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# Multiple Initiation of Bacteriophage T4 DNA Replication: Delaying Effect of Bromodeoxyuridine

KARIN CARLSON[1]

*Department of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104*

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Effects of bromodeoxyuridine (BUdR) substitutions in phage T4 DNA on the initial stages of DNA replication were investigated. Electron microscope studies of partially replicated, light (thymidine-containing) T4 DNA revealed the presence of multiple loops and forks. These DNA preparations had no BUdR in either parental or newly synthesized DNA, and the observations thus show that multiple initiation of DNA replication is a normal event in T4 development and is not caused by the presence of BUdR. A comparison of early replicative stages of light and heavy (BUdR-containing) DNA in cells mixedly infected with light and heavy T4 phage showed that early DNA synthesis occurs preferentially on the light template. Heavy and light parental DNA became associated with the protein complex of replicative DNA with equal efficiency, and there was no effect of BUdR on the net rate of DNA synthesis after infection. Newly synthesized DNA from heavy templates sedimented more slowly through alkaline sucrose gradients than did newly synthesized DNA from light templates and appeared to represent fewer replicative regions per molecule. These data indicate that BUdR substitutions in the DNA caused a slight delay in initiation but that replication of heavy DNA proceeded normally once initiated.

Several lines of evidence indicate that the initiation of bacteriophage T4 DNA replication takes place at multiple sites along a molecule. Utilizing bromodeoxyuridine (BUdR) to separate replicative and nonreplicative DNA moieties, Kozinski and Kozinski (11) observed that partially replicated DNA molecules (PRM), upon shearing to a size smaller than the total length of replicated DNA, did not yield fragments of either parental or hybrid density. (Hybrid DNA is defined as DNA which has completed the first round of replication. In a density-labeled system, hybrid DNA consists of one thymidine [TdR]-containing [light] strand and one BUdR-containing [heavy] strand, and bands between fully light and fully heavy DNA at equal distance from either in a density gradient.) Later investigations by Delius et al. (2) and by Howe et al. (7) gave similar results whether BUdR was present in the parental DNA or in the newly synthesized progeny DNA, and whether the PRM resulted from single or multiple infection. Delius et al. (2) found that PRM mounted for electron microscopy displayed multiple loops and forks, which were interpreted as replicative structures. The analysis of the CsCl banding patterns of the sheared fragments in the preceding paper (7) suggested that there are at least three to six initiation sites in a DNA molecule. Examining the extent of protection from exonuclease I digestion of T4 DNA strands to which small DNA fragments had been hybridized, Howe et al. (7) found less protection by fragments containing the replicated areas of PRM than by random DNA fragments. They concluded that the multiple replicative sites in each DNA molecule were not random.

The fact that these experiments (2, 7, 11) yielded very similar results, irrespective of whether the BUdR label was in the parental strand or in the newly synthesized strand, quite convincingly suggested that the observed multiple initiation was not an artifact due to the presence of BUdR. It is known, however, that

[1] Present address: McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wis. 53706.

BUdR-substituted DNA differs in some physical properties from normal TdR-containing DNA. These differences are manifested in, e.g., increased light sensitivity (21) and higher melting temperature (10). It seemed unlikely to us that such physical properties could interfere with the number of initiation sites for DNA replication, yet it could be argued that the results obtained through the the different experimental approaches outlined above were due to "false" initiations caused by BUdR. The primary objective of this paper is to establish that multiple initiation of DNA replication is a normal event in T4 development, whether or not BUdR is present in the system. This objective is achieved in the first part of the paper, which describes an electron microscope study of replicative DNA from an experiment where no density label was utilized.

In the second part of the paper, a detailed comparison of the replication of BUdR-labeled (heavy) and non-BUdR-labeled (light) DNA is made. This investigation shows that the initiation of replication of heavy DNA is slightly delayed as compared to the initiation of replication of light DNA. Possible mechanisms underlying this observation are discussed.

## MATERIALS AND METHODS

Strains, media, and growth conditions were the same as in the preceding paper (7).

**Isotope labeling procedures.** To obtain heavy (BUdR-substituted) and light (not BUdR-substituted) $^{32}$P-labeled phage of the same specific activity, $^{32}$P-containing light TCG (8) of desired specific activity was prepared and then divided into two parts. To one part, a BUdR "package" giving final concentrations of 200 $\mu$g of BUdR per ml, 5 $\mu$g of fluorodeoxyuridine (FUdR) per ml, and 20 $\mu$g of uracil per ml was added. This package contributed a negligible volume to the medium. *Escherichia coli* bacteria were pelleted from a fresh log-phase broth culture and resuspended in the radioactive media to allow one to two generations growth to a density of $3 \times 10^8$ cells per ml before infection. Radioactive phage was isolated and purified by differential centrifugation as previously described (8). In some experiments, the radioactive phage was further purified by CsCl banding; this step had no noticeable effect on the results. Viability was calculated from the titer of PFU divided by the particle titer. The latter was determined from the $^{32}$P content of the phage (1 $\mu$g of phosphorous = $5 \times 10^{10}$ T4 phage-equivalent units of DNA).

Intracellular DNA was labeled with $^3$H-TdR in the presence of 5 $\mu$g of cold TdR per ml, 5 $\mu$g of FUdR per ml, and 20 $\mu$g of uracil per ml, and with $^{32}$P in the presence of 5 $\mu$g of cold phosphorous (as phosphate) per ml.

