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Early Intermediates in Bacteriophage T4 DNA Replication and Recombination

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We investigated, by density gradients and subsequent electron microscopy, vegetative T4 DNA after single or multiple infection of *Escherichia coli* with wild-type T4. Our results can be summarized as follows. (i) After single infection (i.e., when early intermolecular recombination could not occur), most, if not all, T4 DNA molecules initiated the first round of replication with a single loop. (ii) After multiple infection, recombinational intermediates containing label from both parents first appeared as early as 1 min after the onset of replication, long before all parental DNA molecules had finished their first round and before secondary replication was detectable. (iii) At the same time, in multiple infections only, complex, highly branched concatemeric T4 DNA first appeared. (iv) Molecules in which two loops or several branches were arranged in tandem were only found after multiple infections. (v) Secondary loops within primary loops were seen after both single and multiple infections, but they were rare and many appeared off center. Thus, recombination in wild-type T4-infected cells occurred very early, and the generation of multiple tandem loops or branches in vegetative T4 DNA depended on recombination. These results are consistent with the previous finding (A. Luder and G. Mosig, Proc. Natl. Acad. Sci. U.S.A. **79**:1101–1105, 1982) that most secondary growing points of T4 are not initiated from origin sequences but from recombinational intermediates. By these and previous results, the various DNA molecules that we observed are most readily explained as intermediates in DNA replication and recombination according to a model proposed earlier to explain various other aspects of T4 DNA metabolism (Mosig et al., p. 277–295, *in* D. Ray, ed., *The Initiation of DNA Replication*, Academic Press, Inc., New York, 1981).

Replication of bacteriophage T4 DNA is first initiated within linear DNA molecules at one or several preferred origins (18, 19, 29, 38, 46, 53, 58, 59, 64). Secondary replication forks appear soon thereafter. Eventually they follow each other at average distances of approximately one tenth the T4 genome (6, 54, 85) in highly branched complex structures (3, 6, 17, 31, 39, 52, 59, 60, 64). In recombination-deficient mutants, however, DNA replication is arrested after one or a few rounds and no concatemers are formed (for reviews, see references 12 and 16).

Our previous results indicated that (i) this arrest occurs because most of the secondary growing points have to be initiated from recombinational intermediates (52, 60, 63), (ii) this "recombinational initiation" occurs as soon as the first growing points reach an end of a DNA molecule, and (iii) recombinational initiation is largely responsible for the complexity of replicating T4 DNA mentioned above (18, 52, 59, 60, 63, 64) (see Fig. 1). On the other hand, it has been stated that in T4 DNA no recombination occurs until extensive replication of linear DNA molecules has established a pool of replicas, which then recombine during the latter part of the latent period (12, 20, 30, 44, 45, 55, 78).

Here we show electron micrographs of early replicative T4 DNA intermediates which were obviously also engaged in recombination. These complex DNA intermediates were seen soon after the onset of replication, before the majority of parental chromosomes had completed their first round. At such early times, we found no recombining structures among unreplicated DNA molecules either in the density gradient fractions containing unshifted parental DNA, in control experiments after infection of *Escherichia coli* with solitary T4 chromosomes (see below), or after infection with gene 32 mutants (18). Recombination between unreplicated T4 chromosomes has been observed (4, 5, 11, 13), but it occurs much later.

