Molecular Recombination in T4 Bacteriophage Deoxyribonucleic Acid

III. Formation of Long Single Strands During Recombination

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Received for publication 3 November 1969

Evidence was presented to support the hypothesis that long single strands appearing at late times (15 min after infection) are produced as a result of recombination and not as a continuous elongation during the replication process. The production of long strands does not depend on the multiplicity of infection, and the first long strands appear at the time when 20 to 50 phage equivalent units of deoxyribonucleic (DNA) are synthesized, and not earlier. The addition of chloramphenicol at 5 min, which prevents molecular recombination but allows replication of DNA, prevents the formation of long, single strands. Chloramphenicol added between 8 and 10 min after infection, a time at which molecular recombination is fully expressed and covalent repair of recombinant molecules is allowed, does not prevent formation of long single strands. Cutting of single-strand DNA with a limited amount of endonuclease I allows confirmation that the fast-sedimenting characteristic of intracellular denatured DNA is caused primarily by the length of the strands, and not by the formation of aggregates. The computer simulation of two recombination models indicates the feasibility of random breakage and rejoining of molecules in generating long concatenates.

The deoxyribonucleic acid (DNA) molecules of T4 bacteriophage are circularly permuted and terminally redundant (8, 12). Circular permutation can be created by at least two different mechanisms. One would involve forming a physical circle of the DNA during the replicative process. The second process would require, prior to maturation, the formation of molecules much longer than one phage equivalent unit. This would be concluded by excision of molecules of about one phage equivalent unit in length during the maturation process. The long molecules could be created by some mechanism of replication in which the DNA is continuously elongated, or by a mechanism of recombination, which we favor. Since neutral sucrose gradient analysis does not reliably measure the molecular weight of replicative DNA (6), the presence of long single strands in an alkaline sucrose gradient would be a desirable indication that double-stranded DNA longer than one phage equivalent unit does exist in the intracellular DNA pool.

When newly synthesized DNA is labeled with 3H-thymidine during later stages of infection of Escherichia coli B by bacteriophage T4, some of the label is found in material which sediments faster than reference DNA in an alkaline sucrose gradient (1). It has been postulated that this material represents single-stranded DNA which is longer than the single strands which can be isolated from mature phage. Experiments testing this hypothesis are reported here. Net synthesis of DNA was measured simultaneously with the production of long single strands to determine whether the long single strands were formed during the initial stages of replication or arose at later times, concomitantly with the onset of recombination. Furthermore, it will be demonstrated that one can inhibit long single strand formation without inhibiting DNA synthesis.

Studies to be reported here support the contention that the fast-sedimenting DNA, in alkaline sucrose gradients, is indeed composed of single strands longer than those isolated from mature phage. Estimates of net DNA synthesis concomitant with long single strand production indicate that approximately 20 phage equivalent units of DNA are synthesized before a measurable fraction of intracellular DNA enters single strands.

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exceeding in size the reference mature DNA. Addition of chloramphenicol (CM) to infected cells at a time which leads to the inhibition of recombination inhibits the formation of long single strands even though DNA synthesis is not impaired. Furthermore, an insignificant extent of long single strand production can be detected in cells grown in 5-bromodeoxyuridine (5-BuDR) medium even though this condition allows production of viable phage of normal burst sizes. Two models of recombination will be simulated in the computer, and the results will be evaluated in respect to the feasibility of the formation of long molecules as a result of recombination.

MATERIALS AND METHODS
Most of the procedures for density or isotopic labeling and sucrose or density gradient analysis have been described elsewhere (6). Some sucrose gradients were underlayered with a pad of saturated sucrose to guarantee quantitative recovery of the input radioactivity; these gradients were prepared by underlayering 3.5 ml of 5 to 20% sucrose gradient with 1.0 ml of saturated sucrose with a canulating needle attached to a syringe. The lysozyme-Triton X-100 lysis (LTL) method was performed in the following manner. Cells were suspended in a mixture of 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.05 M NaCl, 0.05 M ethylenediaminetetraacetate (EDTA), and lysozyme (100 μg/ml), pH 8.0, and chilled at 4°C for 10 min. Triton X-100 was added to a final concentration of 1%, and the mixtures were chilled for at least 10 min at 4°C before the addition of alkali. The programming approaches for computer simulation will be outlined in the text.

