Multiple and Specific Initiation of T4 DNA Replication

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Partially replicated T4 DNA molecules (PRM) whose parental or progeny DNA was labeled with bromodeoxyuridine BUdR was analyzed by gradual shearing followed by CsCl banding of the sheared product. Analysis of PRM containing 18- μ m replicated DNA showed that each replicated region was 3- to 6- μ m long, indicating three to 6 replicative sites per molecule. Analysis of PRM containing 9- μ m replicated DNA similarly indicated two to three replicated regions per molecule. DNA from the replicated regions of PRM containing 10- μ m replicated DNA ("donor") was hybridized to DNA from mature phage ("recipient"), and the resulting hybrid was subjected to digestion with exonuclease I. The extent of protection of the recipient and more efficient self-annealing of progeny fragments from PRM indicated that the replicated regions represented 8 to 10 nonrandom locations of the genome. Possible significance of multiple sites for initiation of DNA replication is discussed.

The molecular details of DNA replication have been studied in several different organisms. The initiation of replication has been demonstrated to occur at a single, genetically fixed origin (4, 6, 16, 21, 26, 27, 29, 30, 33, 34). Within a given replicating region synthesis is usually bidirectional (4, 6, 21, 25, 26, 29-32) except in the case of phage P2 in which replication is predominantly unidirectional (27).

A density labeling study of partially replicated molecules (PRM) of T4 DNA performed in this laboratory (12) suggested that the initial stages of replication involved the addition of progeny DNA to the parental template in several discrete patches. This was confirmed in an electron microscope analysis of PRM by Delius, Howe, and Kozinski (2) which showed multiple replicative loops extending bidirectionally. In contrast, Mosig and Werner (23), Mosig (22), and Marsh et al. (20) have reported experiments with phages containing partial T4 genomes leading to the conclusion that replication proceeds unidirectionally from a unique origin.

Since observation by electron microscopy is necessarily limited to a small number of molecules, it is desirable to study further the structure of the partially replicated molecules by an independent technique which deals with a whole population of molecules. The experimental results described in this paper provide independent physical confirmation of our previous

conclusion (2, 12) that the initiation of T4 synthesis occurs at multiple sites along the molecule. In addition, evidence is provided showing the uniqueness of these sites.

MATERIALS AND METHODS

Strains. Bacteriophage T4BO₁,⁺ and *Escherichia coli* B23 were used for all experiments.

Media and growth conditions. Basic media and growth procedures were as described previously (7, 11, 14). All experiments were performed at 37°C at a cell concentration of 3×10^8 cells/ml in TCG medium. "Light" medium is TCG, "heavy" medium is TCG containing 200 μ g of bromodeoxyuridine (BUdR) per ml, 5 μ g of fluorodeoxyuridine (FUdR) per ml, and 20 μ g of uracil per ml.

Isotope labeling procedures. ³²P- or ³H-labeled phage was prepared as described previously (7, 11). Intracellular DNA was labeled with ³H-thymidine (³H-TdR) in the presence of 5 μ g of FUdR per ml, 20 μ g of uracil per ml, and 5 μ g of cold TdR per ml, all added together with the isotope 3 min after infection. Hybridization of intracellular DNA to T4 or *E. coli* DNA shows that at this time there is no bacterial DNA synthesis in multiply infected cells, and added label is retrieved in phage DNA exclusively (9, and confirmed here). ³H-adenine labeling was performed in the presence of 0.2 μ g of cold adenine carrier per ml (the strain employed is not adenine requiring). Under these labeling conditions normal rates of DNA synthesis and normal phage bursts were observed.

Isolation of PRM. "Heavy" PRM were isolated from light cells growing in light medium infected with ³²P-labeled or cold heavy phage. Newly synthesized DNA was labeled with ³H-TdR added after infection.

"Light" PRM were similarly isolated after infection of heavy cells growing in heavy medium with light ^{32}P -labeled or cold phage. In this case newly synthesized DNA was labeled with ^3H -adenine. Infected cells were sampled at various later times, and DNA was purified by sodium dodecyl sulfate (SDS)-Pro-nase-phenol extraction (15) followed by a preparative CsCl banding (14).

The CsCl analysis of intracellular DNA from heavy cells infected with light phage typically showed a gradual shift in the density of both parental and newly synthesized progeny phage DNA. At progressively later times after infection DNA moved from an original parental light location towards the heavy location. A similar shift, though in the opposite direction, was seen in phage DNA from light cells infected with heavy phage.

PRM were isolated between 4 and 6 min after infection. Fractions of the CsCl gradients were isolated which corresponded to a density intermediate between the original location of the parental DNA and the hybrid location (see Fig. 1A and D), showing that the first round of replication had started, but was not yet complete. (In the context of this paper hybrid DNA is defined as DNA composed of one TdR-containing [light] strand and one BudR-containing [heavy] strand. This hybrid will band halfway between fully light and fully heavy DNA in CsCl ["hybrid position"].) At this stage the parental DNA is still integral, and there is not yet any molecular recombination (14). In fact the entire first round of replication takes place in the absence of recombination and without any breakage of the DNA (B. S. Emanuel, 1972, Ph.D. thesis, University of Pennsylvania). The isolated PRM were quite pure and devoid of contaminating hybrid DNA (see Fig. 1B and E). Panels C and F in Fig. 1 show that the newly synthesized DNA is not covalently associated with parental DNA.

Shearing of DNA. Gradual shearing of DNA was performed at $+4^\circ\text{C}$ in 0.15 M NaCl-0.015 M sodium citrate with a Virtis homogenizer, a Raytheon 10-kc/cycle sonic oscillator, or a Branson probe sonic oscillator. Sizes of resulting fragments were confirmed by alkaline sucrose sedimentation together with appropriate reference DNA (T4 or T7).

DNA-DNA hybridization. Hybridization to DNA bound to nitrocellulose membrane filters was performed by the method of Denhardt (3). Hybridization in liquid was performed in 0.45 M NaCl-0.045 M sodium citrate ($3 \times \text{CS}$) at 65°C for 18 h.

Enzymological techniques. Exonuclease I was isolated from *E. coli* and assayed according to the method of Lehman (17) as modified by Buckley (P. J. Buckley, 1972, Ph. D. thesis, University of Pennsylvania).

The enzyme, as purified and assayed here, is devoid of detectable endonucleolytic activity and free of RNase contamination.

