MOI of 9 petite and the other with an equal MOI of complete bacteriophages. Biological controls showed that 100% of the bacteria in both aliquots yielded productive infectious centers. At 3 min after infection, ^{32}P was added to both suspensions (specific activity of 0.5 mCi/mg of P), and at intervals thereafter samples were withdrawn and precipitated with cold 0.3 M trichloroacetic acid, using albumin as a carrier. The process of reprecipitation and washing with acid was repeated several times and then followed by digestion with 1.0 M KOH at 37 C for 18 h. After neutralization and two successive precipitations, the final pellet was dissolved in NH$_4$OH and applied to a fiber-glass disk. Radioactivity was measured in a liquid scintillation counter. The data were normalized to phage-equivalent units of DNA synthesized per infective center and are shown in Fig. 1. Even from the very earliest stages after infection, it is clear that ^{32}P incorporation into DNA pro-

ceeds virtually identically for cells multiply infected with either petite or with complete bacteriophages. No lag of any sort is detectable for cells infected with petites, suggesting that, if any preparatory steps (like recombination) are required prior to replication in those bacteria, such steps must occur efficiently prior to 5 min after infection.

(2) **Timing of DNA replication after multiple infection with petite genomes.** Extensive data have already been published on the replication kinetics of parental DNA from complete bacteriophages (18). The replication of normal T4 DNA starts about 5 min after infection at 37 C. With isotope-labeled light (LL) parental DNA in cells and medium containing the density label bromodeoxyuridine, the first indication of replication observed in a density gradient is the appearance of a small shoulder adjacent to the nonreplicated moiety. This shoulder consists of partially replicated molecules that have not undergone a complete first round of replication. Indeed, in the electron microscope they display numerous replicative loops (7, 12). At 6 to 8 min the parental label assumes an intermediate density, the molecules consisting mostly of one light parental strand and one new heavy strand (HL). At about 10 min after infection, parental DNA becomes fragmented and, due to recombination with heavy progeny molecules, the parental label assumes a density close to pure progeny material (18). The addition of chloramphenicol (CM) at about 5 min allows semiconservative replication of parental molecules but prevents recombination with progeny. Thus, when run in a CsCl density gradient, the replicative parental molecules remain at an intermediate HL location. Addition of CM later than 7 min al-
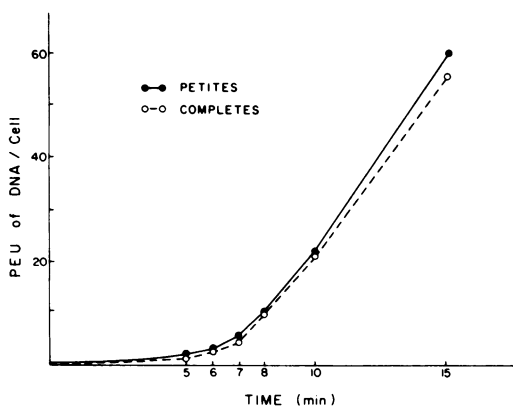

FIG. 1. *Net synthesis of DNA in bacteria infected with petite or with complete bacteriophages at high multiplicity. P.E.U., Phage-equivalent units of DNA.*

lows abundant recombination with progeny molecules, causing parental label to assume a density close to the pure HH location (18).

The experiment now to be described was designed to find out whether any differences in the DNA replication pattern or sequence of events are observed when bacteria are multiply infected with incomplete genomes. Bacteria, pregrown for one generation in bromodeoxyuridine medium, were infected with an MOI of 9, using light ^{32}P-labeled petite bacteriophages. At intervals, samples of the suspension were chilled in LTL-EDTA preparatory to DNA extraction. In addition, at 5, 7, and 10 min after infection, aliquots of the infected suspension were transferred to CM (150 μg/ml). Those moieties exposed to CM were further incubated for an additional 45 min and then were chilled by diluting into ice-cold LTL-EDTA solution for intracellular DNA extraction. Thereafter samples were supplemented with ^{3}H-labeled LL T4 DNA to provide a reference for the light location and were then subjected to a CsCl density equilibrium centrifugation.

The results of this experiment are shown in Fig. 2 and 3. Scrutiny of Fig. 2 shows that the timing of events (appearance of partially replicated molecules at 5 min, of hybrid DNA at 6 min, and of highly recombinant DNA molecules between 10 and 15 min) is not distinguishable from that observed for complete bacteriophage (18). Moreover, addition of CM at 5 min (Fig. 3) prevents parent to progeny recombination, with the parental label remaining at the HL location. Addition of CM at 7 or at 10 min, however, allows abundant recombination, leading to the displacement of the parental label far beyond the HL location.

In summary, the results of these experiments correspond very closely with those obtained earlier for complete phages (18).