To detect early recombinants, we used ^{13}C

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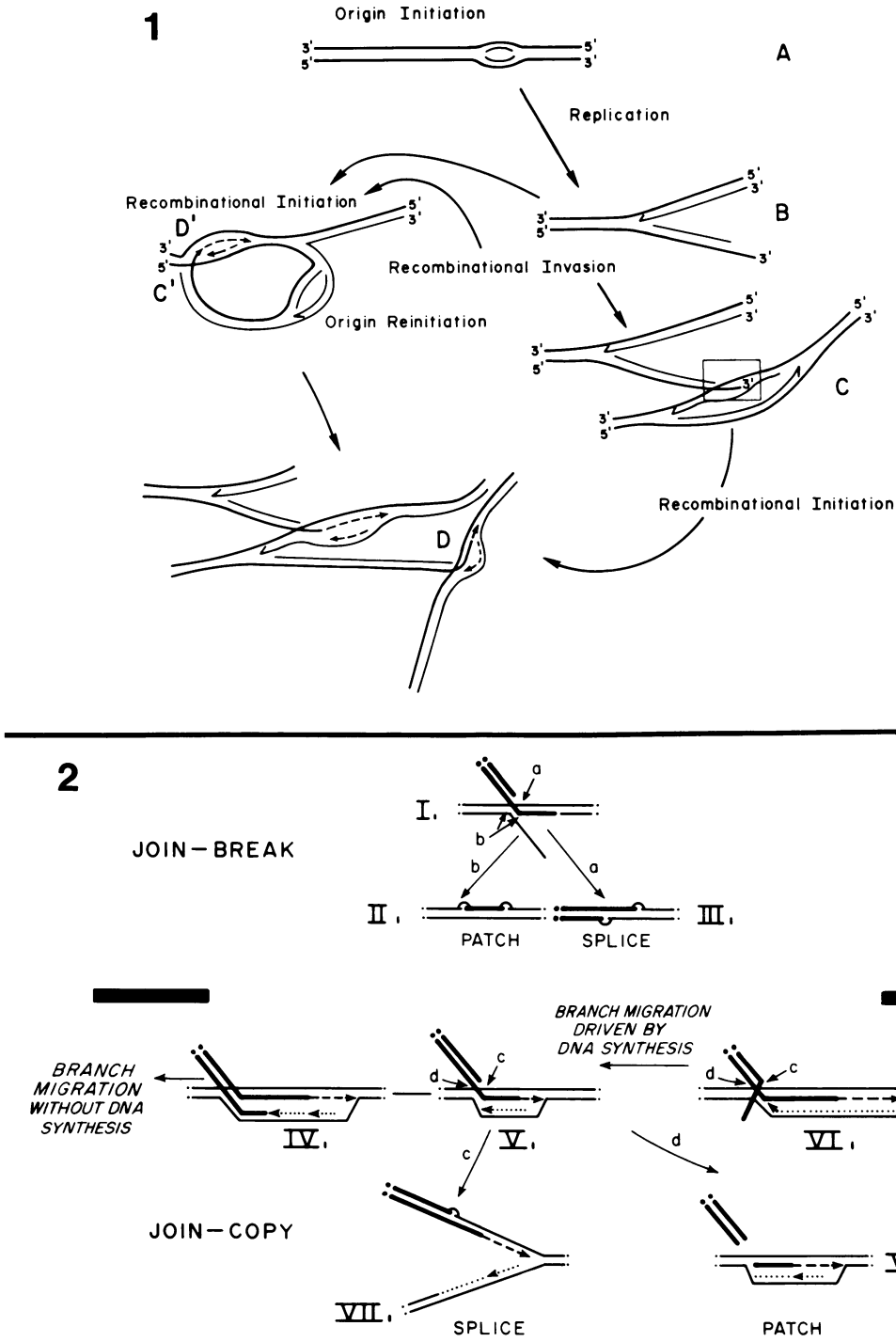

FIG. 1. Model of T4 DNA metabolism. (Panel 1) The first round of replication is initiated at an origin sequence (A). When a growing point reaches one end of the chromosome, the tip of the template for the lagging strand remains single stranded (B) (84; T. Broker, Ph.D. thesis, Stanford University, Stanford, Calif., 1972). This single-stranded segment invades a homologous region of another chromosome (which may be partially replicated) (C) or of the same molecule (C') to give a recombinational fork. Such a recombinational fork can be cut (56) and joined to give covalently linked recombinants (37, 86), and DNA fragments (see panel 2, join-break).

and ^{15}N as heavy density labels in at least one of the parental phage and isolated early replicative intermediates by virtue of their density shift. Three different kinds of infections were done. In the first, unlabeled light *E. coli* cells were infected with equal proportions of ^{32}P-labeled heavy and ^{3}H-labeled light T4 particles. DNA of intermediate density was expected to be enriched for recombinational intermediates (13). In the other two infections, parental and progeny T4 DNAs were differentially labeled with radio- and density isotopes; in one control we infected bacteria with single T4 chromosomes so that early recombination between chromosomes of different parentage was prevented, and in the other control we used multiple infections, i.e., conditions which permit early recombination between chromosomes of different parentage. T4 DNA isolated at different times after infection was fractionated in density gradients and then viewed in the electron microscope. The various types of DNA intermediates which we observed after multiple infections are most readily explained as different intermediates in T4 DNA metabolism (Fig. 1).

(A preliminary account of this work has been published [63].)

MATERIALS AND METHODS

Bacteria. *E. coli* strains S/6, B, and B/5 are maintained in this laboratory.