RESULTS

Density gradient analysis of sheared PRM: multiple replicative sites. The experiments described in this section are focused on a

characterization of the structure of PRM of T4 DNA isolated soon after the initiation of the first round of replication to determine whether the replicating T4 DNA molecule contains one or several replicated regions. These experiments are based upon a comparison of the total length of replicated DNA within a molecule with the length of each replicated region, utilizing density and isotope labels to distinguish newly synthesized DNA from parental-injected DNA. The total length of replicated DNA is calculated from the percent progeny contribution in the replicating DNA, which in turn is calculated from the banding position of the molecules in a CsCl gradient: a 50% progeny contribution is found in hybrid DNA which has completed the first round of replication. Here the total length of newly synthesized DNA is equal to the length of parental DNA. The size of an individual replicated region is estimated by gradual shearing of PRM followed by analysis of the fragments in CsCl density gradients. The method is described in detail in Appendix 1. If the size of the fragments after shearing is smaller than that of an individual replicated region, the progeny label will band as a peak at the hybrid density location (see Fig. 9). On the other hand, if the fragment size is large compared to the size of each replicated region, all the progeny label will be recovered at a density intermediate between the positions of parental and hybrid DNA (see Fig. 9). As shown in Appendix 1, the observed CsCl banding pattern of sheared PRM fragments allows an estimation of the average length of each replicated region when the size of the fragments are known. From a comparison of the total length of newly synthesized progeny DNA (from the total progeny contribution) with the average length of each replicated region, it can be determined whether PRM contains one or several replicated regions.

Partially replicated molecules whose total progeny contribution was 15 to 25% were used to analyze the effect of gradual shearing on the density of PRM. The PRM were derived from heavy cells infected with light cold parental T4 phage with ^3H -adenine added after infection, or, alternatively, from light cells infected with heavy cold parental phage with ^3H -TdR added after infection (Fig. 1). In the latter case PRM derived from singly infected (multiplicity of infection [MOI] 0.1) as well as multiply infected (MOI 6) cells were analyzed. A portion of the PRM was sheared to $\frac{1}{8}$ the length of intact DNA (about 16×10^6 daltons and $7 \mu\text{m}$ in length). Another portion of the PRM was sheared to about 0.2- to $0.5\text{-}\mu\text{m}$ length by sonic treatment. The CsCl analysis of the large PRM fragments ($7 \mu\text{m}$) revealed that most of the

FIG. 1. Isolation of PRM. A 0.7-ml amount of intracellular DNA extracted 6 min after infection was added to 2.6 ml of saturated CsCl in $1 \times$ CS, and the gradient was centrifuged 72 h at 29,600 rpm (Spinco SW50 rotor). The gradients were collected in shell vials, and fractions indicated by arrows in panels A and D were pooled for reanalysis. The four panels to the right show reanalysis of the pooled PRM without shearing: B and E native, and C and F denatured. Note that after denaturation progeny DNA bands at the density of pure heavy (upper panel) or light (lower panel) DNA, thus unassociated with parental DNA. Upper panels, PRM from heavy cells infected with light, cold phage; lower panels, PRM from light cells infected with heavy cold phage; ^3H , newly synthesized DNA (adenine or TdR label); ^{32}P , reference light and heavy DNA from mature phage added prior to banding.

progeny ^3H -label assumed a bimodal distribution between hybrid and parental densities. Similar results were obtained whether replication took place in light (Fig. 2A) or heavy (Fig. 2C) medium. In neither case was any hybrid peak

found. The density gradient analysis of the small fragment (0.2–0.5 μm) showed that progeny DNA from both light (Fig. 2B) and heavy (Fig. 2D) PRM banded at the hybrid location. The multiplicity of infection did not influence the

results in that single infection yielded results (data not shown) identical to those illustrated here for multiple infection. For the sake of brevity, the analysis in the following is based on the banding patterns of light PRM (Fig. 1A, B, and C; Fig. 2A and B). The same arguments are then applied to the heavy PRM (Fig. 1D, E, and F; Fig. 2C and D).

The total progeny contribution in the light PRM is about 25% (Fig. 1B), and the total length of newly replicated DNA per PRM is about $\frac{1}{3}$ phage equivalent unit, or approximately 18 μm . If this mass was present in a single unit, shearing of the PRM to 7 μm , which is much shorter than 18 μm , should have resulted in a substantial portion of the progeny DNA label banding at the hybrid location. The observed lack of a hybrid peak (Fig. 2A) rules out a structure containing a single replicated region, as well as a structure containing multiple, closely clustered, replicative areas, or a structure containing one large replicated area and one or more scattered small regions where the large region accounts for most of the progeny label. Instead this analysis proves that the PRM contains multiple replicative regions whose individual lengths are less than $\frac{1}{3}$ of the length of T4 DNA (or 7 μm).

The analysis of the 0.5- μm fragments provides an upper limit to the number of replicated regions per molecule. For example, if the PRM contained 100 equally spaced replicated regions of about 0.18- μm length each, shearing the PRM to fragments of about 0.5- μm length would still produce a peak in CsCl gradient between the parental and hybrid locations, since each fragment would be composed of replicated and unreplicated regions. On the other hand, such shearing of PRM containing replicative regions significantly larger than 0.5 μm would yield a peak of progeny label at the hybrid density. Panels B and D in Fig. 2 show that the majority of the ^3H -labeled progeny DNA in the 0.5- μm fragments banded at the hybrid location whether the original PRM was light or heavy. This result excludes a PRM structure with a large number of replicated regions, each smaller than 0.5 μm .

Thus, in PRM where the total length of replicated DNA corresponds to about 18 μm in length, the newly synthesized DNA is found as several discrete patches, each on the average more than 0.5- μm , but less than 7- μm long. A comparison of the observed density distribution of the 7- μm fragments (Fig. 2A) with patterns expected for different sizes of individual replicated regions (Appendix 1, see Fig. 10) suggests that the patches are 3- to 6- μm long.

An independent estimate of the size of newly

replicated progeny strands in PRM was obtained by alkaline sucrose gradient sedimentation (Fig. 3A). The relative distance sedimented corresponds to a strand length of 4 to 6 μm . Although the accuracy of this estimate is quite low, due to the relatively large distance to the reference, the value is in good agreement with the length inferred from the gradual shearing experiments. When compared to the total length of newly synthesized progeny DNA (in this case 18 μm), the number of replicative regions can be estimated to be about three to six per molecule.