B. Are recombinants formed among input parental DNA molecules? Experiments described above did not detect any lag either in net DNA synthesis or in the replication pattern of petite parental molecules when compared with molecules from complete T4 particles. This implies that, if any interparental recombination is prerequisite for normal replication of petite parental DNA, it must occur very early and surely within the first 5 min after infection and before a significant amount of progeny DNA has been synthesized. To prove or disprove the possibility of interparental recombination, an experiment was performed identical in construction to that of Kozinski et al. (19), this time, however, using petite bacteriophages as infecting parents. The experiment calls for simultaneous infection of light bacteria in light

medium with a minority of LL ^{32}P-labeled and a majority of cold (or ^{3}H-labeled) HH petites. The primary cause for change in density of parental ^{32}P-labeled LL DNA is expected to be recombination between LL and HH parental genomes.

Before performing this experiment, we felt compelled to confirm two previous observations. One deals with a still unexplained phenomenon that was originally observed by Miller (Ph.D. thesis, Univ. of Pennsylvania, Philadelphia, 1969) and mentioned in the paper of Kozinski et al. (18). Despite the fact that no density label was employed in Miller's experiment, it was observed that, in the interval between 7 and 10 min after infection, injected parental DNA becomes slightly denser than reference DNA. It seemed essential to confirm that this phenomenon occurs for petite bacteriophage genomes also. Second, it seemed essential to repeat the original experiment done with complete phages by Kozinski et al. (19) because that experiment was performed without a ^{3}H-labeled reference. The repetition was important not only as a basis for comparison with multiple-petite infection but also, by inclusion of the additional LL reference, to strengthen the earlier observation that recombination does not normally occur among parental complete genomes.

(1) Density changes of LL parental petite DNA infecting bacteria in the absence of density label. Light bacteria were grown to 3×10^8 cells/ml in light medium and infected with an MOI of 9 LL ^{32}P-labeled petite bacteriophages (no density label added). At 3, 5, 7, and 10 min after infection, samples were withdrawn. After DNA extraction, ^{3}H-labeled LL reference DNA was added and the mixture was subjected to CsCl gradient analysis. The results are shown in Fig. 4. It is immediately apparent that a subtle, but significant, separation (see summed percentages given with the horizontal brackets) of parental label away from the reference DNA

FIG. 2. *Replication of ^{32}P-labeled LL DNA in heavy bacteria and heavy medium after a high MOI with petite bacteriophage. The broken line shows the position of ^{3}H-labeled LL reference T4-DNA. Note the gradual displacement of the ^{32}P moiety into replicative and nonreplicative components.*