Bacteriophage. The T4D wild-type phage particles were grown and assayed by standard procedures (1). φX174 circular single-stranded and duplex DNAs were obtained from R. Sinsheimer (California Institute of Technology) and Miles Laboratories, respectively. PM2 duplex circular DNA was ethyl ether extracted from a phage stock grown by the method of Salditt et al. (71).

Preparation of labeled particles. Parental ^{13}C-, ^{15}N-, ^{32}P-labeled phage particles were grown at 37°C in Tris-minimal medium (35) containing 0.021 M $^{15}NH_4Cl$ (99.5 atom %; Bio-Rad Laboratories), 0.2% [^{13}C]glucose (56 atom %; Merck Sharp & Dohme of Canada, Ltd.), and 1 mCi of $^{32}P_i$ (E. R. Squibb & Sons, Inc.) per mg of phosphorus. They were purified by differential centrifugation as previously described (53).

Density shift experiments. Bacteria were grown and infected in M9 medium (1) as previously described (18). Bacteria, unadsorbed particles, and phage-producing bacteria (infective centers) as well as progeny particles were assayed by standard methods (1). At various times after infection, 2-ml samples were withdrawn, chilled in the presence of 0.01 M NaCN–0.05 M EDTA, and lysed as previously described (18). The samples were handled gently to avoid shearing the DNA. The samples were then dialyzed at 4°C against 0.05 M Tris buffer (pH 7.4) containing 0.02 M EDTA. In our hands, several other published lysis procedures resulted in lower recovery of T4 DNA than did this procedure.

Density gradient analysis of replicated T4 DNA. After dialysis, samples were mixed with a saturated Cs_2SO_4 solution (1.1 ml per 1.4 ml of lysate) and spun in a Spinco type 40 or 50 Ti rotor at 35,000 rpm for approximately 60 h at 10°C. Gradients were fractionated from the bottom of the tube. Samples were spotted onto filter papers, and radioactivity was determined by liquid scintillation counting.

Electron microscopy. The basic formamide spreading technique was used (11, 13) for electron microscopy, exactly as described before (18). In this system, duplex DNA appeared as relatively smooth, uniformly thick fibers, and single-stranded DNA appeared to be kinked and uneven.

Grids were scanned at 5,000× magnification in a Hitachi HU 11B electron microscope operating at 75,000 V. They were photographed at 2,500 to 25,000× magnification on Kodak electron microscope sheet film (no. 4489, 6.5 by 9.0 cm) and screened for suitable spreading and appearance of reference molecules as previously described (18). In no instance was more than a small percentage of micrographs taken from any one sampling of one gradient fraction, a single grid, or a particular grid section.

Negatives were enlarged 2 to 50 times by photographic printing or by projection through a Besselar projector onto tracing paper. They were measured with a map measure (Keuffel and Esser no. 620300 or Numonics 250-116). The PM2 double-stranded circular DNA (10 kilobases, 3.4 μm) (81) present in each negative was used for length calibration; by this calibration, unit-length T4 chromosomes (170 kilobases) (42) should measure 57.8 μm. However, average lengths as short as 52 or 53 μm have been reported for T4 DNA (24, 47). We therefore assume that molecules within the range of these measurements are of unit length. Single-stranded φX174 circular DNA served as an internal standard for single-stranded regions. Although reference molecules were present on all grids, they were not present in all photographs.

(If the invading segment is from the tip of a chromosome, only one additional cut in that chromosome is required to generate a patch-type recombinant.) Alternatively, the invading 3'OH end can be used as a primer to initiate a replication fork (D, D') (see panel 2, join-copy) (52). (For simplicity, C shows only the invasion into another replication loop. We assume that invasions into unreplicated DNA also occur.) Reiteration of similar invasions of a single-stranded termini into homologous regions of other chromosomes and the initiation of replication from these invasions (D) generates the network of branched and looped T4 DNAs whose complexity increases with increasing multiplicities of infection and with time. The initiation of replication forks from recombinational intermediates predicts the precise patterns of recombination and segregation of alleles in terminal regions of the T4 chromosomes that have been observed (52, 61). Eventually, DNA is packaged from these complex concatemers (62, 80). (Panel 2) Plausible ways (see the text) of resolving recombinational intermediates (see inset box in C, panel 1) by join-break mechanisms, by forming replication forks and by cutting with endonuclease VII (56). Symbols: dots at the ends, DNA strands continue; dashed lines, elongation from 3' ends (leading strands); dotted lines, lagging strands; small arrows (a–d), endonuclease cuts; semicircles, ligation, if necessary, after gap filling. Further details are given in the text.