A similar analysis of heavy PRM (Fig. 1D, E, and F; Fig. 2C and D) yields the following results. The average progeny contributions in this case is 14% of the mass, corresponding to a total length of about 9 μm of replicated DNA. The shearing analysis showed that also in this case each replicated area is less than 7 μm and more than 0.5 μm (Fig. 2C and D). The alkaline sucrose gradient analysis (Fig. 3B) indicates that this moiety is similar in size to the progeny DNA from the light PRM. These data, compared to banding patterns expected for different sizes of replicated regions (Appendix 1, Fig. 10), suggests that there are two to three replicative sites per molecule.

One should bear in mind that the experimental results described above cannot exclude the possible existence of additional very short replicated areas which contribute little to the total amount of progeny label in the density gradient.

Hybridization and protection from exonuclease I digestion. In the experiments described below a rather different approach to the question of initiation of DNA replication was employed. The principle underlying these experiments is that, after hybridization of small single-stranded "donor" fragments of DNA to larger single-stranded "recipient" DNA (extracted from T4 phage), double-stranded regions are created on the recipient which protect the recipient from hydrolysis by *E. coli* exonuclease I (see Fig. 4). Depending on the nature of the donor fragments, saturating concentrations of donor DNA will result in complete or less than complete protection of the recipient; if the donor fragments are random, they will effectively cover all parts of the recipient molecules upon hybridization, thus rendering these recipient molecules fully resistant to exonuclease I. On the other hand, if the donor fragments represent a small unique portion of the T4 genome, they will hybridize only to a specific region of the recipient fragments, whereas most of the recipient will remain single-stranded and unprotected, sensitive to exonuclease I. The

FIG. 2. Gradual shearing of PRM. ^3H -labeled PRM isolated from the gradients illustrated in Fig. 1A and D were sheared to about 7- μm length (panels A and C) or 0.2- to 0.5- μm length (panels B and D) and rebanded in CsCl together with ^{32}P -labeled light and heavy reference DNA.

FIG. 3. Alkaline sucrose gradient analysis of PRM. PRM from the gradients illustrated in Fig. 1A and D were sedimented through 5 to 20% sucrose containing 1 M NaCl and 0.2 M NaOH (26,700 rpm, 3 h, SW50) together with integral ^{32}P -labeled DNA from mature T4 phage. A, Light PRM (from Fig. 1A); B, heavy PRM (from Fig. 1D). Strand lengths were estimated from the distance sedimented relative to the distance sedimented by the reference DNA, using the nomogram of Litwin et al. (19).

fraction of exonuclease I-resistant (protected) recipient in the hybrid thus becomes a measure of the randomness or uniqueness of the donor DNA. By using DNA from the replicated areas of PRM as donor in this assay, an estimate of the number of replicative sites may be obtained as well as information whether the replicated regions represent unique portions of the genome. DNA initiated from a random site, single or multiple, should confer as much protection as random donor. A single, unique site for initiation of DNA replication will result in less protection in this assay than multiple, widely spaced, nonrandom initiation sites. The assay is made more sensitive by using as recipient not integral DNA strands, but rather DNA subjected to limited shear, e.g., to yield fragments $\frac{1}{8}$ the size of an intact molecule. DNA from a short single unique replicative area hybridized to DNA strands $\frac{1}{8}$ of the integral size would anneal to only one of the eight recipients (Fig.

4). The recipient protection expected in this case would be about 13%. If the recipient instead were an integral DNA molecule, an average 50% protection would be expected.

Introducing some simplifying assumptions, a relationship between the number of specific sites and the amount of protection can be calculated for any given extent of total replication. It is assumed that all molecules possess the same number of unique, equally sized and spaced replicative sites. It is further assumed that each molecule contains a 15% progeny contribution, or a total of 10 μm replicated DNA. When such small fragments of replicated DNA are hybridized to recipient DNA $\frac{1}{8}$ of the size of integral molecules, the donor fragments will occupy genetically distinct positions which are random with respect to molecular ends of the circularly permuted recipient. Considering all possible permutations the net protection of recipient DNA towards exonuclease I can be

FIG. 4. Schematic representation of random and specific donor DNA fragments hybridized to T4 recipient $\frac{1}{8}$ molecules.

calculated. Figure 5 illustrates the method in the case of four replicative regions. Figure 6 shows a plot of the extent of protection expected versus number of ideal replicative sites. It is obvious from this graph that a large number (more than 16) of quite small replicative areas or even a few large sites and several very small sites would give the same full protection as random DNA. Although the model is based on equal size of all replicated regions, variations in sizes will not affect the observed protection markedly; a small replicated area would yield protection reasonably similar to that from a several-times-larger area.

In the experimental test of the model, a PRM moiety with 15% progeny contribution was used. The PRM were isolated from cells infected with heavy, ^{32}P -labeled phage with ^3H -TdR added after infection. After the first CsCl banding of intracellular DNA the PRM moiety was isolated and sonically treated to 0.2- to 0.5- μm fragments, and the newly replicated (^3H -labeled) portions of the PRM were separated from the unreplicated heavy parental DNA by a second preparative CsCl banding. Fragments of hybrid density (Fig. 7), comprising the replicated DNA, were isolated from this gradient and used as donor in the protection assay. As a control, "random" donor fragments were prepared by sonic treatment of ^3H -labeled mature

phage DNA. Recipient ^{32}P -labeled mature T4 DNA was sheared to $\frac{1}{8}$ molecular length and mixed with the donor fragments. The mixtures were denatured (5 min at 100 C) and allowed to hybridize (at 65 C for 18 h) in $3 \times \text{CS}$. After completion of the hybridization, samples were diluted in exonuclease I assay buffer (17). One portion of these samples was then hydrolyzed by exonuclease I. Another portion was reheated to 100 C, ice-chilled, and then subjected to exonuclease I digestion. This procedure allows a comparison of the amount of hydrolysis of the annealed DNA with the total extent of hydrolysis possible for fully denatured samples, and from this a calculation of net hydrolysis of the donor-recipient hybrid. The extent of protection

FIG. 5. Theoretical protection of recipient $\frac{1}{8}$ molecules from 3'-specific hydrolysis by DNA from four initiation sites, each 5% of the molecular length. The circularly permuted mature recipient molecules sheared to $\frac{1}{8}$ the original size are represented as a circle randomly cut into eight wedges by a pie-cutter. The location of the hybridized donor fragments are specific with respect to the circle (= genetically unique), but random with respect to the cutter. The graph illustrates some possible locations of the hybridized pieces with respect to the cutter, and the extent of recipient protection resulting from each configuration of the depicted strand. Both recipient fragments and donor fragments contain both DNA strands, so that for each illustrated configuration there is a partner configuration with opposite polarity, which is digested from the opposite direction. Considering this, and all other possible configurations not illustrated, an average of 44% of the recipient DNA will be protected in this case.