FIG. 3. *Extent of replication and recombination of parental ^{32}P-labeled LL DNA in the presence of CM. Bacteria infected under conditions similar to those described in Fig. 2 were supplemented with CM (150 μg/ml) at 5, 7, and 10 min after infection. After further incubation of 45 min, the DNA was extracted and subjected to analysis in a CsCl density gradient. The open circles and broken line indicate the position of ^{3}H-labeled LL reference DNA extracted from T4 phages. The arrows indicate the location of the hybrid moiety (HL).*
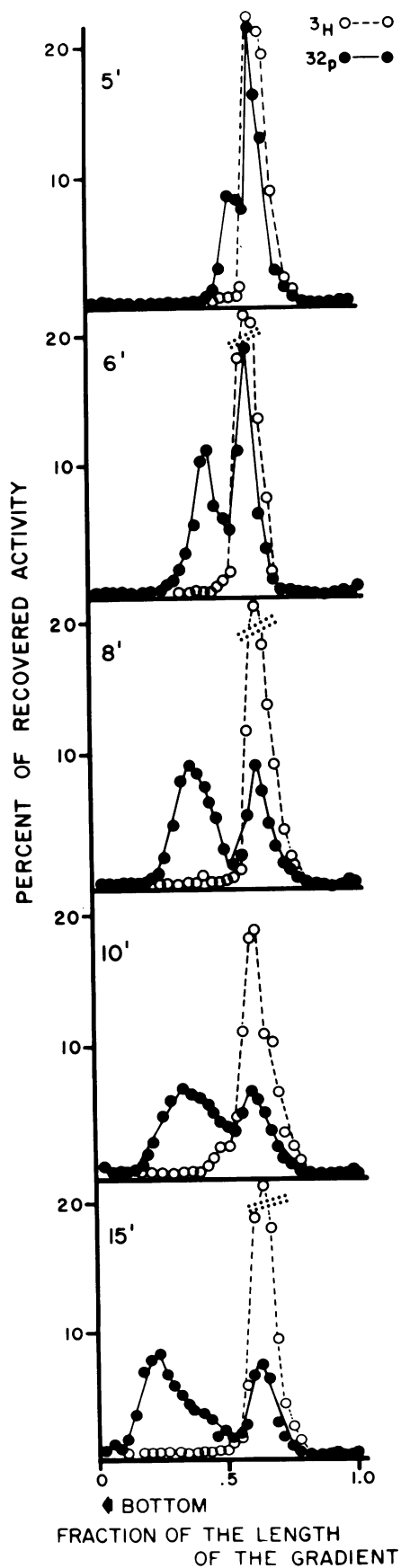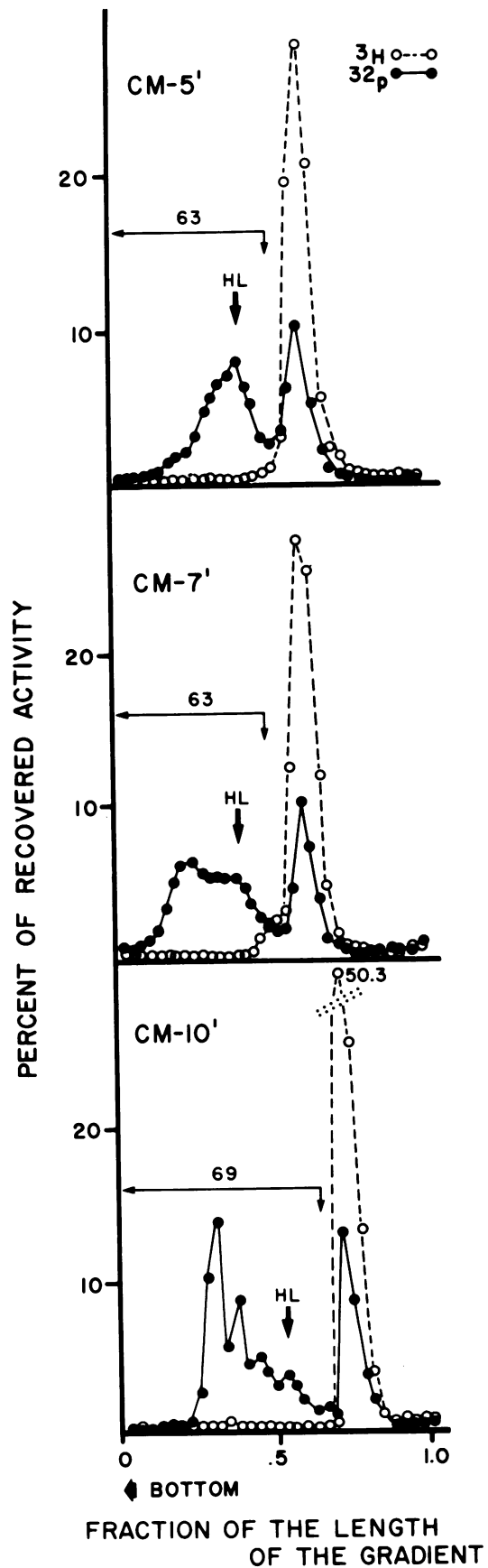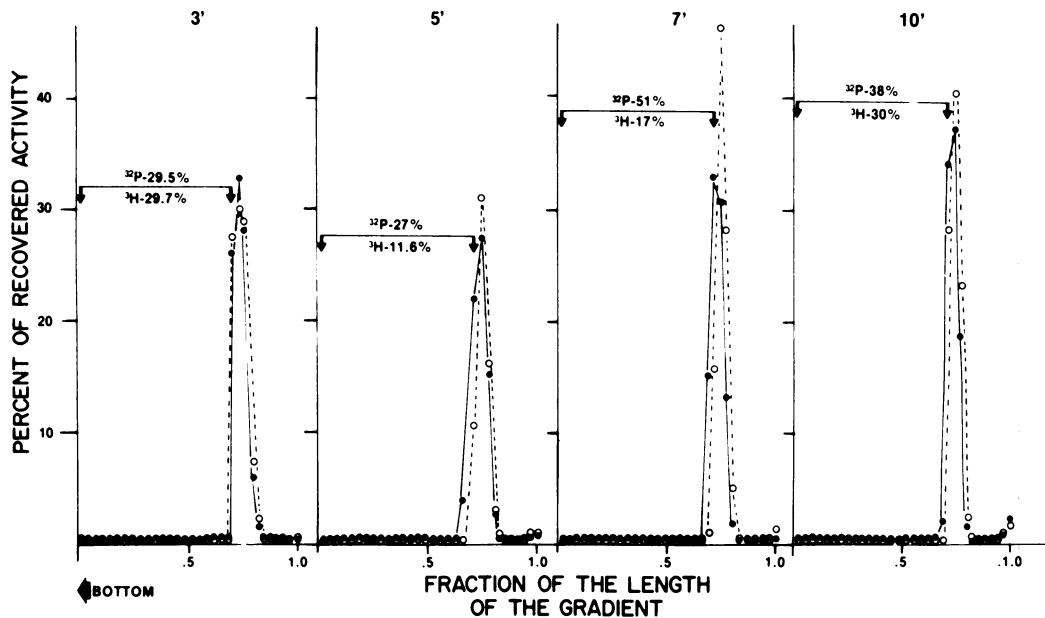
FIG. 2.

FIG. 3.