RESULTS
Net DNA synthesis versus long strand. If long single strands are produced simply as a function of replication, they should be detected before two or three phage equivalent units of DNA per bacterium are synthesized. The following experiment was conducted to determine the amount of DNA synthesized before long single strands can be detected.

_E. coli_ B23 was grown to 3 × 10^8 cells/ml in low phosphate TCG medium. The culture was split into two samples; the first one (part 1) was infected with T4 bacteriophage [multiplicity of infection (MOI) = 5.0] labeled with ^32P at a specific activity of 5.0 mc/mg of P. The second sample was (part 2) infected with cold phage (MOI = 5.0). ^32P at a specific activity of 0.5 mc/mg of P was added to the second sample at 2 min after infection. DNA synthesis was measured by uptake of ^32P to trichloroacetic acid-precipitable, alkali-resistant material (9). At various times after infection, samples from part 1 were transferred to KCN and lysed with lysozyme and sodium lauryl sarcosinate (1) or with lysozyme and Triton X-100. At the same time, samples from part 2 were precipitated with trichloroacetic acid and the amount of DNA synthesized was determined after alkali digestion of the samples by procedure of Schmidt et al. (9). The number of phage equivalent units of DNA synthesized was calculated on the basis that 1 μg of P = 5 × 10^8 phage equivalent units. ^3H reference phage were added to the lysates from part 1; the mixtures were treated with alkali and layered on 5 to 20% sucrose gradients which were underlayered with 1 ml of saturated sucrose. The saturated sucrose pad provided quantitative recovery of the material from the gradients.

Alternatively, if progeny strands were labeled with ^32P incorporated after infection (part 2), ^3H reference phage was added, similarly to part 1, but fractions were collected into conical tubes and were alkali-digested (9) prior to being counted in a scintillation counter at various times after infection (Fig. 1). Figure 2 shows the results of sucrose gradient analysis of lysates obtained at corresponding times after infection. This result is independent of the MOI up to at least 15 phages per bacterium. Figure 2 represents the fate of parental ^32P label. Progeny label was analyzed at the same times, and the patterns obtained in sucrose gradient analysis were virtually indistinguishable from those of parental DNA; therefore, they are not documented in this paper.

Inhibition of long strand formation. Kozinski has shown that addition of CM at about 5 min after infection inhibits recombination without impairing DNA replication (6). If long single strands are formed as a result of recombination, the addition of CM to the infected bacteria at this time should inhibit their assembly. Indeed, if CM is added at 5 min after infection and the cells are incubated for 45 minutes, even though 50 to 100 phage equivalent units of DNA were synthesized (a figure at least twice as large as that when long single strands are first formed during normal infection), no long single strands are found (Fig. 3). This indicates that synthesis can proceed without necessarily forming long single strands. In contrast, addition of CM at 8 to 10 min allows the appearance of fast-sedimenting DNA.

When the infected bacteria were pregrown in 5-BuDR, an unmeasurable amount of long single strands was formed in the absence of CM. When CM was added to bacteria infected in 5-BuDR medium, the amount of long single strands formed was much less than in light medium (Fig. 4). When one notes that 5-BuDR-labeled DNA sediments faster than an unsubstituted DNA molecule of the same size (10), the actual amount of long single strands which were
synthesized in the presence of CM decreases further. However, bacteria infected in 5-BuDR medium liberates 5-BuDR-labeled, viable progeny in normal quantities. It appears, therefore, that the formation of long single strands is not an obligatory intermediate of DNA replication in T4-infected cells.

In all cases, the procedure yielding the greatest amount of long single strands was to add CM at 10 to 12 min after infection, a time when all the enzymes necessary for recombination and repair of recombiant molecules were present and yet a time when maturation was inhibited.

**Length of denatured DNA as a factor primarily responsible for fast sedimentation.** Incubation of the infected bacteria with lysozyme prior to alkaline lysis is imperative; without lysozyme present in the lysing solution, material which sediments much faster than reference DNA in alkaline sucrose gradients can be detected even if CM is added at the moment of infection. When this material is incubated with lysozyme, the parental label is found to sediment identically with the reference DNA. The alkali-resistant, lysozyme-sensitive component presumably is an association of the parental DNA with cell wall material. This association takes place even if CM is added at the moment of infection, even if the parental phage is inactivated with ultraviolet light, and it takes place with amber mutants, deficient in DNA synthesis. Figure 5 illustrates the results of alkaline sucrose gradient analysis of one lysate treated in three ways: (i) lysed in 0.25 M KOH, (ii) lysed by the sarkosyl method, and (iii) lysed by the Triton method. The lysate was E. coli B infected with T4BO1r in the presence of CM and was incubated for 30 min before sampling.