**DNA extraction and gradient analyses.** Cells were lysed either with sodium dodecyl sulfate (SDS) (12) or with lysozyme and Triton X-100 (17). The lysates were digested with Pronase, and the DNA was extracted with phenol (12). CsCl and sucrose gradient centrifugation were performed as previously described (13, 17). $Cs_2SO_4$ equilibrium density gradients contained 1.1 ml of DNA solution ($\leq 2.5 \times 10^9$ *E. coli* cell equivalents of DNA in 0.15 M NaCl-0.015 M EDTA, pH 8.0, or 0.15 M NaCl-0.015 M sodium-citrate) per 1.5 ml of saturated $Cs_2SO_4$ in water; these gradients were spun for 72 h at 25,000 rpm in a Spinco SW50 rotor. Fractions to be reanalyzed were pooled and dialyzed 4 to 6 h against 0.15 M NaCl-0.015 M sodium-citrate (for gradient reanalysis), or against 1 mM EDTA, pH 7.5 (for electron microscopy).

**Electron microscopy.** DNA was mounted for electron microscopy by using the formamide technique of Westmoreland et al. (22) as modified by Davis et al. (1) and by Delius et al. (3). The hyperphase consisted of 0.05 to 0.3 $\mu$g of DNA per ml in 0.1 M Tris-0.01 M EDTA (pH 8.5), 0.1 mg of cytochrome *c* per ml, and 40% formamide. The hypophase contained 10% formamide in 10 mM Tris-1 mM EDTA, pH 7.5. The buffers were adjusted with HCl. The grids were observed in a Siemens Ia electron microscope using an accelerating voltage of 60 kV and an instrument magnification of 4,000 to 10,000.

## RESULTS

**Observations of multiple loops in non-BUdR-labeled replicative T4 DNA by electron microscopy.** The use of BUdR-labeled infecting phage allowed not only a very efficient separation of phage DNA from bacterial DNA, but also the separation of non-replicative phage DNA from the activity replicating moiety (2). This ascertained a good proportion of replicative structures on the electron microscope grids (2). In the absence of a density label, the removal of bacterial DNA is more difficult, and it is not possible to separate nonreplicative and replicative phage DNA.

We found that a fairly shallow $Cs_2SO_4$ gradient of the type described by Erikson and Szybalski (5) gives adequate separation of non-BUdR-labeled T4 DNA and *E. coli* DNA (Fig. 1). It is possible to isolate light phage DNA virtually free from contaminating bacterial DNA in preparative gradients of this type. The high molecular weight of the bacterial DNA, which persists approximately until the time when hybrid phage DNA appears, results in a very viscous band of bacterial DNA in the gradient that is easily noticed during fractionation. These viscous fractions are then avoided when the radioactive peak of phage DNA is pooled for reanalysis.

Samples of light T4 DNA for electron microscopy were obtained after infection of light bacteria with light $^{32}$P-labeled phage at a multiplicity of 10 particles per cell. The culture was divided into two parts. Part A was left without

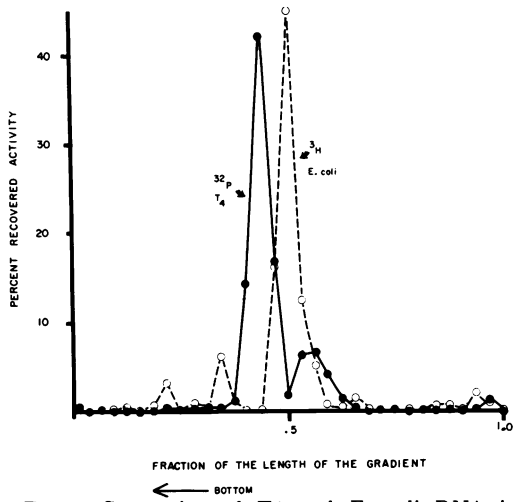


FIG. 1. *Separation of T4 and E. coli DNA in $Cs_2SO_4$. Bacteria were grown for one generation in light TCG containing $^3$H-TdR, FUdR, and uracil to a density of 1.5 × $10^8$/ml. They were sedimented and resuspended in light TCG containing 100 μg of cold TdR per ml, and incubation was continued. At a cell density of 3 × $10^8$, the culture was infected with light, $^{32}$P-labeled T4 phage. Five minutes after infection, the cells were lysed, and DNA was extracted and banded in $Cs_2SO_4$.*

further additions. BUdR, FUdR, and uracil were added to part B immediately after infection. At various later times, samples were withdrawn, and intracellular DNA was extracted and banded in $Cs_2SO_4$. The analysis of part B showed normal timing of the intracellular development, i.e., PRM were found 5 to 6 min after infection, and the first hybrid was detected 7 min after infection. Samples isolated from part A 4 to 6 min after infection were chosen for electron microscopy.

From the $Cs_2SO_4$ gradients, fractions corresponding to the peak of phage DNA including one or two fractions on the heavy side of the peak, but avoiding the light side of the peak, were pooled, and after dialysis the DNA was mounted for electron microscopy. Although most of the observed molecules did not show any evidence of having started replication (cf. Fig. 4 and Table 1), replicative structures proved frequent enough to be studied. Figure 2 shows some representative molecules. Multiple loops and forks were seen which were similar to those observed during the early stages of replication of heavy DNA (2). A statistical comparison of the structures observed here with those seen in the heavy PRM (2) is not possible due to the complex structure of integral, replicative T4 DNA which leads to a pronounced tendency of tangling. Electron microscope observations are heavily biased in favor of the few untangled molecules that are found which do not necessarily form a representative sample of the population. Thus, while there are no doubts that multiple replicative areas are found in non-BUdR-labeled PRM as in BUdR-labeled PRM, no conclusions can be based on a comparison of our light PRM with the heavy PRM studied by Delius et al. (2), even though the two moieties were isolated from very similar systems at comparable times after infection.