FRACTION OF THE LENGTH OF THE GRADIENT

FRACTION OF THE LENGTH OF THE GRADIENT

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FIG. 4. *Density changes of LL parental DNA from petite phages multiplying in the absence of density label. Note that after 3 min, parental ³²P-labeled DNA (filled circles and solid line) becomes displaced away from ³H-labeled LL reference (open circles and broken line).*

occurs at about 5 to 7 min after infection. This confirms previous observations for complete bacteriophages (18; Miller, Ph.D. thesis), and it provides a base line for interpretation of experiments in which the question of recombination between HH and LL parental DNA molecules will be directly confronted.

At present we are unable to specify the reason for this change in density. It may be, as suggested earlier (18), that the displacement is due to the appearance of single-stranded segments preceding molecular recombination. Alternatively, it might result from association of traces of primer or mRNA. This phenomenon is being pursued further.

(2) Complete bacteriophages: lack of interparental recombination between LL radioactive and HH nonradioactive coinfecting DNA. Light bacteria, grown to 3×10^8 cells/ml, were coinfected in light medium with an MOI of 1 LL, ³²P-labeled and 9 HH, nonradioactive complete particles. As in the previous experiment, samples were withdrawn at intervals, DNA was extracted, LL ³H-labeled reference DNA was added to each extract, and the mixtures were analyzed in a CsCl gradient. The results are presented in Fig. 5. It is evident from inspection of the figure that at no time after infection is there any evidence of recombination between LL and HH coinfecting DNAs. The slight shoulder appearing at 9 min after infec-

tion, when there are already 15 phage equivalents of progeny DNA synthesized per cell (see Fig. 1), does not differ significantly from corresponding data observed in bacteria infected in the absence of any density label (for example, in Fig. 4), and the amount of displacement in the 7-min sample is, for that matter, less in Fig. 5 than in Fig. 4. The slight density shift cannot, therefore, be attributed to interparental recombination but rather to a phenomenon independent of the density label used, as was observed in the preceding experiment.

(3) Petite bacteriophages: lack of interparental recombination between LL radioactive and HH nonradioactive coinfecting DNA. This experiment was performed in a manner similar to the preceding one, except for two changes. Petite phage were used in this experiment for both infecting parents. Moreover, both parents in this experiment carried radioactive labels: the LL minority phages were labeled with ³²P, and the HH majority phages were labeled with [³H]adenine. This was done not only to provide full exposure of any possible interparental recombination but also to disclose the first initiation of replication that should manifest itself by a change in density of the HH ³H-labeled DNA. By judiciously determining the amounts of reference label, it should be possible to recognize slight changes in the densities of either of the parental labels.

In the experiment, bacteria were infected with an MOI of 0.7 of LL ^{32}P-labeled and 9 HH ^{3}H-labeled petite bacteriophage (specific activities, 1.0 mCi of ^{32}P/mg of P and 3.0 mCi of ^{3}H/mg of adenine). At intervals, samples were collected and intracellular DNA was extracted. The resulting extracts were analyzed by CsCl gradient analysis in three ways (see Fig. 6 and 7): (i) with no reference added (left panels, Fig. 6), permitting analysis of gross density changes — mostly shifting of ^{3}H-labeled HH DNA due to replication; (ii) with addition of ^{3}H-labeled LL reference (right panels, Fig. 6) in quantity exceeding the total ^{3}H parental label by 100-fold — in effect, submerging proportionally the ^{3}H label in the HH parental DNA but allowing recognition of subtle density changes in the ^{32}P-labeled LL parental DNA; (iii) with addition of ^{32}P-labeled HH DNA (Fig. 7), providing a reference that aids in recognition of the initial partial replication of the ^{3}H-labeled HH parental moiety. In contrast to the large quantity of ^{3}H-labeled LL DNA added for reference shown in the right panels of Fig. 6, only a small amount of ^{32}P-labeled HH DNA was used for reference as shown in Fig. 7. This stems from the necessity of avoiding coincidence of a large amount of ^{32}P with ^{3}H, since ^{32}P transfers about 5% of its counts to the ^{3}H channel in our counting system. In addition, the centrifuge speed and gradient profile were altered to locate the heavy moiety closer to the middle of the gradient than in the other two analyses on this sample, thereby increasing the resolution of the samples of greatest interest. This, in turn, caused the light moiety to band very closely to the top of the gradient.

It is seen that replication began between 3 and 5 min since the ^{3}H-labeled parental DNA, that at 3 min is quite closely coincident with the HH reference, has, for the most part, moved to a position of slightly lower density (Fig. 7). This is in agreement with the results presented in Fig. 2, which also showed that replication had begun by 5 min. Because there is no difference in the rates of DNA synthesis after complete versus petite infections (Fig. 1), any recombination required for replication of petite genomes must have occurred prior to 5 min. This should be revealed by a shift in density of the ^{32}P label of the minority parent toward a position of greater density (right panels, Fig. 6). Table 1 compares the displacements observed at 5, 7, and 8 min after infection in this experiment with that seen in the experiment without density label (Fig. 4). The data given represent the accumulated percentages of the [3]H (density reference) label and of the [32]P

(LL parental) label at the point where the two curves cross. Comparison of these values shows no significant difference in density shift and indicates that the small displacement observed in this experiment is attributable to a density shift that is independent of the presence of the BU label. It is, therefore, concluded that interparental recombination is unnecessary for initiation of replication of the fractional genomes of petite phages and that it does not occur during the early stages of infection.