One explanation of the lysozyme-resistant, fast-sedimenting material could be that the replicating pool of phage DNA is such a tangled mass of nucleic acid that during alkali denaturation individual single strands become trapped in a "chicken wire-like" mesh which would sediment as an aggregate in alkaline sucrose gradient. A second explanation might be that the association with cell wall material which is sensitive to lysozyme when the trapped DNA is of conservative nature might be resistant when the DNA is in replicative form, since replicative DNA may have a structure which facilitates the association with cell wall material. Recombinant DNA is known to have "puffs," "gaps," and a highly tangled structure (3, 6); this structure might maintain an association with cell wall material which could be more intimate and resistant to lysozyme. This would impart a fast-sedimenting characteristic to the DNA. Two experiments argue against this hypothesis.

First, fast-sedimenting, denatured DNA can be isolated from an alkaline sucrose gradient, dialyzed, and heated for 2 min at 95 C in standard saline-citrate containing 1% formaldehyde, and the material will still sediment as a fast-sedimenting component. A partially renatured, mesh-like structure might be expected to decompose at melting temperatures in the presence of formaldehyde.

Second, if the fast-sedimenting, denatured DNA is incubated with endonuclease I so that the DNA receives, on the average, one to two single strand breaks per phage equivalent unit of length, the fast-sedimenting DNA is cut to short fragments as predicted for a linear molecule. The molecular-weight distribution of the fragments of a molecule which has been randomly cut can be
predicted; furthermore, the sedimentation pattern of these fragments in a sucrose gradient has been simulated in a computer (7). Figure 6 illustrates the predicted distribution of fragments produced by cutting molecules of different length (L = phage equivalents of length) with various average numbers of cuts (λ = number of cuts) per phage equivalent unit of DNA. When one to two cuts per phage equivalent unit have degraded the target molecules, the products all have approximately the same distribution regardless of the original length of the molecule. After one to two cuts per phage equivalent unit of DNA, the products of size 1 molecules will sediment in a sucrose gradient similarly to those of sizes 3, 5, or 15, for example. However, if the target molecules

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**Fig. 2.** Alkaline sucrose gradient analysis of intracellular parental 32P-labeled DNA. Samples from part i of the experiment (described in Fig. 1) were transferred at various times after infection to KCN, chilled, and lysed with lysozyme and Triton X-100. 3H reference phage were added to the lysates and the mixtures were treated with alkali and were layered on 5 to 20% alkaline sucrose gradients which were underlayered with 1.0 ml of saturated sucrose. 3H = reference, integral DNA.
are not linear single-stranded DNA, the products of cutting may not be expected to sediment similarly to reference DNA treated in an identical manner. Figure 7 illustrates the principle of this experiment. If the fast-sedimenting material acquires its sedimentation characteristics from a structure resembling a "chicken wire-like" mesh, it would not be expected to fall completely apart after one to two cuts per phage equivalent unit of length. Also, if the labeled DNA were attached to some lysozyme-resistant, alkali-resistant material, a portion of the label would still sediment fast after cutting. With these ideas under consideration, the following experiment was conducted to test the hypothesis that the fast-sedimenting material was composed of single strands longer than those isolated from mature phage.

_E. coli_ B23 was grown to 3 × 10^8 cells/ml in high phosphate TCG medium and infected with _T4BOir_ (MOI = 5.0) labeled with ^32P at a specific activity of 3.0. Chloramphenicol (100 µg/ml) was added to the infected cells 10 min after infection, and the cells were incubated for 45 min at 37 C. The cells were lysed by the lysozyme-Triton method; ^3H reference DNA was added to the lysates, and the mixture was incubated for 20 min with 0.25 M KOH. The mixture was analyzed in an alkaline sucrose gradient which had been underlayered with a pad of saturated sucrose. ^32P-labeled DNA reaching the pad was isolated, dialyzed against 0.07 M Tris (pH 7.6) and supplemented with single-stranded ^3H reference DNA. This ^32P-labeled DNA was considered to be apparently long single strands. Part of the mixture was reanalyzed in an alkaline sucrose gradient (Fig. 8A); this served as a control which showed that fast sedimenting strands retained their characteristics after the isolation procedure. Part of the mixture was heated to 95 C for 2 min in standard saline-citrate containing 1% formaldehyde; this part was analyzed on a 5 to 20% sucrose gradient containing 1% formaldehyde (Fig. 8B). One can see that such heat treatment does not drastically affect the sedimentation characteristic of the ^32P-labeled DNA. Another part of the fast-sedimenting moiety was incu-
FRACTION OF THE LENGTH OF THE GRADIENT