With these reservations in mind, a comparison suggested that the replicative structures in the light PRM were larger in comparison to those seen in heavy PRM (2), and most loops observed were found in molecules having more than one replicative structure. Such differences, if true, could result simply from differences in timing, if the light PRM represents a somewhat later stage than the heavy PRM of Delius et al. (2). On the other hand, the observations could reflect a further advanced replication and a higher incidence of multiple replicative areas in non-BUdR-labeled PRM than what is found in BUdR-labeled PRM isolated at the same time after infection. If this were true, BUdR interferes with replication not by causing "false" initiations, but by inhibiting early DNA synthesis.

**Comparison of the initial replication of heavy and light DNA: biased uptake of $^3$H-TdR to light phage DNA.** To explore the possibility that BUdR interferes with the initiation of DNA replication, PRM from cells mixedly infected with light and heavy phage were studied. This procedure allows an accurate comparison of the initial events in replication of the two DNA types. PRM were analyzed by density and velocity gradients, in addition to electron microscopy, to avoid the observational bias and compare the entire populations of light and heavy parental DNA in the cells.

Light bacteria were infected with heavy and light $^{32}$P-labeled phage of the same specific activity of $^{32}$P at multiplicity of infection (MOI) of 5 of each phage type. Three minutes after infection, $^3$H-TdR was added to the culture to label newly synthesized DNA. Previous investigations have showed that $^3$H-TdR added at this time is recovered mostly in phage DNA (7, 9). At various later times, the intracellular DNA was extracted from the infected cells for analysis. One portion of extracted DNA was banded in CsCl to estimate the extent of replication of the heavy parental DNA (this gradient type provides a better separation of BUdR-labeled and non-BUdR-labeled DNA than does $Cs_2SO_4$), as well as the relative amounts of

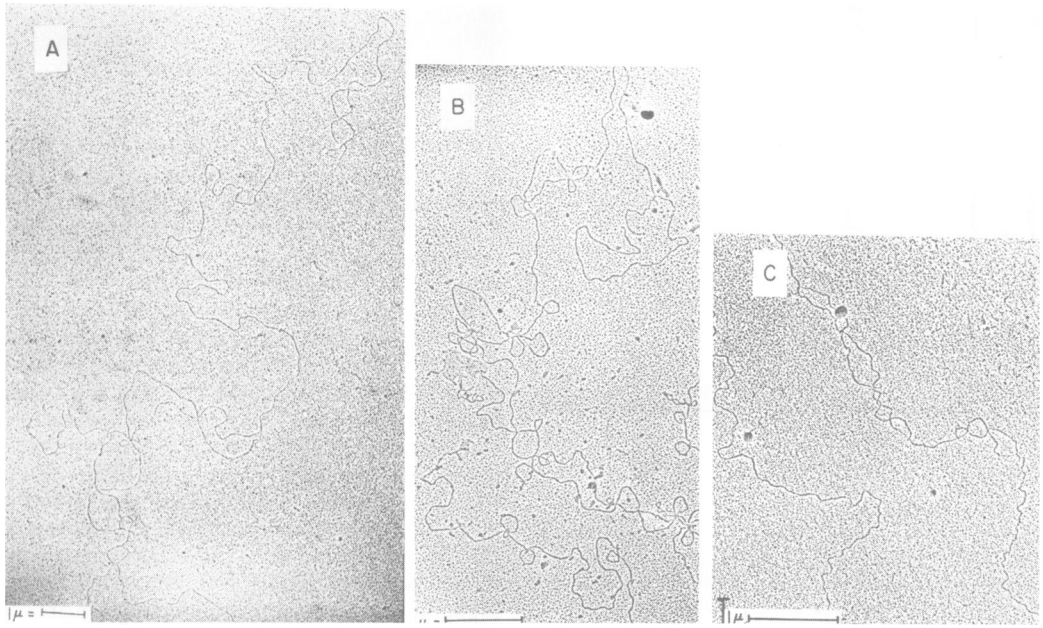


FIG. 2. *Electron micrographs of partially replicated light T4 DNA obtained 4 min (A and C) or 5 min (B) after infection. Note multiple loops as well as terminal forks in the molecules.*

$^3$H-labeled newly synthesized DNA associated with either parental type of DNA. Another portion was banded in $Cs_2SO_4$ to enable isolation of PRM for reanalysis.

The majority of the heavy parental DNA does not initiate replication until about 6 min after infection (Fig. 3, solid lines, $^{32}$P). Already at 7 min after infection there is significantly less $^{32}$P in the heavy area than originally, and 10 min after infection there is virtually no $^{32}$P in the heavy location. This proves that all the heavy parental DNA did participate in replication.

The newly synthesized DNA (Fig. 3, broken lines, $^3$H) shows a bimodal distribution, as expected. There is, however, significantly more $^3$H in the light region of the gradient than there is in the heavy region. This is particularly conspicuous in the earliest samples where there is not yet any noticeable replication of the bulk heavy parental DNA, suggesting a bias in $^3$H-uptake, or DNA synthesis, which favors the light parental DNA. A quantitative expression of the bias is calculated in Table 1. The activities of $^3$H and $^{32}$P recovered in the gradients in the fractions indicated in the panels of Fig. 3 were summed, and the sums were expressed as ratios between light and heavy regions. Column 5 in Table 1 shows the ratio between $^{32}$P in the light area and $^{32}$P in the heavy area; this ratio should equal 1, since an equal multiplicity of heavy and light particles of identical specific activity was used to infect the cells. The fact that the ratio indeed is very close to 1 shows that there has been no preferential losses of one replicative moiety. Column 7 shows the ratios of $^3$H/$^{32}$P in the light region divided by the same ratios in the heavy region. This provides a measurement of the relative amount of newly synthesized DNA associated with either parental type; a ratio of 1 would indicate that there is equal synthesis per unit mass with heavy or light template. The observed ratios of 2 to 8 indicate a strong preference in the $^3$H-TdR uptake, favoring the light parental.