A comment should be made regarding the peak of ^{3}H label from the HH majority parent, which appears in the 5-min and later extracts and is located at a slightly higher density (1.72) than that of the ^{32}P-labeled LL parental DNA. This peak is thought to represent a loose association of some of the ^{3}H-labeled HH parental T4 DNA with host DNA that is present at a high concentration in the extract. It seems impossible that it is a product of recombination between the HH and LL parental T4 molecules for several reasons. (i) When samples from this peak are rerun at a lower concentration, the label returns to the density location characteristic of the HH DNA (1.80). (ii) The peak is observed consistently, even when bacteria are infected with HH phage exclusively. (iii) It seems quantitatively impossible to account for the movement of about 8% of the HH label to a density location, which indicates that the composition must include about 80% LL DNA. In this experiment the MOI of the HH parent was 9, and the 8% HH label, therefore, represents about 0.72 petite-phage equivalents of DNA per cell. To move 0.72 equivalents to the location of the peak in question would require four times as much LL DNA or 2.9 petite-phage equivalents. That amount is about four times the input, which in this experiment was only 0.7 LL phage per cell. Furthermore, inspection of Fig. 6 shows that the majority of the ^{32}P label of the LL parent remained at the original LL density location. It is concluded, therefore, that the ^{3}H label from the HH parent present at the intermediate density of 1.72 owes its location to some mechanism other than interparental recombination.

C. Fate of the parental label of petite bacteriophage DNA in the resulting progeny phages. This experiment will provide information about the fate of the parental labels used in the previous experiment. Three observations are described: (i) that the label of petite parental DNA is transferred to progeny with an efficiency similar to that experienced with complete phages, (ii) that both heavy and light labels are transferred equally, and (iii) that
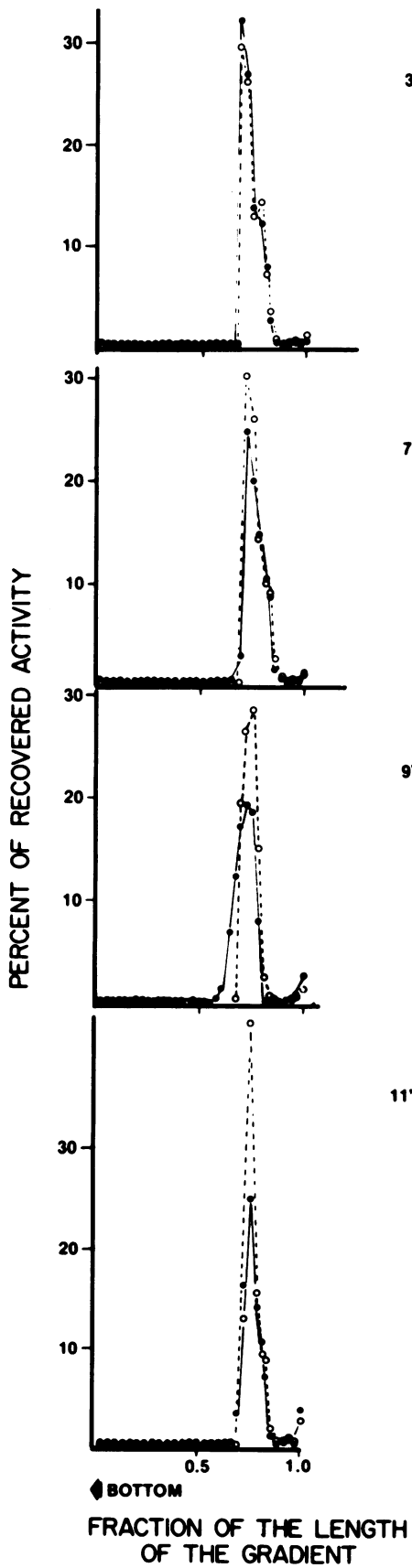
PERCENT OF RECOVERED ACTIVITY

3'

7'

9'

11'

0.5   1.0

◆ BOTTOM

FRACTION OF THE LENGTH
OF THE GRADIENT

FIG. 5.