RESULTS

Infection with differentially labeled parental particles revealed early replicative intermediates which were also recombining. *E. coli* B. cells growing in unlabeled medium were infected with equal proportions of ^{3}H-labeled (light) and ^{13}C-, ^{15}N-, ^{32}P-labeled (heavy) wild-type T4 particles (multiplicity of infection, 5 of each) at 37°C and lysed at various times after infection. Unsheared DNA samples isolated from the infected bacteria were fractionated in neutral Cs_2SO_4 gradients.

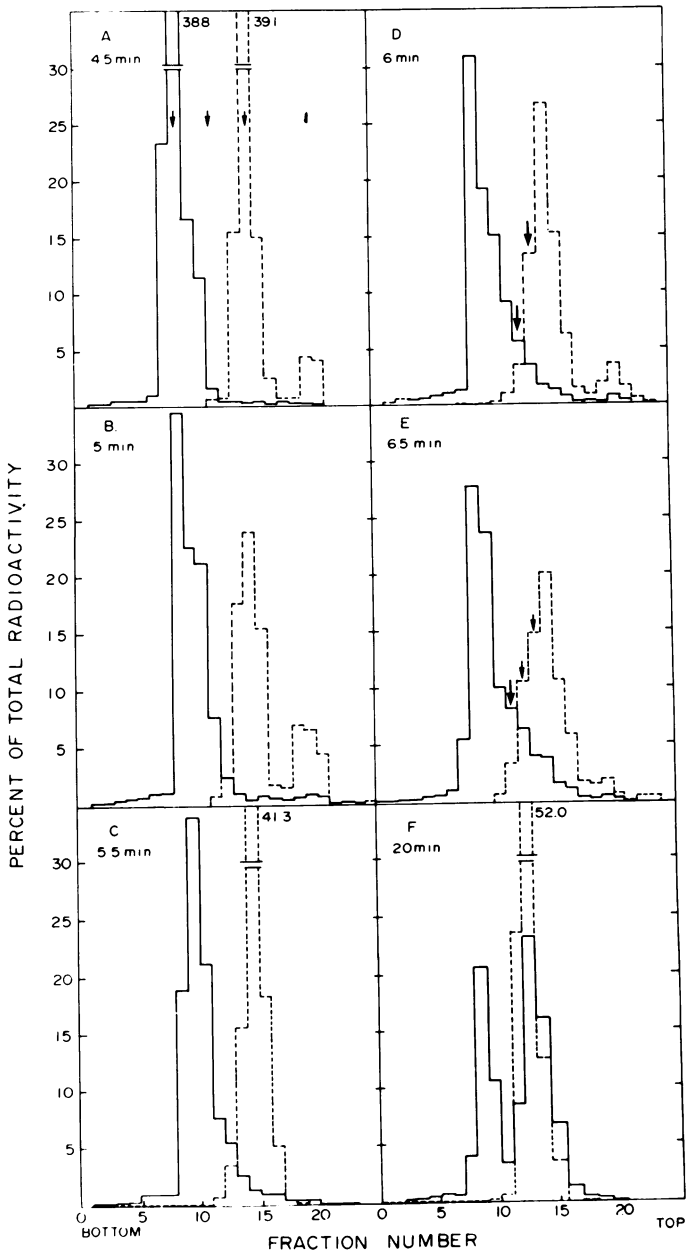

FIG. 2. Distribution in neutral Cs_2SO_4 gradients of unsheared T4 DNA isolated at different times after infection of *E. coli* B cells with an average of five ^{3}H-labeled and five ^{15}N-, ^{13}C-, ^{32}P-labeled T4D particles per bacterium. The arrows in panel A indicate positions of heavy-heavy (parental ^{15}N ^{13}C ^{32}P), heavy-light (hybrid), and light-light (parental ^{3}H) T4 DNAs and of bacterial DNA, respectively. The position of (viscous) bacterial DNA served as a density marker in all gradients. The arrows in panels D and E indicate the positions in which most recombining molecules were found. Symbols: ———, ^{32}P; – – – –, ^{3}H.

Since light parental DNA cannot become denser by replication in light medium, a shift of light parental ^{3}H label toward heavier densities suggested that recombination with molecules containing heavy isotopes had occurred. On the other hand, the parental DNA containing heavy isotopes can shift towards lighter densities as a result of either replication or recombination or both.

Figure 2 shows representative density profiles of T4 DNAs isolated at different times after infection. At 4.5 or 5 min after infection there was some apparent replication, i.e., some ^{32}P shift toward lighter density, but little or no recombination, i.e., no ^{3}H shift toward heavier density (Fig. 2A and B). Starting at 5.5 min after infection, however, increasing proportions of ^{3}H label appeared at denser positions than parental light DNA (Fig. 2C through F).