The most obvious explanation for the biased $^3$H-uptake is that more newly synthesized phage DNA is associated with the light parental DNA. While there is little doubt that $^3$H-TdR uptake to heavy DNA represents replication of this heavy DNA, several factors may interfere with the $^3$H-uptake in the region of light phage DNA and obscure the results.

Reanalysis of the $^3$H-labeled light moiety would show whether the $^3$H-TdR uptake represents only light phage DNA replication. Factors which may influence the uptake of $^3$H-TdR will be dealt with one by one.

**(i) Synthesis of $^3$H-labeled bacterial DNA.** The very low fraction of surviving cells at the time of addition of $^3$H-TdR (Table 1, column 2) and previous studies where $^3$H-TdR was added at this time after infection (7, 9) suggest that little residual bacterial synthesis should be expected. If, however, a significant amount of

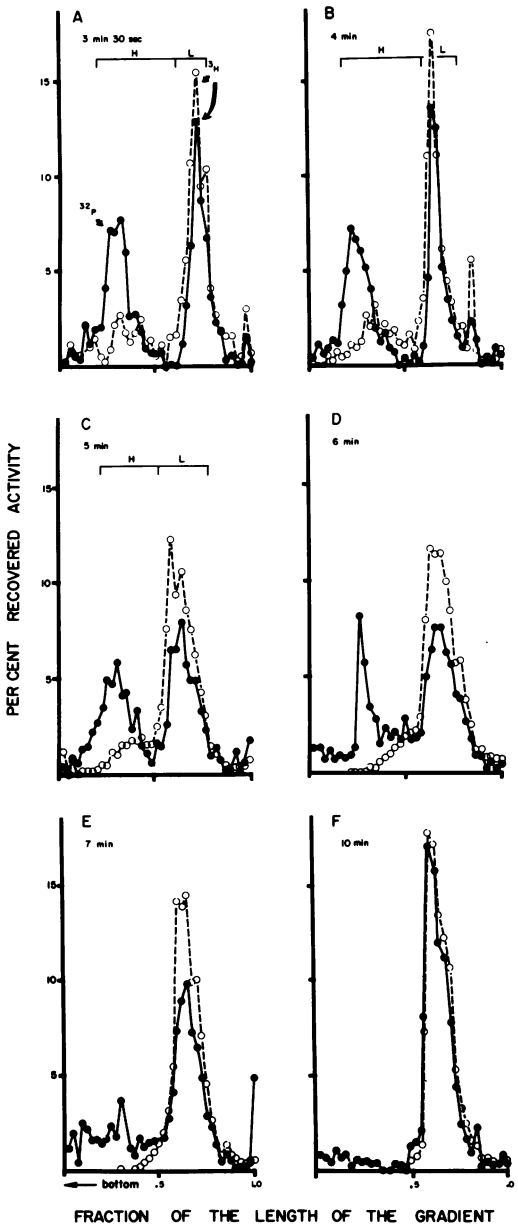


FIG. 3. *CsCl analyses of DNA from cells mixedly infected with heavy and light phage. Light bacteria were infected with $^{32}$P-labeled (sp act 0.5 mCi/mg, P) light and heavy T4 phage at an MOI of 5 particles of each kind. $^{3}$H-TdR (sp act 80 mCi/mg, TdR) was added after infection to label newly synthesized DNA. Samples were taken at times indicated in the figure, and DNA was extracted from the cells with SDS-Pronase-phenol and banded in CsCl (72 h, 29,600 rpm, Spinco SW50 rotor). No references were added. The activities of $^{3}$H and $^{32}$P recovered in the fractions marked H and L in the panels were summed, and specific activities were calculated (Table 1).*

bacterial DNA was synthesized after the addition of the isotope, this DNA would band in a position of the gradient close to the band of light phage DNA and contribute to the total $^{3}$H counts in this area. Three different tests were employed to verify the phage nature of the $^{3}$H-labeled light DNA.

"Light PRM" were isolated from the gradient illustrated in Fig. 4. One portion of this light PRM was reanalyzed in $Cs_2SO_4$. If a significant fraction of the $^{3}$H-label represented bacterial DNA, a pattern resembling that seen in Fig. 1 would be expected. Panel C in Fig. 4 shows that this is not the case. $^{3}$H-labeled material and $^{32}$P-labeled light phage DNA overlap perfectly. Moreover, the specific activity in the overlapping peaks is the same as in the original analysis (panel A of Table 1).

Another portion of the pooled light DNA was tested for reactivity with antibodies against $\alpha$-glucosylated DNA. It was shown previously that newly synthesized T4 DNA from PRM is equally as reactive with these antibodies as is DNA from mature phages (7). The $^{3}$H-labeled moiety in the light PRM was as well precipitated by these antibodies as was the parental $^{32}$P-labeled DNA (Table 2).

A third portion of the light PRM was hybridized to T4 phage DNA immobilized on nitrocellulose membrane filters. No differences were observed in the extent of hybridization of the $^{3}$H-labeled DNA and of the parental DNA (Table 2). Taken together, these results prove that there is no significant contribution by bacterial DNA to the total $^{3}$H-uptake in the light region of the gradient.

**(ii) Completion of the second round of replication of heavy DNA.** This would result in $^{3}$H-TdR uptake to the light region of the gradient even though the first round was initiated on heavy matrix. Considering the fact that at the time when PRM are isolated less than 5% of the heavy parental has completed the first round of replication (Fig. 3, 4A), it seems unlikely that any significant proportion of $^{3}$H-uptake can be due to a very small fraction of rapidly replicating DNA. This remote possibility was tested, however, in one experiment (expt 3 in Table 1) by infecting a portion of the culture with heavy phage alone. Intracellular DNA from this culture was analyzed by CsCl centrifugation at the same times after infection as the samples from the mixed infection. There was no measurable synthesis of light phage DNA until 8 to 10 min after infection. Thus, no heavy DNA has completed a second round of replication at the times when the biased $^{3}$H-TdR uptake is observed.