NO REFERENCE

³H LL REFERENCE ADDED

3'   3'

5'   5'

7'   7'

8'   8'

PERCENT OF RECOVERED ACTIVITY

.5   1.0   .5   1.0

◆BOTTOM

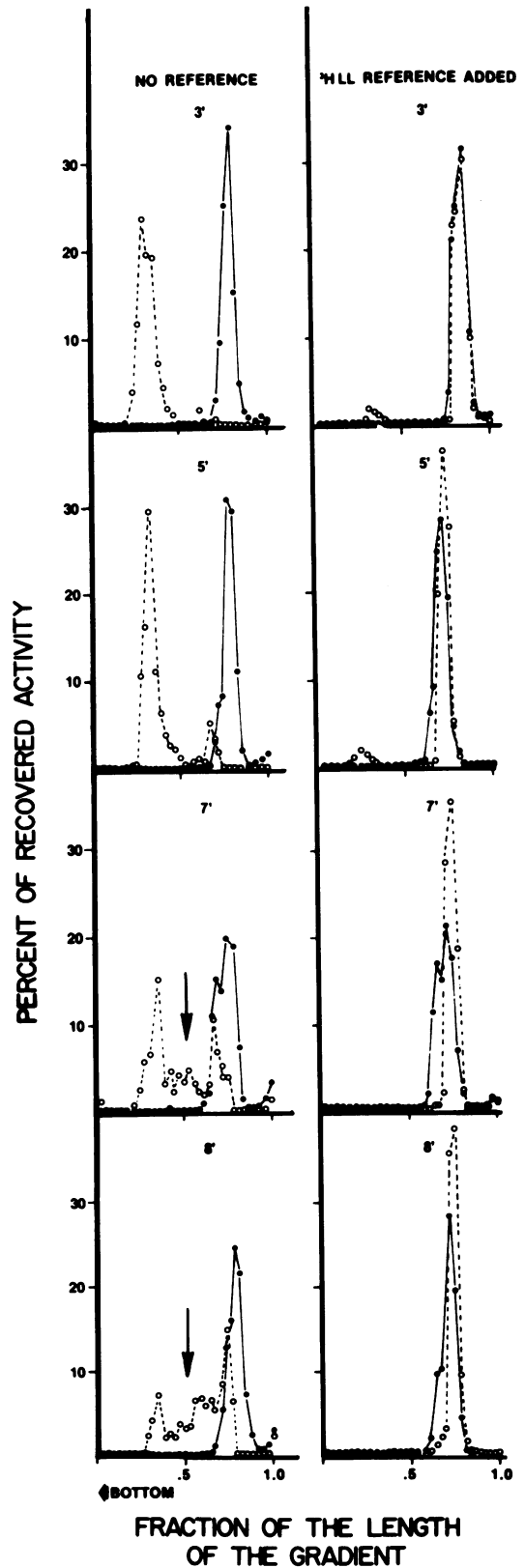FRACTION OF THE LENGTH
OF THE GRADIENT

FIG. 6.

both parental labels are distributed between complete and petite progeny phages.

As part of experiment 3 described in section B, a fraction of the bacterial suspension, simultaneously infected with light and heavy petite bacteriophages, was allowed to lyse. The amount of label transferred from parents (^{32}P from LL and ^{3}H from HH) to progeny was estimated. Furthermore, the types of phage particle (i. e., petite or complete) to which the labels were transferred were identified.

The efficiency of transfer of both labels from parent to progeny will be considered first. At 3 min after infection, an aliquot of the suspension was precipitated with trichloroacetic acid. The amount of ^{3}H and ^{32}P recovered in the precipitate was defined as 100% of the input label and is given in line (i) of Table 2. (It should be noted that no significant amount of label was lost in the supernatant of this precipitation.) At 120 min after infection, chloroform was added to the remainder of the suspension to insure complete lysis, and DNase was then added. A sample of the DNase-treated lysate was withdrawn and precipitated with trichloroacetic acid, estimating the DNase-resistant or encapsidated label (line [ii], Table 2). The remainder of the DNase-treated suspension was centrifuged at low speed to remove debris, and another sample was withdrawn from the supernate for acid precipitation (line [iii], Table 2). Finally, the remaining supernatant was centrifuged through two cycles of alternating high and low speeds to purify the progeny phages. A sample of the final purified suspension was also precipitated with acid (line [iv], Table 2).

Inspection of Table 2 indicates a transfer of about 40% of both parental labels to the DNase-

FIG. 7. *Analysis of the fate of ^{3}H-labeled HH parental DNA at 3 and 5 min after infection. The same extracts documented in the upper panels of Fig. 6 were supplemented with a small quantity of ^{32}P-labeled HH reference DNA and then analyzed under conditions identical to those described in the legend to Fig. 6. After fractionation, however, the upper third (light region) of the gradient was eliminated from the figure and the calculations. Thus, only the bottom 21 to 23 fractions were subjected to counting and integration in this figure. That was done because the great majority of the ^{3}H bands in the vicinity of the heavy location. The procedure adopted thus allows a meaningful comparison with ^{32}P-labeled HH reference. Note the displacement of the ^{3}H parental label away from the ^{32}P-labeled HH reference. The extent to which replication of the partially replicated molecules has proceeded is indicated by the degree of displacement. Evidently, by 5 min, most of the parental molecules have been partially replicated but still contain a majority of heavy parental DNA and a minority of light, newly synthesized material.*

TABLE 1. *Early density shift of parental DNA observed after mixed infection by HH (MOI = 9) and ^{32}P-labeled LL (MOI = 0.7) petite genomes (Fig. 6, right panels) compared with the displacement seen in the complete absence of density label (Fig. 4)*

Time of DNA extraction (min)	^{3}H:^{32}P	
	Data from Fig. 6	Data from Fig. 4
5	8:20[a]	12:27
7	10:37	17:51
8	17:38	30:38

[a] The numbers given represent the percentages of ^{3}H (T4 DNA as a density reference) and ^{32}P (LL parental label) accumulated up to and including the point of the gradient in which the two curves intersect (see also arrows in Fig. 4).