Fig. 4. Effect of CM on formation of long single strands produced in bacteria infected in 5-BUdR medium. Cells were grown for two generations in 5-BUdR medium and were infected with 32P-labeled T4BO1r. CM was added at various times after infection, and the cultures were incubated for 45 min. The cells were lysed, and alkaline sucrose gradient analysis of the intracellular, 32P-labeled DNA was performed with the 3H integral DNA as a reference.

bated with a very low concentration of endonuclease I. As an internal control, 3H-labeled mature phage DNA in denatured form was added prior to digestion. After treatment with endonuclease I, the mixture was incubated with 0.25 M KOH–0.02 M EDTA for 20 min and then analyzed by alkaline sucrose gradient sedimentation (Fig. 8D). It is obvious from this graph that the 32P- and 3H-labeled DNA sedimented in a similar fashion after treatment with endonuclease I. The apparently long single strands were cut to the size fragments predicted for linear molecules. To test the size of resulting fragments, fresh 32P reference DNA was added in excess to the mixture treated with endonuclease I and the mixture was analyzed in an alkaline sucrose gradient (Fig. 8C). By comparing the distance sedimented by the 32P reference DNA (D1) with the distance sedimented by the 3H DNA (D2), one can determine the number of single-strand scissions received by the DNA per phage equivalent unit of DNA. This can be done by comparing Fig. 8C with Fig. 7 or by using the graph of number of scissions (λ) versus D2/D1 of Litwin, Shahn, and Kozinski (7).

The number of scissions which the DNA received from the endonuclease I was between one and two per phage equivalent unit of DNA. This experiment supports the contention that the fast-sedimenting DNA is actually composed of single strands longer than one phage equivalent unit and argues against the possibility of the artefacts discussed before.

Simultaneous maturation of progeny and parental phage DNA. Some theories of the in vivo replication of T4 bacteriophage DNA predict that labeled parental DNA should appear in mature progeny phage at a rate different from that of labeled progeny DNA. For example, if T4 phage DNA replicated in a continuously elongating concatenate (11), 50% of the original parental label would be extended out at one end. If phage DNA then is encapsulated at this end, 50% of the parental label would be encapsulated in the initial stages of maturation. The “rolling circle” model predicts, on the other hand, that some of the parental DNA resides in a sanctuary attached to
the cell membrane or resides as the core of the rolling circle (2). Parental label would, in this case, be expected to mature at a rate slower than that of progeny phage. In contrast, the possibility that recombination disperses all parental material uniformly throughout the pool of replicative DNA should guarantee that both parental and progeny DNA in the pool should have equal chance of becoming incorporated into maturing phages. To test this hypothesis, parental phage DNA was labeled with \(^{32}\)P and progeny phage DNA was labeled with tritiated thymidine, and then the percentage of each label which became resistant to deoxyribonuclease was determined as a function of time after infection. The experiment was conducted in the following manner. A culture of *E. coli* B23 was grown at 37 C to 3 \(\times\) 10^8 cells/ml in high phosphate TCG medium supplemented with 5 \(\mu\)g of thymidine per ml, 5 \(\mu\)g of 5-fluorodeoxyuridine per ml, and 25 \(\mu\)g of uridine per ml. The culture was infected at an MOI of 5.0 with T4 bacteriophage labeled with \(^{32}\)P at a specific activity of 0.3 mc/mg of P. At 11 min after infection, \(^3\)H-thymidine was added to the culture at a final specific activity of 2 mc/mg of thymidine. At 12 min after infection, the infected cells were chilled, centrifuged, washed with and suspended in high phosphate TCG medium supplemented with 100 \(\mu\)g of cold thymidine per ml. The incubation of the cells at 37 C was continued, and at intervals thereafter samples of the infected bacteria were chilled; one portion of each sample was tested for the presence of mature progeny phage, and one portion was lysed by the lysozyme-Triton method. The lysates were incubated with pancreatic deoxyribonuclease; before and after treatment with deoxyribonuclease, a portion was precipitated with trichloroacetic acid, transferred to a glass-fiber filter, dried, and counted for radioactivity. (A separate test showed that in a mixture