TABLE 1. *Biased uptake of $^3H$-thymidine to light T4 DNA*[a]

| Expt | Fraction of surviving cells | Viability L/ viability H | Time[b] after infection | $^{32}P$ L/ $^{32}P$ H | $^3H/^{32}P$ | | $(^3H/^{32}P)$ L/ $(^3H/^{32}P)$ H |
|---|---|---|---|---|---|---|---|
| | | | | | H | L | |
| 1[c] | $6.5 \times 10^{-4}$ | 1.6 | 3 min 30 s | 1.06 | 0.87 | 2.35 | 2.7 |
| | | | 4 min | 1.03 | 4.42 | 7.58 | 1.8 |
| | | | 5 min | 1.14 | 7.9 | 27.6 | 3.5 |
| 2 | $4 \times 10^{-4}$ | 2.2 | 3 min 30 s | 0.98 | 0.062 | 0.312 | 4.1 |
| | | | 4 min[d] | 1.01 | 0.262 | 1.22 | 4.7 |
| | | | 4 min 30 s | 1.05 | 0.362 | 1.53 | 4.2 |
| | | | 5 min | 1.07 | 1.36 | 6.35 | 4.7 |
| 3 | $3 \times 10^{-3}$ | 2.7 | 4 min | 0.96 | 0.174 | 0.268 | 1.5 |
| | | | 5 min[e] | 0.97 | 0.373 | 2.03 | 5.4 |
| | | | 6 min | 0.89 | 0.813 | 6.20 | 7.6 |

[a] L, Light; H, heavy.

[b] The first hybrid from heavy templates appeared 5 min after infection in experiments 1 and 2, and 6 min after infection in experiment 3.

[c] See Fig. 3.

[d] See Table 3.

[e] See Fig. 4 and 5 and Table 3.

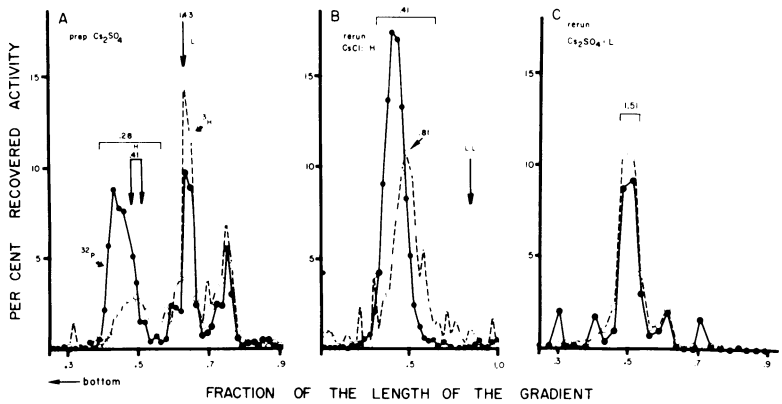


FIG. 4. *Isolation of heavy and light PRM from mixedly infected cells. Intracellular DNA obtained, as described in the legend to Fig. 3, 5 min after infection was banded in $Cs_2SO_4$. The CsCl analysis of this sample is summarized in Table 1 (expt 3, 5 min). Fractions indicated by the arrows marked H and L in panel A were pooled for further study. Panel B shows the reanalysis of heavy PRM in CsCl, and panel C shows the reanalysis of light PRM in $Cs_2SO_4$. $^3H$ and $^{32}P$ represent progeny and parental DNA, respectively; no references were added. Numbers in the panels refer to the ratio of $^3H/^{32}P$ in the indicated fractions. The presence of a third peak lighter than the main peak of light phage DNA in panel A is an artifact due to the presence of a large quantity of very viscous bacterial DNA which bands between the main peak of light phage DNA and the auxiliary peak. Reanalysis of DNA from the auxliary peak showed that only light phage DNA is present in this moiety (data not shown).*

**(iii) Lower viability of heavy phages.** While light phages always are 80 to 100% viable, the viability of BUdR-substituted phages is often somewhat lower. In these experiments, the difference was about twofold (Table 1, column 3). However, a low viability does not necessarily mean that the phages remain "dead" under conditions of multiple infection. We invariably observe that simultaneously infecting viable phage will rescue the "dead" phages and promote their replication in multiple infections. This is demonstrated in the experiment illustrated in Fig. 3, where all the heavy parental DNA replicated although only 46% of the heavy phage particles were able to form plaques. One may assume, for sake of argument, that this rescue takes place relatively late after infection (although there are no proofs, or even reasons, that this should be so). The bias factor can then be normalized with respect to experimental differences in the viability ratio. This yields a "corrected" bias close to 2 for the amount of synthesis just prior the appearance of the first hybrid in all experiments.

Considering these three points, it can be concluded that there is at least twice as much

TABLE 2. *Hybridization and antiserum precipitation of light PRM*

| Input[a] | Sp act of $^3H/^{32}P$[b] | |
|---|---|---|
| | α-Glucosylated DNA[c] | Phage DNA[d] |
| 1.00 | 1.32 | 1.11 |

[a] Trichloroacetic acid-precipitable counts per minute.

[b] Normalized to $^3H/^{32}P$ in the input = 1.00.

[c] Precipitated with antiserum against α-glycosylated T4 DNA according to the method of McNicol and Goldberg (submitted for publication).

[d] Hybridized to nitrocellulose filters charged with T4 DNA according to the method of Denhardt (4).

phage DNA synthesis on a light template as there is on a heavy template in mixedly infected cells early after infection, suggesting that heavy DNA is a poorer substrate for the DNA replication machinery than is normal light DNA.