Although some replication or recombination may have remained undetected at the earliest times, it was clear that recombination started within 1 min after the onset of primary DNA replication and that it occurred before there was detectable secondary DNA replication. The absence of density shift in the samples taken earlier showed that there was little or no unspecific association of the two parental DNAs. This was confirmed by analysis of sheared DNA samples (data not shown) (R. Dannenberg, Ph.D. thesis, Vanderbilt University, Nashville, Tenn., 1979) by electron microscopy and by the results of control experiments described below.

DNA which banded at different densities was then inspected in the electron microscope. DNA banding at heavy parental density had not replicated or recombined, since none of the more than 1,000 unit-length chromosomes that were inspected from parental density fractions of all infections showed any loops or branches. Fractions of intermediate density (Fig. 2D and E, arrows) were expected to be enriched for (i) partially replicated heavy molecules which became lighter by incorporation of light, nonradioactive DNA precursors and (ii) recombinational intermediates in which (replicated or unreplicated) light DNA segments were recombining with ^{32}P-labeled heavy DNA segments.

It soon became obvious throughout these experiments and the controls described below that unreplicated T4 DNA (from the heavy parental density position) could be spread easily without being broken (Fig. 3). In contrast, most partially replicated or recombining molecules (selected from gradient fractions of intermediate density) appeared to be entangled or broken in spite of isolation and spreading conditions identical to those used for unreplicated DNA. This was consistent with our model (Fig. 1). Already at 6 min after infection, many molecules resembled

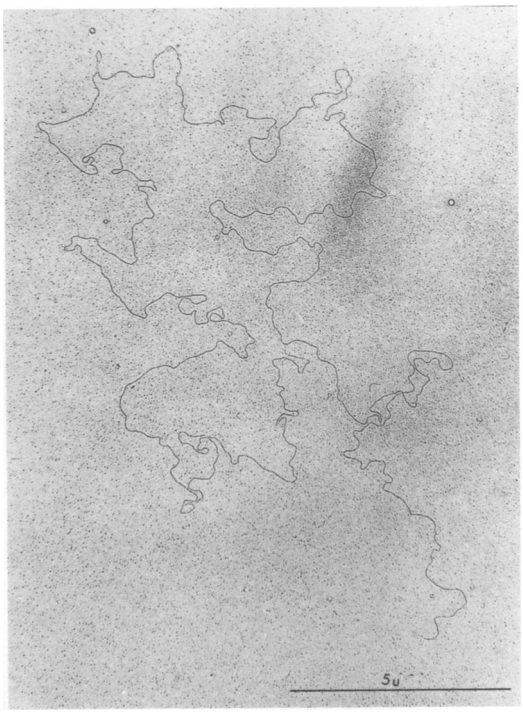

FIG. 3. Unit-length (57.0 μm, 177,000 base pairs) linear duplex T4 DNA spread from the dense parental peak fraction of the gradient shown in Fig. 2E. Bar, 5 μm.

in branching, but not yet in size and complexity, the networks first seen by others (39, 41) late after infection. Figure 4 shows one of the simpler examples of such structures. Not all branches could be explained as simple overlaps. Since such structures were not found early after single infection (see below), we concluded that the complexity was caused by replication and, in part, by formation of recombinational intermediates. Many of the branch points in these complexes had single-stranded whiskers, as do recombinational branch points seen after infection under replication-deficient conditions (11, 13). These will be discussed in more detail later.

More than 500 DNA molecules of intermediate density, isolated 6 or 6.5 min after infection, were viewed in the electron microscope. Of these, 20% were more complex than linear duplex. Since many DNA complexes were entangled and, at best, difficult to interpret, we show here only a few examples of those molecules in which individual DNA strands could be traced along most of their length. We shall only briefly mention simple replicative intermediates (see Table 1) and then focus on those types of molecules which have not been previously described by others (19) in early replicating T4 DNA (e.g., isolated 6 or 6.5 min after infection).