**FRACTION OF THE LENGTH OF THE GRADIENT**

Fig. 5. Effect of lysing procedure on sedimentation characteristics of parental DNA in alkaline sucrose gradients. *E. coli* B23 cells were infected with \(^{32}\)P-labeled T4BO, and CM was added at the moment of infection. The cultures were incubated for 30 min and chilled in NaCl-EDTA. The suspensions were divided into three parts; each part was lysed by a different method: A, lysed in 0.25 M KOH; B, lysed by the sarkosyl method (1); and C, lysed by the Triton method. \(^3\)H reference DNA was added to the lysates, and the mixtures were treated with alkali. The alkali-treated material was layered on 5 to 20% alkaline sucrose gradients underlayered with a pad of saturated sucrose and analyzed as usual. \(^3\)H = reference, integral DNA.
of $^3$H-labeled DNA and $^32$P-labeled phage which was treated in a reconstruction experiment, the $^3$H was rendered completely soluble in 0.3 M trichloroacetic acid, whereas the $^32$P was completely insoluble; i.e., the procedure truly discriminated between DNA outside and inside the phage head. In Fig. 9, the percentage of each label which became resistant to deoxyribonuclease is plotted, as well as the production of mature progeny phage. It is apparent from this graph that the $^32$P and $^3$H become resistant to deoxyribonuclease at almost identical rates; this indicates that the parental and progeny DNA formed prior to maturation become enclosed in the phage head at the same rate.

Computer simulation of recombination and the

Fig. 6. Predicted size distributions of DNA fragments resulting from random breaks. The graphs presented in this figure were produced by the method of Litwin, Shahn, and Kozinski (7). The graphs represent computer-simulated sedimentation patterns of fragments of long molecules. The fragments were produced by cutting different-length "molecules" ($L =$ phage equivalent units of length) with various average numbers of cuts per phage equivalent unit of DNA ($\lambda =$ cuts per phage equivalent unit of DNA).
**Production of long molecules.** Evidence presented in this paper supports the hypothesis previously expressed (6) that recombination between fragments of DNA may lead to the formation of long molecules and ultimately to the production of circularly permuted, mature DNA molecules. It remains to be proven, however, how feasible this mechanism is, from a stochastic point of view.

This question can be approached by the method of computer simulation. The T4 DNA molecule can be represented by a series of numbers (2 x 10^6) arranged in a circularly permuted manner and endowed with a 5% terminal redundancy (7). Two basic hypothetical modes of recombination can be considered: (model 1, cut and strip model) one which would demand double-strand cutting and enzymatic removal of opposite strands, resulting, in effect, in the two original neighbors being unable to recombine with each other [a model similar to that postulated by Thomas (11)]; and (model 2, diagonal cut model) one which will generate recombining fragments by a diagonal cut between two nicks located in the trans position. Nicks or cuts can be introduced into the simulated molecules by a random-number generator by a procedure previously described (7). The program involved in simulating recombination in a simulated cell is as follows. A population of simulated bacteria are infected with a poissonly distributed MOI of 12 phages, 2 light and 10 heavy. (The difference in the density in
the context of this paper plays no role, but will be used for analysis of density classes of simulated recombinants presented elsewhere.) Each of the infecting phage DNA receives a randomly distributed number of nicks or cuts assigned by the random-number generator. After completion of this step, the program calls for separation of each infecting molecule into subunits either by (model 1) introduction of a cut on the opposite strand, which will be followed by the removal of 400 "nucleotides" on two opposite strands (let's say, from simulated 5' end of strands) or (model 2) by performing a diagonal cut between two closest translocated nicks. The resulting fragments are characterized according to their size, number, and sequence of numbers within the open complementary area, a sequence, which being unique, offers no ambiguity. The listing of the resulting subunits and their terminal composition is stored in the memory of the machine. The next step is a simulated recombination which proceeds as follows. The computer retrieves at random one of the fragments and matches it against a randomly retrieved additional fragment out of the pool of fragments in the simulated bacterium. If there is no homology between the two fragments of at least 12 bases, the second fragment is transferred back to the pool. The machine proceeds to pick at random a third subunit, checking it for possible homology. If homology is indeed found, a dimer (recombinant) is formed which is returned to the pool in which further random matching operations are performed until all such possibilities have been exhausted. After completion of the recombinational process, recombinant molecules are characterized according to their lengths, densities, possible unmatched areas, and sizes of gaps and "whiskers." The results of these characterizations are stored in the memory of the machine. After completion of this matching process within the bacterium, the computer keeps tally of the resulting population of fragments and recombinants and proceeds to evaluate another bacterium in a similar manner. The simulation usually involves $10^4$ to $10^6$ simulated bacteria. Upon completion of the experiment, the results are retrieved from the memory of the machine and can be represented in a variety of ways. The machine may be instructed to withdraw the molecules from one bacterium and draw the molecular configurations of the recombinants (Fig. 10). Alternatively, the machine may be instructed to subdivide the resulting fragments in the entire experiment according to their molecular lengths and draw the results as simulated sucrose gradient distribution (7).