FIG. 6. Theoretical protection of recipient $\frac{1}{8}$ molecules from 3'-specific hydrolysis by donor DNA from varying numbers of initiation sites. The total amount of replication equals 20% of the molecular length (10 μ m) and is, in each case, divided equally among the number of sites indicated; the replicative sites are uniformly spaced.

of the recipient was then calculated from the net hydrolysis of ^{32}P -labeled material. In addition the extent of self protection among donor fragments was calculated from the hydrolysis of ^3H -labeled material.

Table 1 shows the extent of recipient protection obtained by using a constant, low amount of recipient DNA to which varying amounts of random donor DNA was hybridized. It can be seen that a 40-fold excess of donor fragments saturates the recipient, and a further increase in the ratio of donor to recipient has little effect upon the extent of protection.

Table 2 shows the extent of recipient protection obtained by saturating amounts of donor DNA from PRM fragments. Although in this assay ^{32}P was present both in the recipient DNA and in the parental strands of the PRM fragments, the latter contributed less than 10^{-4} of the total ^{32}P counts in the donor-recipient mixture. Thus, the hydrolysis of ^{32}P -labeled material from the hybrid yields a fair estimate of the extent of solubilization of the recipient. Two different PRM preparations were tested, and with each preparation six to nine separate annealings and digestions were performed. Both PRM preparations gave less protection of the recipient than did the control random DNA; the observed protection with PRM donor being about 75% as effective as that seen with random DNA.

Comparing this result with that expected for different modes of initiation (see above), ran-

dom initiation, a large number of small unique replicative sites, and a single unique initiation site are all excluded. Instead, the data suggest that newly produced DNA is initiated from a limited number of specific sites. A comparison of the observed recipient protection with that theoretically predicted (Fig. 6) for equally spaced, unique replication sites leads to an estimate of eight to ten such sites per molecule. This estimate is, of course, subject to the limitations introduced into the model, which will be elaborated in Discussion.

The extent of self protection among the donor fragments (Table 3) lends additional support to the conclusion that initiation sites are multiple and specific. If one assumes that donor fragments isolated from PRM represent random areas of the genome, the PRM donor fragments should self anneal to the same extent as random donor fragments derived from mature phage

FIG. 7. Isolation of replicated DNA from PRM for the exonuclease protection assay. ^3H -labeled PRM obtained from light cells infected with heavy, ^{32}P -labeled phage with ^3H -TdR added after infection were isolated from CsCl (see Fig. 1D), sonically treated to about 0.2- to 0.5- μ m length and rebanded in CsCl . Fractions corresponding to the peak of hybrid (replicated) material were pooled and used in the assay.

TABLE 1. *Hydrolysis by exonuclease I of recipient 7- μ m fragments of mature T4 DNA to which random 0.5- μ m fragments of donor DNA are hybridized*

Donor DNA concn (μ g/ml)	Donor excess ^a	Protection (%) ^b
2.28	407	87.6
0.228	40.7	80.9
0.114	20.3	61.6
0.0228	4.07	15.0
0.0	0.0	17.0

^a Donor DNA concentration divided by recipient DNA concentration. The latter was 0.0056 μ g/ml.

^b 100% (1-net hydrolysis), where the net hydrolysis is the fraction ³²P-labeled recipient solubilized from the donor-recipient hybrid divided by the fraction recipient solubilized after denaturation of the hybrid.

TABLE 2. *Hydrolysis by exonuclease I of 7- μ m recipient DNA fragments from mature T4 phage to which PRM donor DNA is hybridized*

Expt	Sample ^a	Protection (%) ^b	Protection by PRM/protection by control ^c
I	Control ^d	61.4 $\pm$ 4.2	0.766 $\pm$ 0.285
	PRM ^e	47.0 $\pm$ 17.4	
II	Control ^d	69.5 $\pm$ 4.1	0.72 $\pm$ 0.211
	PRM ^e	49.8 $\pm$ 14.14	

^a Recipient concentration was 0.0047 μ g/ml, and donor concentration 1 μ g/ml (213-fold donor excess).

^b Calculated as in Table 1. Average of 6 (Expt I) or 9 (Expt II) determinations $\pm$ standard deviation.

^c Average $\pm$ standard deviation.

^d DNA from mature phage sonically treated to 0.5- μ m length.

^e The replicated portions of PRM (see Fig. 7).

TABLE 3. *Self hybridization of donor fragments comprising the replicated portions of PRM^a*

Expt	Sample ^b	Protection (%) ^c	Protection of PRM/protection of control ^d
I	Control	37.6 $\pm$ 4.8	2.05 $\pm$ 0.27
	PRM	74.9 $\pm$ 2.8	
II	Control	53.5 $\pm$ 7.0	1.37 $\pm$ 0.36
	PRM	71.6 $\pm$ 11.6	

^a Hydrolysis of donor-recipient hybrids by exonuclease I as in Tables 1 and 2, except that here the hydrolysis of ³H-labeled donor material is considered.

^b See Table 2.

^c 100% (1-net hydrolysis), where the net hydrolysis is the fraction of ³H-labeled donor solubilized from the donor-recipient hybrid divided by the fraction donor solubilized after denaturation of the hybrid. Average of 6 (Expt I) or 9 (Expt II) determinations $\pm$ standard deviation.

^d Average $\pm$ standard deviation.

and thus should be equally resistant, upon annealing, to hydrolysis by exonuclease I. However, if the PRM donor fragments represent specific areas of the T4 molecule, self hybridization among progeny fragments should be more efficient than among random fragments. Thus such PRM donor fragments, after annealing, would be more resistant to exonuclease I hydrolysis than the random fragments. Table 3 demonstrates that upon self annealing the random donor fragments show an almost twofold greater susceptibility to exonuclease I hydrolysis than the fragments from the hybrid moiety of sonically treated PRM at identical DNA concentrations.