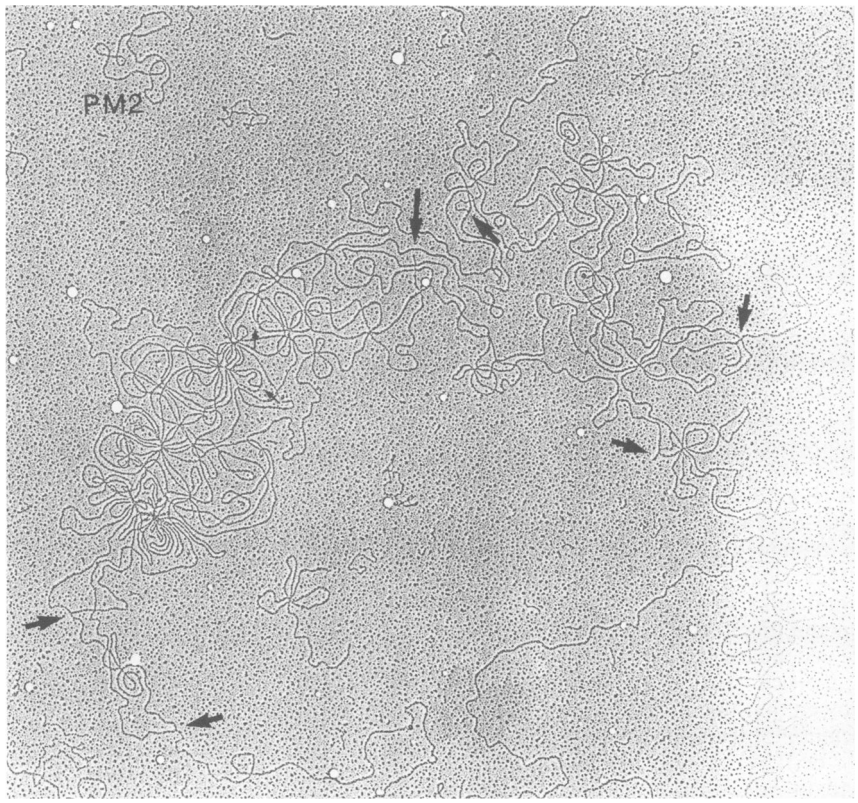

FIG. 4. Complex branched molecule from a shifted fraction (arrows) of the DNA shown in Fig. 2E. Arrows indicate branch points.

Most DNA molecules isolated at later times resembled the complex structures described by others (6, 39). Such increased complexity is predicted by our model (Fig. 1).

Figure 5 shows two representative examples of typical DNA loops, which are most readily explained as simple replicative intermediates (18, 19, 63) since they resemble the DNA loops seen after single infection (see below) or in recombination-deficient T4 mutants (18). In many of these loops, one or both branch points appeared to be partially single stranded, as was expected for replication forks (23, 68, 72). Some of the branch points (Fig. 5b) showed, instead, short protruding single-stranded whiskers, which could be explained by in vitro branch migration of the leading strand (19, 49) or by in vivo branch migration during recombination (11, 13) (see below).

More complex structures that could not have been generated by replication of linear chromosomes alone but must also have experienced some kind of recombination are shown in Fig. 6–8. Several molecules were longer than unit length. The longest (238,200 base pairs) contained one protruding single-stranded whisker, which could be a remnant from an invasion by another molecule (Dannenberg, Ph.D. thesis, Fig. 16).

Figure 6 shows an example of the invasion of a replication loop (A-B) by the single-stranded terminus of another molecule (C). One of the forks of the replication loop (A) formed a complex structure. Similar fork structures were seen in several other molecules. They may represent an alternative arrangement of DNA strands at an origin region, as was postulated for the origin region of lambdoid phages (36). Figure 7 is an example of a tip of a forked molecule (I) invading another molecule (II) at branch point A. Figure 8a shows the most complex branched structure that was still interpretable. Traced in a certain way (Fig. 8b), this molecule represented a circle of 54 μm (i.e., the length of the T4 genome without terminal redundancy), from which protruded several branches of variable lengths. Thus, this molecule resembled a firewheel, a structure which was proposed by Werner (85) to explain the close proximity of growing points in replicating T4 DNA. The lengths of the branches did not follow the predictions for rolling circle initiation from a single origin, as