**Comparison of the initial replication of heavy and light DNA: delayed initiation of heavy DNA replication.** There are several conceivable mechanisms which would lead to a lower net DNA synthesis during the early stages of replication of heavy DNA. Initiation of replication may be delayed, while the rate of elongation is unaffected. There may be fewer initiation sites in heavy DNA, while both initiation and elongation proceed at normal rates. The number of initiation sites and the rate of initiation may be normal, while elongation proceeds more slowly. The BUdR substitution may also, of course, affect two or three of these events simultaneously. An estimation of the size of individual replicative regions, as well as a determination of the rate of net DNA synthesis with and without BUdR, will allow a choice between these alternatives.

**(i) Size of replicative regions.** Light and heavy PRM from the $Cs_2SO_4$ gradient illustrated in Fig. 4 were examined by electron microscopy. This study confirmed the previously voiced suspicion that replicative loops in heavy PRM were smaller and that multiple replicative regions less numerous than what was observed in the light PRM without, however, providing any definite proofs for that notion. The observational bias in microscopy of T4 DNA molecules, which was mentioned previously, necessitated the use of an additional, unbiased technique to verify this suspicion. The pooled light and heavy PRM moieties were sedimented through alkaline sucrose gradients together with $^{32}P$-labeled T7 reference DNA (Fig. 5, Table 3). The results indicate that newly synthesized DNA in the heavy PRM is indeed smaller than that in the light PRM by a factor of 1.6 to 1.8. The fact that the relative difference in size between light and heavy PRM seems to remain fairly constant (Table 3) suggests that it is the initiation which is delayed, while the net replication rate is similar in the two cases. This is also suggested by the similar

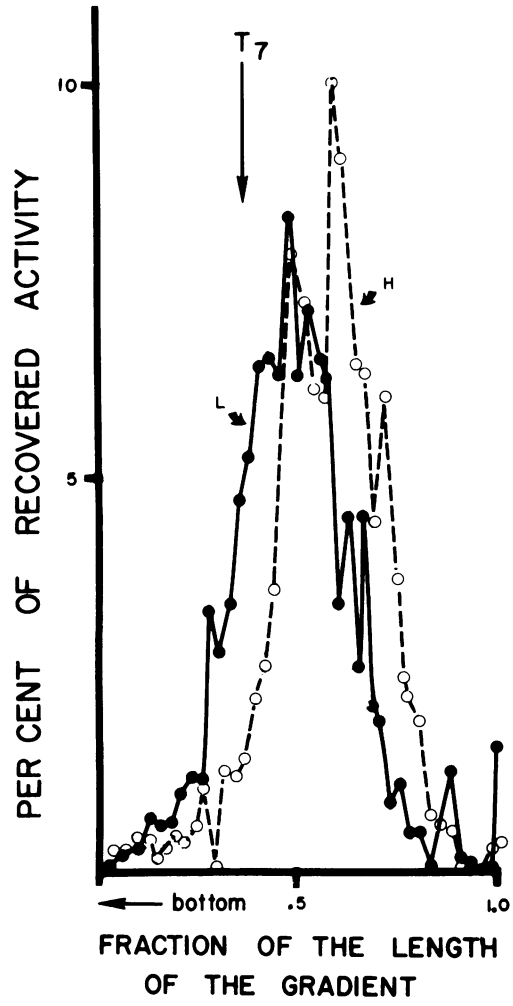


FIG. 5. *Alkaline sucrose gradient analysis of newly synthesized DNA from heavy and light PRM. The PRM moieties from the gradients illustrated in Fig. 4 were sedimented through separate 5 to 20% alkaline sucrose gradients together with $^{32}P$-labeled T7 reference DNA (35,000 rpm, 3 h, Spinco SW50 rotor). The two sedimentation patterns were then superimposed to facilitate comparison between the two moieties by using the position of the T7 marker to align the two gradients. The figure shows this superimposed pattern. Solid lines, Progeny DNA from light PRM; broken lines, progeny DNA from heavy PRM. Molecular weights were calculated from the relative distance sedimented using the nomogram of Litwin et al. (14).*

TABLE 3. *Molecular weights[a] of single strands of newly synthesized DNA from heavy and light PRM*

| Sample | Heavy PRM mol wt ($\times 10^6$) | Light PRM mol wt ($\times 10^6$) |
|---|---|---|
| Expt 2, 4 min | 3.3 | 5.8 |
| Expt 3, 5 min | 4.6 | 7.2 |

[a] Calculated according to Litwin et al. (14).

rate of $^3$H-TdR uptake to light and heavy DNA (Table 1, column 6).

**(ii) Net DNA synthesis.** Previous work has suggested that the rate of net DNA synthesis is not affected by the presence of BUdR (A. W. Kozinski, unpublished data). The following experiment verified this. Light or heavy bacteria were infected with cold, light T4 phage. To both cultures $^{32}$P was added 5 min after infection, and at various later times the amount of newly synthesized DNA was calculated from the trichloroacetic acid-precipitable, alkali-resistant counts. A third permutation of this experiment, mimicking the mixed infection experiments, would have been to measure DNA synthesis in light cells infected with heavy phage. In this case, however, an effect of BUdR could be expected only during the initial rounds of replication, since at later times newly synthesized light DNA will be more abundant than the heavy parental molecules, and late synthesis will therefore predominantly utilize newly synthesized, light templates. By comparing, instead, replication in BUdR-medium with the replication in light medium, any BUdR effect will be repeated in each round of replication and will be noticed more easily (Fig. 6). There were no significant differences in the net rate of DNA synthesis in the two cultures.

Taken together, these results support the first of the three alternatives. The initiation of replication of heavy DNA is delayed in comparison to initiation of light DNA replication, but there is no difference in replication rate, once synthesis has been started.