FIG. 5. *Analysis in CsCl density gradient of ^{32}P label (from complete LL parent) after coinfection with a majority of BU-labeled complete HH phages in light medium and light cells. Solid circles and solid lines show the distribution of ^{32}P in the gradient, and open circles and broken lines indicate the ^{3}H-labeled LL reference. Note that the ^{32}P label moved only about as much as observed in the total absence of density label using petite phages (as seen in Fig. 4).*

FIG. 6. *CsCl density gradient analysis of intracellular DNA in an experiment designed to demonstrate both replication of ^{3}H-labeled HH DNA and recombination among petite parents. Cells were infected with an average of 9 ^{3}H-labeled HH and 0.7 ^{32}P-labeled LL petite phages. DNA was extracted at 3, 5, 7, and 8 min after infection and analyzed with (right column) and without (left column) ^{3}H-labeled LL reference DNA. Note in left column the evidently normal replication of the HH parental DNA, indicated by the shift of ^{3}H (open circles, broken lines) toward hybrid density and beyond. In the right column note the failure of ^{32}P to move toward higher density more than was observed (Fig. 4) in the absence of any density label.*
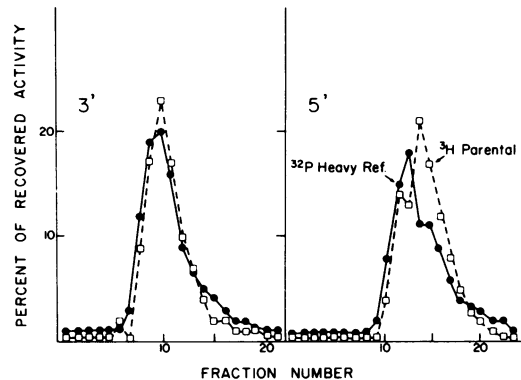
resistant fraction and about 30% to the purified progeny phage. Importantly, for the experiments described in the previous section, both heavy and light (^{3}H and ^{32}P, respectively) were transferred about equally. (If the slight preponderance of ^{3}H transfer seen here is meaningful,

it should be called to attention that it is carried in the BU-labeled parental DNA.) In any event, the amount of transfer does not differ significantly from the extensive data compiled previously for the transfer of parent label to progeny phages during a growth cycle of complete bacteriophage.

The purified progeny bacteriophages were further analyzed in a sucrose gradient for distribution of label among the two visible phage bands. Complete and petite particles can be separated with less than 0.05% completes in the petite fraction. There was no attempt to isolate the giant phage moiety: first, because it constitutes a minority class of the total phage mass, and, second, that class does not form a visible band in sucrose gradients due to the sizable variation in the length of giants (9). The contents of both parental labels reappearing in either complete or in petite particles are summarized in Table 3. It is seen that both labels are distributed equally, with about 20% of each label in the complete moiety and about 80% in the petite fraction.

DISCUSSION

The experiments with which this paper deals focus on the question whether recombination occurs among the several parental genomes that coinfect a single bacterium. The data presented appear to force rejection of that possibility. That conclusion, in turn, becomes important to our interpretation of MRA of UV-inactivated phages (23) as well as MRC of the terminally deficient genomes of petite phages. The assumption is frequently made that the mechanism leading to reactivation is due to recombinations among the damaged input phages that

TABLE 2. *Equality in transfer to progeny of LL (^{32}P) and HH (^{3}H) parental DNA after mixed multiple infection*

Sample	Recovery (%)		^{3}H/^{32}P
	^{3}H	^{32}P	
(i) 3 min after infection	100	100	1.00
(ii) Lysate after DNase treatment	41	38	1.08
(iii) Low-speed supernatant of lysate after DNase	38.5	33	1.17
(iv) Purified progeny phage	31	26	1.19

TABLE 3. *Distribution of parental DNA labels to petite and complete progeny phages*

Progeny fraction	^{3}H	^{32}P	^{3}H/^{32}P
Petite moiety	0.78	0.80	0.98
Complete moiety	0.22	0.20	1.10

result in at least one complete undamaged genome, after which a normal pattern of events would ensue and viable progeny phage would be produced (see page 1080 in reference 6 and page 263 in reference 24). Barricelli (2) has argued that the probability of accumulating all the needed crossovers to eliminate every UV lesion to yield one undamaged functional genome is far too small to account for the high efficiency of MRA. In his hypothesis, the irradiated phages would replicate the segments between UV damages, and the resulting partial replicas would be joined by overlaps in homologous regions. Multireplica genomes would, thus, be available for packaging into phage heads. This hypothesis was received with considerable skepticism and ultimately disregarded when it became clear that most T-even genomes consist of double-stranded molecules with no single-strand interruptions (28).