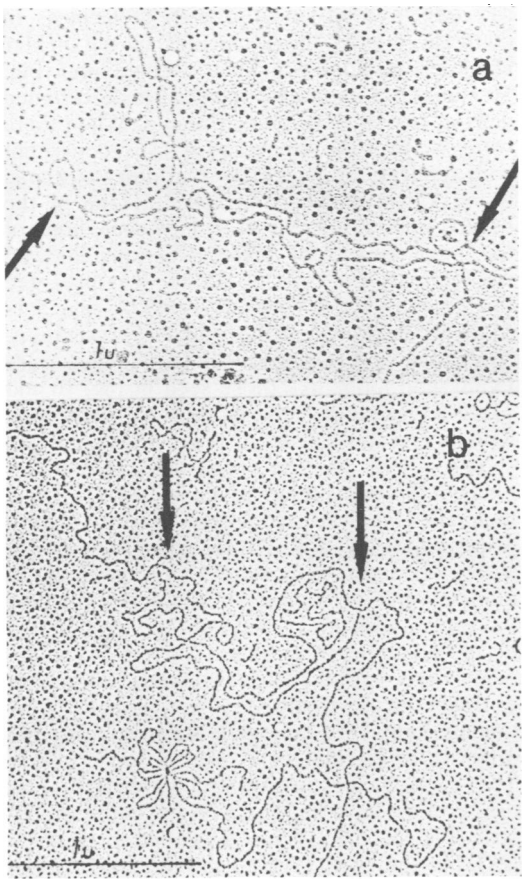

FIG. 5. Loops found in shifted fractions of the DNA shown in Fig. 2E. Arrows indicate branch points. (a) Loop arms (3.3 μm, 9,700 base pairs); total molecule length, 31 μm (approximately 91,200 base pairs). (b) Loop arms (2.8 μm, 8,200 base pairs); the total molecule measured 45 μm (13,200 base pairs). Arrows indicate branch points.

originally proposed (27, 85). Most likely some branches represent recombinational intermediates or replication forks initiated from such intermediates (Fig. 1). In addition to the branches, there were two loops within the circle. These loops could have been initiated from two different origins. It was, however, equally likely that forks of one of the loops were initiated by recombination between the terminal redundancies (Fig. 1, panel 1, C′).

The electron micrographs and the density shift measurements showed that recombination started within 1 min or less after the onset of replication. Some replication was required for recombination at this early time, since no recombining structures were found among the molecules of parental density; however, very limited replication was sufficient. Since replication and recombination are so intimately related, it was impossible to tell whether a given loop initiated from an origin or from a recombinational intermediate. Therefore we asked whether the earliest replicative intermediates seen after single infection were different from those seen after multiple infection, since there was no opportunity for recombination between molecules of different parentage after single infection (only the terminal redundancies can recombine).

T4 DNA isolated early after single infection showed neither multiple tandem loops nor branches. To reduce early recombination, we infected light *E. coli* B cells with single ^{13}C-, ^{15}N-, ^{32}P-labeled particles in the presence of ^{3}H-labeled thymidine so that more than 90% of the infected bacteria received only one T4 chromosome. Thus, parental ^{32}P label could acquire hybrid density only by replication. It could become lighter by repeated reinitiation from the same origin, by recombination with light-light progeny DNA, or when recombination between (replicated) terminal redundancies established new replication forks. Altogether, 12 independent infections were done with less than 0.1 phage particle used per bacterium.

A representative set of neutral Cs_2SO_4 gradients of unsheared DNA are shown in Fig. 9.

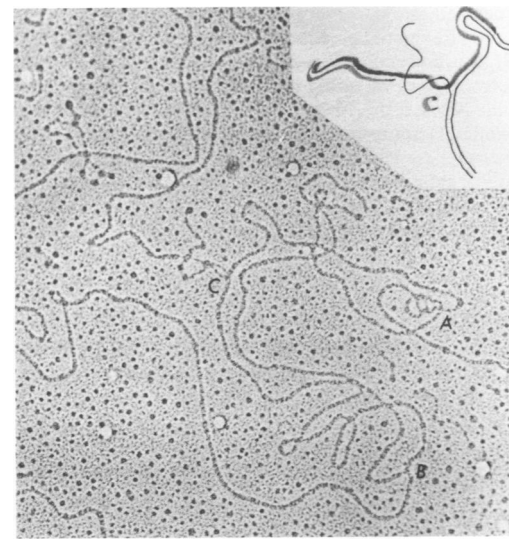

FIG. 6. Replicating and recombining DNA molecule of intermediate density (see Fig. 2E). (A and B) Branch points of replication loop; (C) site of single-strand invasion. Length of the molecule containing the replication loop, 30.3 μm (89,100 base pairs); length of each loop arm between A and B, 3.0 μm (8,800 base pairs). The total invading single-stranded segment measured approximately 0.74 μm. Inset: One plausible interpretation of pairing at the site of invasion that results in displacement of one single-stranded segment from the replicated loop.