In this case, the dispersion of intact molecules in sucrose gradient in our laboratory. The simulation of both models of recombination reveals that whereas the model invoking cutting and denuding the ends, "cut and strip model" proposed by Thomas (11), is not compatible with the formation of long molecules and, for that matter, produces recombinants of short size, the model of diagonal cut produces a sizeable proportion of recombinant molecules which are larger than size one. Significantly, the ends of the participating molecules were not allowed to be engaged in recombinational events, i.e., to be partially denuded. Even without this option, the distribution of observed sizes of recombinants, for a limited number of nicks and limited number of infecting molecules, resembles pretty well the distributions observed in an in vivo experiment.

We do want to emphasize that computer simulation proves the stochastical feasibility of the diagonal cut-type recombination as a mechanism generating long molecules. Scrutiny of the drawing represented in Fig. 10 shows that recombinant molecules obtained by this method contain numerous single-stranded gaps and "whiskers." Those could quite feasibly be filled or excised, respectively, by the proper enzymes. Indeed, electron microscopy of recombinant molecules isolated from CsCl reveals a sizeable amount of "whiskers" and branches (3).

**DISCUSSION**

T4 bacteriophage DNA is circularly permutated and terminally redundant (8, 12). Circular permutation could be achieved by a number of different mechanisms. One mechanism could involve the formation of a circular DNA molecule during the replication process. A second mechanism could involve a long molecule of intracellular phage DNA which would be cut into units approximately one phage equivalent unit in length during the maturation process. The long molecule could be created either through replication of progeny DNA or through recombination. Observation of fast-sedimenting DNA in an alkaline sucrose gradient indicated that long molecules may indeed exist at later times after infection.

Experiments were undertaken to demonstrate that this fast-sedimenting DNA was actually composed of single strands longer than one phage equivalent unit. Because neutral sucrose gradient analysis had been shown to be an unreliable indicator of molecular weight of replicative DNA (6), it was feared that analysis at pH 12.5 might also be unreliable. For example, it was conceivable that DNA sedimented fast in the sucrose gradient at pH 12.5 not because of its length but because of its association with some lysozyme-resistant,
alkali-resistant material, or because individual single strands in the pool of replicative DNA could not, for any number of reasons, be disassociated. Two experiments presented here argue in favor of the hypothesis that the fast-sedimenting DNA was actually composed of long single strands. First, the DNA still sedimented "fast" after reisolation and heating to 95°C. Second, and more important, the apparently long single strands were cut to the size fragments predicted by computer analysis for linear molecules exposed to a low concentration of endonuclease I (a concentration which gave one to two cuts per phage equivalent unit of length). On the basis of any of the proposed artefacts, a residual, fast-sedimenting core of this DNA moiety should have remained after only one to two cuts per phage equivalent unit of single stranded DNA.

Having supported the contention that the fast-sedimenting DNA, at pH 12.5, was indeed long single strands, the next question investigated was whether the long single strands were produced by a mechanism of replication or by recombination. A computer simulation had in fact shown that long molecules could be produced by a mechanism of random breakage and reunion. This should be followed by efficient repair of the single-strand intersections in the polynucleotide chain of the recombinant molecules.