**(iii) DNA-protein complexes.** The delayed initiation could reflect a delayed formation of the protein-complex of replicative DNA (6, 12, 17). Alternatively, the rate-limiting step could be a later event, for instance the attachment of replicative enzymes to the DNA, or the separation of parental strands necessary to start synthesis of new progeny DNA strands.

The "complex" is formed around 2 to 5 min after infection (15) between parental phage DNA and components of the bacterial cell, which probably includes parts of the cell membrane (16). Formation of this complex is essential for the initiation of DNA replication (17). Complexes may be isolated conveniently by virtue of their very rapid sedimentation through low-salt sucrose gradients (17). An experiment was performed to test whether light and heavy DNA entered this complex at the same time.

Light cells were infected with heavy and light $^{32}$P-labeled phage as previously, though no $^3$H-TdR was added, and samples were withdrawn at intervals after infection for analysis. Part of the infected cells were lysed with lysozyme and Triton X-100, and the lysate was sedimented through a low-salt sucrose gradient. Another part of the cells was lysed with SDS, and DNA was extracted and analyzed in CsCl. Fractions of complexed DNA were isolated from

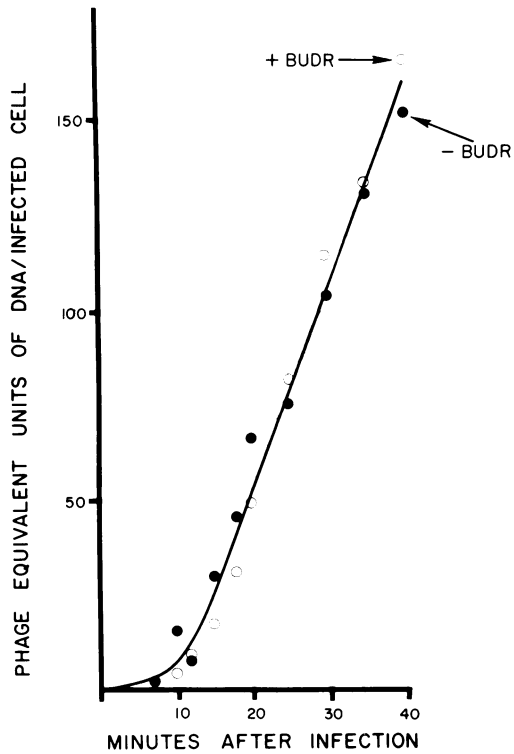


FIG. 6. *Net DNA synthesis in E. coli infected by T4 in the absence or presence of BUdR. E. coli was pregrown for one generation in light or heavy TCG medium containing 5 μg of phosphorus (as phosphate) per ml, to a density of 3 × 10$^8$ cells/ml. The two cultures were infected with cold, light T4 phage (MOI 5). Five minutes after infection, $^{32}$P was added (sp act 0.5 mCi/mg, P). At this time, 0.1% of the cells survived. At the times indicated in the figure, samples were withdrawn, and the extent of DNA synthesis per infected cell was calculated from the alkali-resistant, trichloroacetic acid-precipitable counts (20), knowing that 1 μg of P corresponds to 5 × 10$^{10}$ T4 equivalents of DNA.*

the sucrose gradient and extracted with Pronase and phenol, and the DNA was banded in CsCl. This complexed DNA revealed the same 50:50 proportion of heavy and light DNA that was found in the total cell lysate, also at early times after infection when only part of the injected DNA had entered the complex (data not shown).

This shows that light and heavy DNA display no differences in the rate of complex formation, and the cause for the delay in initiation of replication must be sought elsewhere. Some possible alternatives are discussed below.

## DISCUSSION

Several previous investigations where BUdR was utilized to facilitate isolation of replicative T4 DNA have documented that the initiation of DNA replication takes place at multiple sites. This was evident from experiments involving shearing of partially replicated molecules (7, 11), from an electron microscope study of partially replicated molecules (2), and from an experiment where fragments corresponding to the replicated portions of partically replicated molecules were hybridized to random T4 DNA fragments and the hybrid was subsequently digested with *E. coli* exonuclease I (7). The results of the electron microscope study documented in the first part of this paper show that multiple loops are found in replicative molecules containing no BUdR (neither in parental nor in newly synthesized DNA), indicating that multiple initiation of DNA replication is a normal event in T4 development. Although only circumstantial evidence support the interpretation that the observed loops and forks result from replication, we feel that this conclusion is well justified. Nonreplicative DNA, either from mature phages or from nonpermissive cells infected with $D^0$ amber mutants, do not display any loops or forks of this type (2; Karin Carlson, unpublished observations). It is unlikely that the observed structures result from molecular recombination, since the analysis of part B (BUdR added after infection) showed that the first round of replication was not completed at the time of sampling. We do not observe any recombination in this system until well after the first appearance of hybrid DNA (5, 13).

In the second group of experiments, the possibility that BUdR affects initiation in some different manner was explored by using mixed infection to allow the study of replication of both heavy and light DNA in the same cells. Phage DNA synthesis early after infection was found preferentially on light, TdR-containing templates, and to a lesser extent on BUdR-containing templates. The preference amounted to a two- to a eightfold bias favoring the light template.

Among the various plausible explanations for this bias, a slower rate of initiation of heavy DNA replication followed by a net rate of DNA synthesis similar to what is observed with a light template is favored by evidence documented in Fig. 5 and 6 and in Table 3. Heavy and light parental DNA entered the replicative complex of phage DNA and various cell components with the same efficiency, suggesting that the initiation event itself is the target for BUdR inhibition. We cannot rigorously exclude the possibility that fewer initiation sites are utilized in heavy DNA, although the fact that similar rates of net DNA synthesis were observed in the presence and absence of BUdR does not favor this hypothesis. Results described in the preceding paper (7) also suggest that there are no differences in the number of replicative sites per molecule in heavy or light PRM.