DISCUSSION

The experimental results documented in this paper provide evidence that PRM of T4 DNA, isolated shortly after the initiation of the first round of replication, contain multiple replicative sites. The evidence also suggests that these sites are specific. Two different experimental approaches were employed which yielded similar results. In the first approach PRM with labels of different density in the parental and progeny DNA, containing a total length of 9 or 18 μ m of newly synthesized progeny DNA, were characterized by CsCl density gradient analysis after gradual shearing. The absence of a peak at the hybrid density location in gradients of PRM sheared to about 7 μ m in length ruled out the possibility that DNA was synthesized as a single piece at one site on the parental molecule. Instead, the density distribution of this sheared PRM revealed that DNA synthesis occurred at multiple sites and that the nascent DNA at these sites was smaller than 7 μ m. Density gradient analysis of the PRM sheared to a size of about 0.2 to 0.5 μ m showed that the newly synthesized DNA from these multiple sites was larger than 0.5 μ m since the progeny label now was recovered at the hybrid density. This result excludes the presence of a very large number of small replicative regions scattered throughout the molecule.

An estimate of the number of initiation sites is possible by comparison of the experimental results (Fig. 2) with hypothetical banding patterns of sheared PRM models described in Appendix I. The observed distribution pattern of the light PRM (Fig. 2A) is bimodal, showing that the replicated areas are not of uniform size. A comparison with the patterns shown in Fig. 10 suggests that this PRM moiety contains a mixture of regions approximately 3 μ m and some approximately 6 μ m long, the former being more numerous than the latter. This is corroborated by the alkaline sucrose gradient

analysis of this PRM (Fig. 3A). These regions may, of course, be distributed among individual molecules in several ways to yield the following overall results. Some molecules may have three large regions, others six small regions, and still others any combinations between the two extremes yielding a total replicated length of 18 μm per molecule. The differences in sizes may indicate differences in growth rates of individual loops, or, more likely, sequential initiation.

On the basis of the comparison of the hypothetical banding pattern in Fig. 10 with the experimentally observed distributions one can predict that the average number of replicated regions in the light PRM is between three and six.

The density distribution of heavy PRM fragments after shearing (Fig. 2C) approaches that of the model where replicative areas are 3- μm long (see Fig. 10). The distribution of these fragments is more homogenous than that of light PRM fragments, but the shape of the peak indicates again that replicated areas are not uniform in size. In the heavy PRM the total replicated area is on the average 9 μm . The predominance of 3- μm regions in this moiety suggests that each molecule at this stage of replication has on the average slightly more than three replicated regions.

The accuracy of this estimate of the number of replicative sites per molecule is, of course, affected by the assumptions used in the theoretical analysis. (It is implicit in the model described in Appendix I that there are no "hot spots" in PRM more sensitive to shear than others. One may suspect, for instance, that branching points may be more susceptible to shear than normal duplex DNA. This suspicion is, however, not corroborated by electron microscope studies of sheared PRM; fragments of approximately 7- μm length displayed [intact] loops as were found in the nonsheared molecules [2].) In the PRM model it was assumed that within a given molecule replicative regions are equally sized and spaced. Electron micrographs of PRM (2) show that the replicative loops can vary in size and are often unequally spaced along the molecules (2). This would, however, lead to an underestimate, rather than to an overestimate, of the number of initiation sites; small replicative regions, which do not contribute significantly to the total progeny radioactivity, remain virtually unnoticeable in the density gradients. A cluster of small regions, closely spaced, would not be distinguishable from a single replicated region by this method of analysis. The progressive replication at multiple replicative sites in PRM will also yield an

underestimate of the number of replicative regions. In regions where initiation sites are clustered, many small replicative regions may merge into one large replicative loop. An example of clustering and variation in replicative loop size within a single molecule is shown in Fig. 8.

In spite of the shortcomings of this method of analysis, it should provide a reasonable minimal approximation of the number of replicative sites per T4 DNA molecule. We conclude, therefore, that the number of sites of initiation of DNA replication within a molecule of T4 DNA is at least three to six, but more likely somewhat greater.

The second experimental approach to this problem provided further evidence for multiple replicative sites in T4 DNA and, in addition, suggested that these sites are not random. Exonuclease I hydrolysis of recipient DNA strands to which newly replicated DNA from PRM had been hybridized showed that the amount of protection of the recipient from the actions of the enzyme was definitely not consistent with one, specific initiation of T4 DNA synthesis (Table 2). Instead, the results of these experiments suggest that T4 DNA replication is initiated at multiple, but specific points along the genome.

For reasons similar to those discussed in the preceding section, it is difficult, using this particular technique, to determine just how many initiation sites for DNA replication are present in a molecule of T4 DNA. Keeping in mind the assumptions concerning the size and distribution of initiation sites applied to the analysis in Fig. 5 and 6, comparison of this model with the experimental results leads one to predict that T4 PRM molecules containing a total replicated area of 10 μm have about eight to nine specific replicative sites. As was the case in the density gradient analysis of sheared PRM, an uneven spacing of replicative areas as well as variations in sizes of replicative loops will interfere with the interpretations. If two or more such loops were located close to one another or were clustered, analysis by exonuclease I hydrolysis of recipient DNA hybridized with donor fragments from molecules with such clustered replicative areas could not resolve the true number. Such a configuration would protect the recipient molecule in a manner similar to a single replicated area. In some respects, however, the two experimental techniques described in this paper complement each other, and when they are considered together one can arrive at a reasonable estimate for the number of replicative sites on a molecule of T4 DNA.

FIG. 8. *Electron micrograph of a partially replicated T4 DNA molecule. The molecule measures approximately 55 μm in length and was isolated in a manner analogous to that described in the text. The inset is a schematic representation of the molecule showing two "loops" and a former "loop" which has replicated to the end of the molecule.*

The shearing method of analysis led to the conclusion that there were at least three to six replicative sites per molecule. It was mentioned, in connection with these experiments, that this method could not detect some small, widely spaced, replicative sites which together did not

contribute significantly to the total progeny radioactivity. Hydrolysis by exonuclease I of the donor-recipient complexes, on the other hand, is a sensitive method for the detection of small scattered fragments of DNA. Such small scattered replicated regions would provide protec-

tion of the recipient similar to that from larger areas. Therefore, the estimate of three to six replicative sites, obtained by the shearing method, and about eight by the exonuclease protection method, do not appear to be gross underestimates. The difference in the estimates may in part be due to technical differences in the two experimental approaches; in the density gradient analysis only regions contributing significantly to the total mass of progeny DNA are detected, whereas the exonuclease protection assay measures all replicative areas virtually independent of their size. This suggests that in PRM, with a total replicated area of 9 to 10 μm (or 14 to 15% progeny contribution), about five replicated areas are too small to be detected by the gradual shearing assay. One would expect then that, in later PRM, growth at these regions would permit their detection by gradual shearing. The fact that PRM with 25% progeny contribution appear to have a larger number of replicated areas by the shearing assay than does PRM with 14% progeny contribution is in agreement with this assumption.