If the long single strands were being produced as a result of recombination, one should expect, first of all, to see the appearance of long single
phages (no replication allowed). The infecting phages were distributed poissonly and received 4, 6, or 8 nicks (also of graph) of percent distributed poissonly). For 6-nicks class, two models of cut and strip model. in line). The cut-and-strip model is not have results should be it labeled by uptake of 3H-thymidine 5 at time would be expected to inhibit the production of long single strands without affecting replication of DNA. Addition of CM around 5 min after infection was known to inhibit recombination without inhibiting replication (6). In fact, addition of CM at 5 min inhibited the production of long single strands, as predicted, even though 45 phage equivalent units of DNA were synthesized in this particular experiment. (The maximum figure, not reported here, can be as high as 100 phage equivalent units.)

Many of the experiments documented in this paper have been performed with a parental label; it should be emphasized here that most of the results have been confirmed also for progeny DNA labeled by uptake of 3H-thymidin or 32P. The inhibition of long single strands by CM without inhibiting DNA replication is important when considering another unlikely but possible explanation of the previous results. If there were 20 hypothetical “sites” for replication in the cell (as might be inferred from the fact that 20 phage equivalent units of DNA are synthesized before any long single strands are formed), and if all the sites had to be occupied by a phage DNA molecule before any long single strands were produced, then it would not be surprising that no long single strands were produced during the first rounds of replication. However, since long single strands are not produced when recombination is inhibited even though 50 to 100 phage equivalent units of DNA are synthesized, this hypothesis is very unlikely.

The absence of long single strands produced by phage-infected bacteria in 5-BUdR medium argues against the hypothesis that long single strands are an obligatory intermediate of DNA replication.
Several results presented here and elsewhere bear directly on various published models of DNA replication. In any model explaining the replication of T4 bacteriophage DNA, the following experimental results should be taken into consideration. A pulse of $^3$H-thymidine incorporated in the initial stages of replication is not covalently bonded to the parental DNA; i.e., parental $^{32}$P and progeny $^3$H labels separate in an alkaline sucrose gradient and are represented by both T4 bands (4). An interesting permutation of this result is that a pulse of $^3$H-thymidine from 3 to 6.25 min after infection is incorporated into a piece of progeny DNA the same size as a piece labeled from 6 to 6.25 min; the size of this piece is about that of the fragment Kozinski called FSBP (3). The size of the fragment labeled from 3 to 3.25 min, however, is much smaller. None of this pulse-labeled progeny DNA was covalently attached to parental DNA. It should be noted that these results were obtained by infection with a $^{32}$P-labeled parental phage, so the integrity of the parental DNA was always assured in these experiments; i.e., the size of the progeny pieces could not have been caused by some random nicking by an enzyme such as endonuclease I, nor could the bulk of the progeny DNA have been separated from parental material by some random-nicking process.

Any model explaining the replication of T4 bacteriophage DNA should be consistent with the result that there is no preferential maturation of parental DNA into progeny phage. This proposal is based on the result that the increase in percentage of labeled DNA which becomes resistant to deoxyribonuclease as maturation proceeds is the same whether the labeled DNA is of parental or progeny origin; the progeny DNA was labeled from 11 to 12 min after infection with a pulse of $^3$H-thymidine. This experiment discriminates against a model based on a continuously elongating concatenate of progeny DNA. This model predicts that when maturation proceeds from the end, 50% of the parental label should be encapsulated immediately at the beginning of the maturation process. Even if parental label were recombined away from the end of the elongating concatenate, some residual label would mature preferentially. Predictions of the rolling circle model (2) of DNA replication do not agree with this result either; no sanctuaries such as the core of the rolling circle or the end attached at a membrane can be occupied by parental label. In addition, there are, apart from physical-chemical controversies, genetic phenomena which are not included in the model of the continuously elongating concatenate, such as unquestionable fact of recombination and the phenomenon of clonal distribution of mutants or recombinants. The best hypothesis fitting the data is that recombination distributes parental label randomly through the replicative pool, so all replicative DNA has an equal chance of being encapsulated. Thus the mechanism generating long molecules is a molecular recombination, a phenomenon ultimately determining circular permutation in T4 DNA.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB 13048 and by Public Health Service grant CA 10055 from the National Cancer Institute. The computer evaluation of data was performed in the University of Pennsylvania Medical School Computer Center, supported by Public Health Service grant FR 15-06. One of the authors (R.C.M.) was supported by Public Health Service grant GM-00694-09 awarded to the Graduate Group on Molecular Biology, University of Pennsylvania.

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