An estimate of the average number of replicative areas in heavy and light PRM from the mixedly infected cells may be obtained from the total amount of newly synthesized DNA and the average size of individual replicative areas (7). Although an estimate of the size of the latter from sucrose gradients is not as sensitive as the shearing assay used by Howe et al. (7), strand lengths calculated this way agree both with the average loop size seen in the electron microscope (2) and with the average size of replicated regions calculated from the density of sheared PRM (7). The fact that only replicated regions contributing significantly to the total radioactivity are observable by this technique will possibly lead to an underestimate of the number of regions.

The total progeny contribution in the isolated heavy PRM (Fig. 4B) is 14.7%, corresponding to about $22 \times 10^6$ daltons of replicated DNA per molecule. The average molecular weights of single progeny strands is $4.6 \times 10^6$ (Fig. 6, Table 3), which suggests that there are on the average two to three replicative sites per heavy PRM. In the case of the light DNA, some additional assumptions must be introduced before the number of replicative areas can be calculated, since we cannot directly measure the total mass of progeny DNA per molecule, or the proportion of replicative molecules in the total population.

The specific activity of $^3H/^{32}P$ in the isolated heavy and light moieties (Fig. 4B and C) provides an estimate of the total amount of newly synthesized DNA in the light DNA, since

the same extent of $^{3}$H-TdR uptake corresponds to the same quantity of DNA synthesis with either template. In both cases some of the $^{32}$P undoubtedly represents unreplicated DNA. The electron microscopy of both heavy and light moieties revealed that the majority of the observed molecules lacked any replicative structures. This introduces an error in the calculations, which will, however, lead to an additional underestimate of the number of replicated regions in light PRM. While isolation of heavy PRM separates replicative DNA from nonreplicated DNA by virtue of their different densities, no such separation is achieved in isolation of light (non-density-labeled) DNA. The $^{3}$H/$^{32}$P ratio in the light moiety is therefore probably "diluted out" by nonreplicative molecules (having only $^{32}$P label) to a larger extent than the ratio in the isolated heavy PRM, from which a large fraction of nonreplicative DNA has been removed. The specific activities of $^{3}$H/$^{32}$P of 0.81 in the peak of isolated heavy PRM and 1.53 in the isolated light DNA moiety (Fig. 4) indicate that light PRM contain at least twice as much newly synthesized DNA per replicative molecule as does heavy PRM. Thus, there is at least $44 \times 10^{6}$ daltons of replicated DNA per molecule. Since the average molecular weight of single strands of newly synthesized DNA is $7.2 \times 10^{6}$ (Fig. 5, Table 3), there are at least three replicative regions per molecule.

A different estimate may be obtained by comparing specific activities in the first fractionation of intracellular DNA (Fig. 3). If initiation of heavy templates is somewhat delayed in comparison to initiation of light templates, one would expect a higher proportion of nonreplicated DNA in the heavy region of the gradient than in the light region. A comparison of $^{3}$H/$^{32}$P ratios in the heavy and light regions (Table 1) may, therefore, yield an overestimate of the number of light replicative regions. The 5.4-fold higher specific activity of $^{3}$H/$^{32}$P in the light moiety (Fig. 3, 4A; Table 1) suggests that there is $120 \times 10^{6}$ daltons of replicated DNA per light molecule, or eight replicative sites. The true number may lie in between these two extremes.

These calculations suggest that there are two to eight initiation sites in T4 DNA, a number which is in good agreement with the estimate obtained in the preceding paper (7). The fact that there appears to be fewer replicative regions in our heavy PRM than in the light PRM suggests that within each molecule individual sites may be initiated sequentially. "Early" PRM (represented by the heavy PRM in this case) will then have fewer replicative regions than "late" PRM (represented by the light PRM here). The "silent" replicative sites in the early PRM may be uninitiated, or initiated so recently that no significant amount of newly synthesized DNA in the preceding paper (7) also suggested sequential initiation of individual replicative regions. As discussed above, we consider such sequential initiation more likely than a complete lack of initiation of some sites in heavy DNA.

We conclude that initiation of DNA replication is delayed by the BUdR substitution in the DNA template, but the number of initiation sites per molecule and replication per se are most likely not affected. Judging from the rate of uptake of $^{3}$H-TdR to heavy and light DNA (Table 1, column 6), the delay corresponds to about one minute of development at 37 C.

There are several alternative explanations to the delay in initiation. There may be a delayed attachment of replicative enzymes at the initiation sites if the BUdR residues impair the recognition by these enzymes. Another possibility is that the initial separation of parental strands to allow insertion of new bases requires more effort when the DNA is substituted with BUdR than is necessary with normal light DNA. While we have no data to support either of these alternatives or exclude the possibility of still some other mechanism of action, known properties of BUdR-substituted DNA may provide some hints. The melting temperature of BUdR-substituted DNA is about 10 C higher than that of light DNA (10). Thus, the separation of the two heavy template strands in the initial stages of DNA replication may be less efficient than the separation of light DNA strands.

Mosig and Werner (19), Mosig (18), and Marsh et al. (15) have obtained data which were interpreted as indicating unidirectional replication from a single, genetically defined origin. Their data are more fully discussed in the preceding paper (7). While our data are not contradictory to specific initiation points or a sequential initiation, events which could mimic single specific initiation when viewed at a later stage of development, we find replication from multiple origins a normal mode of DNA synthesis during the first round of replication of T4 DNA molecules.

### Addendum

During the preparation of this manuscript, it came to our attention that multiple loops in replicative non-BUdR-labeled T4 DNA have been observed also by Y. Naot and C. Shalitin (personal communication).

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