Both experimental approaches rest on the validity of nucleotide labeling and density shifts as tools to analyze early replicative stages of DNA, and thus on the assumption that short fragments of T4 DNA do not differ in average nucleotide composition from the whole chromosome and that newly synthesized DNA does not differ in buoyant density from bulk phage DNA. The experiments described in Appendix 2 show that this assumption is valid.

The data obtained from the two different experimental approaches described in this paper are in agreement with the previous results of Kozinski and Kozinski (12) and of Delius et al. (2) showing multiple replicative sites in T4 DNA. The evidence presented here also shows that these replicative sites are specific.

A different conclusion has been reached by Mosig and co-workers. Utilizing small T4 particles containing incomplete chromosomes to study DNA replication, Mosig and Werner (23) postulated that T4 DNA replication is initiated from a unique origin and that replication is unidirectional. Mosig (22) and Marsh et al. (20) subsequently mapped this origin in the vicinity of gene 43 by using two experimental approaches, marker rescue of complete *am*⁻ chromosomes by incomplete *am*⁺ chromosomes and genetic transformation by newly synthesized DNA from cells singly infected with phages containing partial genomes. The relative efficiency of marker rescue as well as the frequency of transformation was found to decrease with the distance from gene 43 clockwise around the

genetic map, supporting the conclusion that replication originates at gene 43 and proceeds unidirectionally along the molecule.

Several factors must be considered in evaluating these diverging lines of evidence. Weaknesses in our analyses have been pointed out above; these, however, tend to underestimate rather than overestimate the number of initiation sites. The marker rescue study by Mosig (22) analyzed the final result of replication in the resulting viable mature phage and thus may be difficult to compare with our investigations of the first round of replication. The replication gradient which was observed by Mosig and Werner (23) and by Marsh et al. (20) after single infection with phage containing incomplete chromosomes may be explained by the fact that genes coding for enzymes necessary for phage DNA replication are clustered around gene 43 (5), so that incomplete chromosomes lacking this essential area would be expected to replicate very poorly in single infection, even if several origins were available in the incomplete chromosome. Short fragments of T4 DNA formed after infection of nonpermissive cells with a ligase-deficient amber mutant (FSBP, or first step breakage product, 2×10^7 daltons or 8-9 μm in length) are known to replicate very efficiently (8, 13), despite the fact that four-fifths of them undoubtedly lack a resident gene 43. The presence of genetically complementing fragments in the cells assures the production of the essential gene products in this case.

The transformation assay utilized by Marsh et al. (20) to study DNA replication is not well understood; in particular the mechanism of entry of DNA into the cells and of the ensuing recombination between transforming DNA and DNA from the helper phage are unknown. The overall efficiency of the transformation is quite low, and depends not only on the concentration of transforming DNA, but also on its size (L. A. McNicol, submitted for publication). This size effect varies from gene to gene, producing a gradient with minimal sensitivity to fragmentation around gene 43 and increasing sensitivity in the clockwise direction around the genetic map. The reasons for this gradient in shear sensitivity are as little understood as the transformation itself. At the present stage of knowledge, then, transformation experiments are difficult to interpret, and the transformation gradients with fragments of newly synthesized DNA, which were observed by Marsh et al. (20), may or may not reflect true differences in amounts of DNA copies of different genes.

Initiation of replication at several nonrandom points along the DNA molecule may ultimately

result in a single, unidirectionally moving replication fork, if the initiation points are somewhat clustered. Such unidirectionality of later stages in replication would be enhanced if the replication sites were initiated sequentially. Sequential initiation was considered previously in our discussion of the shearing and exonuclease I experiments and is also suggested by the fact that electron microscopy shows wide variations in loop sizes within the same molecules (Fig. 8). Evidence which will be presented in a later publication (J. Virol., submitted for publication) is in agreement with this hypothesis. Sequential initiation could occur, for instance, if attachment of a specific site on the molecule to some structure, such as the cell membrane, were to trigger the primary initiation of DNA synthesis at that site. Furthermore, transient circularization of the DNA molecules followed by breakage at a unique point would create a uniform, nonpermuted substrate, upon which sequential initiation, beginning at a preferred site, could take place. Although the distribution of the replicative loops in T4 molecules (2) do not show any obvious pattern repeated from molecule to molecule, this analysis is hampered by technical difficulties in electron microscopy of large DNA molecules like T4. A somewhat variable sequential initiation of the specific sites is not in contradiction with our observations.

It should be emphasized that the analysis in this and preceding papers (2, 12) refers only to the first round of replication. We have no evidence supporting a similar mechanism at later stages of replication, after the onset of molecular recombination. On the contrary, electron microscope observations of such late replicative and recombinant DNA have shown highly tangled, branched molecules devoid of any internal loops (8; K. Carlson, unpublished observations).

One may speculate at this point about the existence of multiple points of initiation for DNA replication in various organisms and their significance in the scheme of biological evolution. Evolution among viruses, for example, might take place by genetic recombination. A bacteriophage A would recombine with an autonomously replicating extrachromosomal genome, such as a bacterial plasmid or the DNA of another phage B. Advantageous portions of the plasmid (or phage B DNA) can be retained by phage A and become genetically stable, whereas subsequent recombination, or deletion, could serve to eliminate the undesired or redundant portions. Since the bacteriophage A possesses a site(s) for initiation of DNA replication, and the plasmid DNA (or phage B DNA)

presumably also contains such a site(s), the organism resulting from this recombination now contains two (or more) independent sites for initiation of DNA replication. Repetition of this process could result in the evolution of small viruses to larger viruses containing multiple initiation sites. T4 bacteriophage could be the product of such a scheme of evolution. It has quite a large genome for a bacteriophage. Evidence presented here indicates that T4 phage DNA does contain multiple and specific sites for the initiation of DNA synthesis, whereas DNA of those smaller phages (such as lambda, P2 and T7), which have been investigated with respect to this point, appear to contain only a single site for initiation of DNA replication (4, 26, 27, 29).