The genomes of petite T4 phages are, in fact, partial genomes, and they do replicate repeatedly after both single (14) and multiple infection (section A of Results). Because in the latter case they do so without interparental recombination (section B, experiment 3 of Results) and produce normal as well as petite progeny phages (Table 2), we propose that they must indeed be undergoing MRC according to a partial replica model. We would modify Barricelli's (2) model for MRA only by proposing that the progeny partial replicas not only join but also finish the process of recombinant formation in overlapping regions by removing redundancies and forming covalently linked continuous strands of DNA. A possible scheme for such events is described by Broker and Doermann (5). The continuous molecule so formed would then serve as the target for encapsidation and the production of mature phage. It does not seem unreasonable to suggest that UV-damaged phages achieve MRA by a similar mechanism. Rayssiguier (Ph.D. thesis; personal communication) analyzed the genetic composition of MRA single bursts resulting from infection by only one phage of each of two UV-irradiated parents that were genetically differentiated at 35 well-distributed sites. In the present context, the significant observation was that, among the 77 MRA progenies tested, no recombinant clones were found that were as large as the parental clones observed in similar bursts originating from unirradiated parents. She concluded that viable phage do not arise from the replication of a lesion-free genome derived by recombination among the UV-damaged parent molecules, but could more easily be explained by a partial-replica model. If the hypothesis proves tenable, that MRA and MRC produce

viable phages by recombination among partial replicas of T4 genomes, then the role of partial replicas in T4 recombination generally should be reconsidered.

The experiments described here that support the partial-replica model, at least for petite genomes, will now be summarized. (i) Net synthesis of DNA and the replication of parental genomes after multiple infection with petites are both indistinguishable from DNA synthesis and replication after multiple infection with normal genomes (section A). This shows that any interparental recombination (that might be required for normal replication of petites) would have to occur efficiently and prior to 5 min after infection, because at that time replication is already proceeding at an effective rate. (ii) No interparental recombination occurs prior to 5 min, nor, for that matter, is it detectable at later times (section B, experiment 3). It is therfore unnecessary. (iii) The DNA of petite parental genomes is transferred to progeny particles with the same efficiency generally observed for the parental DNA of complete phages. Furthermore, it is transferred to both petite and to normal progeny. This indicates that all petite genomes participate normally in the intracellular processes. (iv) Both the HH (BU substituted) and the LL DNA of petites are transferred to progeny with about equal efficiency. It seems unlikely, therefore, that the labeling regime has influenced the results.

The behavior of petite DNA molecules resembles that of completes in many ways; in fact, the two types have not so far been found to differ, except in their physical length. The rates of DNA synthesis in multiple infection, for example, are amazingly similar (Fig. 1). Also, the total absence of interparental recombination is characteristic of both, and both kinds of multiple infections yield petite and complete phages in proportions typical of the mutant ptg191. Another result in which replication of petites is indistinguishable from that of completes is also worth mentioning. In a control experiment in which no density label of any kind was used, a small shift in density of parental radioisotope was observed 5 to 7 min after multiple infection by petite particles. This repeats the earlier observation (as yet not understood) of Miller (18; Ph.D. thesis) made with complete phages and serves here only to point up another characteristic in which petite genomes behave normally.

The apparently normal behavior of the partial replicas produced in petite infections makes it tempting to speculate on the possibility that partial replicas may play a more general role in T4 infections than has heretofore been widely appreciated. The high frequency of recombina-

tion observed in T4, whose map length has been variously estimated from 800 (10) to 2,000 (27) recombination units, may well be related to a partial replica mechanism since repeated copies of fractional genomes would amplify the number of DNA termini, and the ends of DNA molecules are known to be recombinogenic (10).

It is also interesting in this connection that, under certain conditions, the replication of T4 DNA can, indeed, be seen to occur in segments shorter than one genome equivalent. With ligase-negative mutants it has been observed that, in the early stages of infection, both parental and progeny DNA assume sizes equivalent to about 0.2 genome length in neutral as well as in alkaline sucrose gradients (13, 17). The addition of CM at 5 min (which prevents endonucleolytic nicking) allows preservation of this class of subunits, abundant replication of DNA, and, upon removal of CM, maturation of viable phage progeny from each cell (21). Since a large body of data shows that DNA synthesized after CM removal consists of very short pieces (Kozinski, unpublished data), the complete genomes in those progeny particles must have resulted from recombination of partial replicas. Perhaps even more intriguing is the observation that, in wild-type infections, uptake of [3H]thymidine to progeny DNA is, for about the first 7 min after infection, limited to single strands of about 0.2 genome length (Miller, Ph.D. thesis), possibly reflecting the same phenomenon as was observed with the ligase-defective infections.

Because of these observations, we feel justified in the speculation that partial replicas may play a general role in replication and recombination of T4 genomes and are not necessarily limited to such phenomena as MRC and MRA.

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