One additional phenomenon associated with DNA replication in T4 phage might serve as suggestive evidence for this hypothetical scheme of viral evolution. As mentioned above, the parental DNA of ligase-deficient amber mutants of T4 phage is broken to FSBP fragments of about 2×10^7 daltons in nonpermissive cells (8). Similar size fragments, which however are not as well characterized, are found in several other T4 systems (10, 28; K. Carlson, 1971, Ph.D. Thesis, University of Pennsylvania). The FSBP fragments are uniform in size and, at the time of their formation, devoid of internal breaks. Their size indicates that each intact molecule produces six to seven FSBP fragments, a number in remarkable agreement with the number of initiation sites for DNA replication deduced from the present investigation. The property of FSBP which is relevant for this discussion is that these fragments replicate autonomously (8, 13). It is tempting to use FSBP as an illustration of the evolutionary past history of the T4 genome. If T4 bacteriophage were an advanced recombinant, containing a number of previously autonomous genetic elements, the ability of the individual FSBP fragments to initiate and sustain replication and to mature into viable progeny phage under suitable conditions might be viewed in this context as a recapitulation of T4 phylogeny.

This model predicts that highly evolved DNA molecules should contain several possible sites for initiation of DNA replication. However, this appears not to be true for *E. coli* (21, 25, 32). It is likely that the existence of such multiple initiation sites would be disadvantageous for the regulated growth of this organism. Consequently, its extra initiation sites might either be suppressed by a protein, e.g., anti-initiator, or be deleted by further recombinational events. Evidence pointing to the existence of such an anti-initiator protein in *E. coli* has recently been described by Blau and Mordoh (1).

Multiple, independent initiation sites in a DNA molecule might offer an advantage for virulent phage to overcome possibly the effect of host anti-initiators which would prevent their replication. Foreign genomes entering a cell may or may not respond to these resident anti-initiators. Whereas the latter case would lead to virulent growth and the death of the infected cell, the former would lead to an integrated prophage, or episome.

Several cases are known where a DNA molecule may be replicated from an auxiliary or foreign origin when its normal origin is suppressed or nonfunctional. In bacterial cells lysogenic for various temperate phages the phage initiation site is repressed and the prophage genome is replicated from the host origin. Similarly, integrated episomes are replicated passively along with the host chromosome. If the bacterial DNA replication is prevented by a temperature-sensitive mutation on the *dna A* gene, replication of the *E. coli* DNA chromosome may come under the control of the integrated episome, or prophage (Y. Hirota, J. Mordoh, I. Schleffer, and F. Jacob. Fed. Proc., p. 1422-1427, 1972; references 18, 24), possibly by utilizing the episome (or prophage) origin to initiate the entire chromosome.

In accord with the hypotheses discussed above, one can postulate that a possible mechanism for viral oncogenesis is the introduction of viral initiation site(s) into host DNA. Such initiation sites might be immune to normal host control mechanisms (anti-initiator?). The initiation of DNA replication from such viral initiation site(s) would lead to uncontrolled, unrestricted, DNA synthesis, cell division, and ultimately the de-differentiation and neoplasia. To our knowledge such a hypothesis has not been proposed.

There is no evidence either for acquisition of multiple points of initiation of DNA synthesis by recombinational evolution, or for oncogenesis by viral control of the regulation of cell DNA replication, and it is not the intent of this discussion to produce such evidence. However, speculations of this nature can be useful not only in the synthesis of ideas, but also as a possible guide for future experimentation.

APPENDIX

1. **Analytical approach for obtaining simulated CsCl banding patterns of sheared idealized PRM containing varying lengths of replicated DNA.** A PRM model is considered where parental strands and progeny strands have different densities (specifically, parental is "light" and progeny is "heavy"; an identical model could of course be derived from the reversed density label) (see Fig. 9). Only progeny strands are radioactive. This PRM is sheared by a cutter with two blades separated by a distance M which advances from one end of the PRM toward the other end by increments of distance l , producing a finite number of nonidentical fragments. The distance l is very small compared to M .

In the model, it is assumed that: (i) multiple replicated regions are spaced evenly along the molecule, and (ii) all replicated regions are of equal length L .

The fate of all fragments containing heavy radioactive (progeny) DNA is considered as follows.

(A) **Replicative regions larger than the size of sheared fragments ($L > M$).** Considering Fig. 9 (upper model), two classes of fragments are produced: (a) Fragments with hybrid density. The total number of fragments in this class is:

$$n_a = 1 + \frac{L - M}{l} \quad (1)$$

FIG. 9. Hypothetical PRM structures, on which the analysis in Appendix 1 is based.

(b) Fragments with densities intermediate between those of parental and hybrid fragments. The total number of fragments in this class is:

$$n_b = \frac{2(M-l)}{l} \quad (2)$$

Fragments in the first class are uniformly radioactive. The amount of radioactivity found in this class is:

$$r_a = M \times n_a = M \left(1 + \frac{L-M}{l} \right) \quad (3)$$

Fragments in the second class will contain radioactivity in proportion to their density. Those having a density closest to that of hybrid DNA will have most radioactivity, whereas those of parental density lack radioactive material. Consequently, the average contribution per fragment in this class will be $\frac{M}{2}$ and the total radioactivity:

$$r_b = \frac{M}{2} \times n_b = \frac{M(M-l)}{l} \quad (4)$$

From equations 3 and 4 we obtain the fraction of radioactivity expected at the hybrid density:

$$R_{\text{hybrid}} = \frac{r_a}{r_a + r_b} = \frac{L-M+l}{L} \quad (5)$$

Remaining radioactivity will be distributed uniformly among fragments with densities intermediate between those of parental and hybrid fragments. The fraction of radioactivity in the two fragments (one at each fork) in class (b) with the density closest to hybrid density will be approximately:

$$R = 2 \times \frac{R_{\text{hybrid}}}{n_a} \quad (6)$$

and remaining fragments in this class will form a linearly decreasing gradient of radioactivity from the density closest to hybrid to parental density.

(B) **Replicated regions equal to or smaller than sheared fragments ($L \leq M$).** Again two classes are found: (c) Those containing a whole replicated region, having uniform density. The number of fragments in this class is (see Fig. 9, lower model):

$$n_c = 1 + \frac{M-L}{l} \quad (7)$$

and their density is:

$$\rho_c = \frac{M}{M+L} \rho_{\text{par}} + \frac{L}{M+L} \rho_{\text{prog}} \quad (8)$$

(d) Those containing less than a whole replicated region, having densities distributed between those of parental and of class (c) fragments. The number of fragments in this class is:

$$n_d = \frac{2(L-l)}{l} \quad (9)$$

The weighting factors for the amount of radioactivity associated with class (c) and class (d) fragments is obtained in the same way as in case A; they are L and

$L/2$ respectively. From equations 7 and 9 the fraction of radioactivity expected at the greatest density is:

$$R_{\text{max}\rho} = \frac{L \times n_c}{L \times n_c + \frac{L}{2} \times n_d} = \frac{M-L+l}{M} \quad (10)$$

The fraction of radioactivity in the two fragments in class (d) with the density closest to that of fragments in class (c) is:

$$R = 2 \times \frac{R_{\text{max}}}{n_c} \quad (11)$$

and remaining fragments in this class form a linearly decreasing gradient of radioactivity from this density to parental density.

Utilizing equations 5, 6, 8, 10, and 11 we can calculate the expected density distribution of radioactive fragments produced by gradual shearing. Distributions expected for 3- μ m, 6- μ m, 9- μ m, and 18- μ m long replicative regions after shearing PRM to fragments 7- μ m long are illustrated in Fig. 10.

2. Random nucleotide distribution within 0.04- μ m length of T4 DNA and identical buoyant den-

FIG. 10. Hypothetical banding patterns for PRM containing varying numbers of replicative sites per molecule. Density distributions of fragments containing progeny label were calculated from equations 5, 6, 10, and 11 in Appendix 1. Values of the parameters were $M = 7 \mu\text{m}$, $l = 0.1 \mu\text{m}$, $\rho_{\text{par}} = 1.70 \text{ g/cm}^3$, and $\rho_{\text{prog}} = 1.80 \text{ g/cm}^3$. Different values of L were used as indicated in the figure.

sity of newly synthesized DNA in PRM and mature T4 DNA. The interpretation of experiments described in this paper rests critically on the assumption that short stretches of phage DNA synthesized during the initial stages of replication do not differ in buoyant density from bulk phage DNA. The validity of this assumption was verified as follows:

(i) **Nucleotide distribution.** The distribution of base pairs within a DNA molecule is, obviously, nonrandom. On a relatively short length of DNA, representation of all bases might resemble the average base composition of the entire molecule. On the other hand, the possibility of consecutive, repetitious runs of base pairs, and the accumulation in larger than average quantities of any of the base pairs cannot be excluded. During labeling of short replicative fragments with BUdR or ^3H -TdR, incorporation of those labels might be affected if some of the short fragments are considerably different in base composition than the others. This can, in effect, invalidate the application of BUdR incorporation as a density label in short pulses, or ^3H -TdR incorporation as a measure of synthesis. Moreover, the existence of short segments of DNA, having nonaverage base composition, may make the CsCl distribution of products of sonic treatment uninterpretable.

To test whether such extensive nonrandomness occurs in T4 DNA, phage DNA was labeled simultaneously with BUdR (fed for two generations prior infection and during the lytic phage growth), ^3H -adenine, and ^{32}P -orthophosphate (added after infection). Of the two isotopes simultaneously incorporated into the phage DNA, ^{32}P has equal probability to be incorporated with any of the four nucleotides, while ^3H is incorporated into adenylic acid only and thus marks adenine-thymine (A-T) pairs, or, in this case, adenine-bromouracil (A-BU) pairs, exclusively. The presence of BU instead of T increases the density of A-BU rich fragments to far greater than that of G-C rich fragments. Therefore, a nonrandom distribution of base pairs over short lengths of DNA will be manifested in a different density distribution of fragments containing ^3H (A-BU rich) and ^{32}P (total population). The increased density of fragments rich in A-BU will cause an increase in the specific activity of $^3\text{H}/^{32}\text{P}$ towards the bottom of the gradient.

The deoxyadenylic and bromodeoxyuridylic residues in an A-BU-rich fragment may be equally represented on both strands. Alternatively, one strand may be richer in A and the other correspondingly in BU. Denaturation of the fragments will permit a distinction between the two alternatives, since strands richer in A (^3H -labeled) and poorer in BU will after denaturation change position in the CsCl gradient from a denser than average (average represented by ^{32}P) to lighter than average.

The heavy phage resulting from the triple-labeling experiment (^3H -ade, ^{32}P -P_i and BUdR) were purified by two cycles of differential centrifugation (11), and its DNA was extracted with phenol. Figure 11 shows

FIG. 11. CsCl analysis of DNA from phage labeled simultaneously with ^3H -adenine, BUdR, and ^{32}P -P_i. A, Integral, native DNA; B, DNA sonically treated to about 10^6 daltons, native; C, same DNA as in B,

denatured. Note the perfect overlap of the isotope in all panels. Recovered activities and ratios between isotopes are tabulated in Table 4 to facilitate evaluation of the data.

[illegible]

TABLE 5. *Precipitation of PRM with antibodies against α - or β -glucosylated DNA^a*

Sample	DNA Precipitated (% of input)	
	α^b	β^c
Parental (³² P)	61.0	34.6
Progeny (³ H)	57.1	34.1

^a According to McNicol and Goldberg (J. Mol. Biol. submitted for publication), the sera used for these experiments were extensively cross-absorbed and are completely specific for the two glucosyl anomers.

^b Antibodies against α -glucosylated T4 DNA.

^c Antibodies against β -glucosylated T4 DNA.

remove bacterial DNA. The PRM were then sonically treated, denatured, and precipitated with antibodies against α - or β -glucosylated T4 DNA (22). Table 5 shows that the newly synthesized ³H-labeled DNA and the parental ³²P-labeled DNA were precipitated to equal extent.

(iii) **Incorporation of BUdR into heavy DNA.** Different efficiencies in BUdR uptake at early and late times after infection may similarly interfere with buoyant density analysis. However, the density gradient analysis of denatured PRM (Fig. 1C) showed that newly synthesized heavy DNA co-banded with the heavy reference DNA extracted from mature phages, proving equal efficiency of BUdR uptake. This is corroborated by the fact that sonic treatment of PRM where the newly synthesized DNA is labeled with BUdR produced fragments banding at hybrid density, as defined by the banding positions of heavy and light DNA from mature phage (Fig. 2B).

The combined results described in this Appendix demonstrate that there are no differences in buoyant density between newly synthesized DNA in PRM and mature DNA, which could affect the analysis of early replication by methods employed here.

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