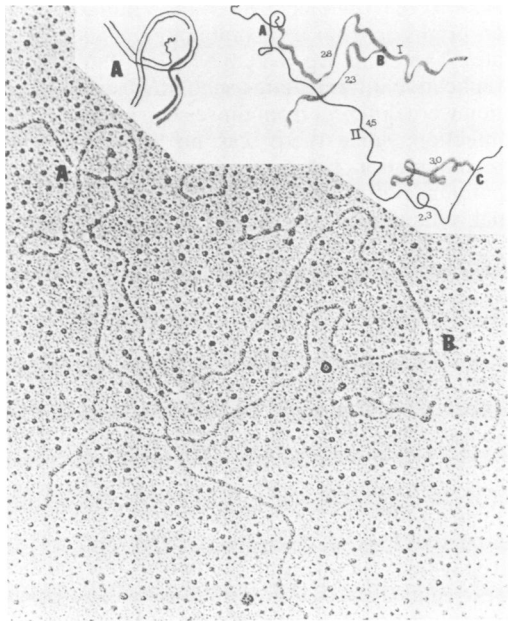

FIG. 7. Part of a multiply branched molecule isolated from shifted fractions of the DNA shown in Fig. 2E. One plausible interpretation of the structure shown is as follows: the rightward replication fork of molecule I is located at B. The leftward replication point of molecule I has reached an end and thereby generated an open fork. The single-stranded terminus of one branch of this fork is invading another molecule (II) at a homologous region (A). (Most likely, a third molecule was invading molecule II at another branch point [C; see the inset]. For the sake of clarity, this segment is not reproduced here. Arabic numbers in the inset represent the distance [in micrometers] between ends or possible branch points. The unequal lengths of the branches originating at C [2.3 and 3.0 μm] indicate that this is not a simple replication loop.)

Both sheared (data not shown) (Dannenberg, Ph.D. thesis) and unsheared samples showed clearly that some replication had occurred by 5 min after infection. Progressive shift of the parental ^{32}P label continued until 12 min after infection; i.e., more slowly than after multiple infections (compare Fig. 9 with 2 and 13). The slower replication could not be explained by lower amounts of replication proteins, since early proteins are overproduced after single compared with multiple infection (65). This suggests that in multiple infections, recombinational initiation accelerated replication of some of the parental DNA.

DNA samples of various densities isolated between 5 and 7 min after infection were spread and inspected in the electron microscope. As expected, all unreplicated DNA at parental density appeared as linear duplex molecules. In contrast, 3% of the molecules in the lighter shoulder fractions contained a single loop (Fig. 10 and 11). Some loops showed single-stranded gaps at one or both branch points. When there were two gaps they appeared in *trans*, consistent with replicative loops viewed in other systems. Some loops showed a whisker at one of the branch points (Fig. 11, a–c). However, none of the molecules from these infections showed whiskers at both ends of a loop. It is plausible that some of these whiskers could be remnants of an RNA primer at the origin of replication. Altogether, 110 replicative intermediates were found with loops like those seen in Fig. 10 and 11. The frequency distributions of their total lengths and the lengths of replication loops are shown in Fig. 12. The proportions of different kinds of loops are summarized in Table 1. A few molecules showed secondary loops within a primary loop (see Fig. 17). However, none of the molecules contained more than one loop in tandem, and no simple or multiply branched molecules like those seen in the coinfection described above were found. Since many molecules were shorter than unit length, we cannot exclude the possibility that we missed a few tandem loops. We conclude, however, that the majority of the molecules used a single origin. The few molecules, if any, which initiated at two origins must have had these origins more widely spaced than the origins described previously (29), but they could have used the major origin between genes *dda* and *dam* that we found and the minor origin near *uvs* y (52a).

DNA isolated early after multiple infection with wild-type T4 particles contained multiple loops and branches. Light bacteria were infected with an average of 10 ^{13}C-, ^{15}N-, ^{32}P-labeled particles in the presence of [^{3}H]thymidine, and replication was monitored by density shift of the ^{32}P label and by ^{3}H incorporation. As in the coinfection experiments described above, these conditions maximized opportunities for recombination between different replicating molecules (although recombinants were not expected to be enriched at intermediate densities). Replication as measured by density shift or [^{3}H]thymidine incorporation started between 4 and 5 min after infection (Fig. 13). (The density shift of the sheared DNA [Dannenberg, Ph.D. thesis] is not shown here.) DNA isolated 5 or 6 min after infection was spread from the light shoulder fractions and inspected in the electron microscope.

Approximately 5% of the molecules from these shoulder fractions contained loops or branches. The majority showed a single loop. Many but not all of these loops looked similar to the simple replication loops seen after single

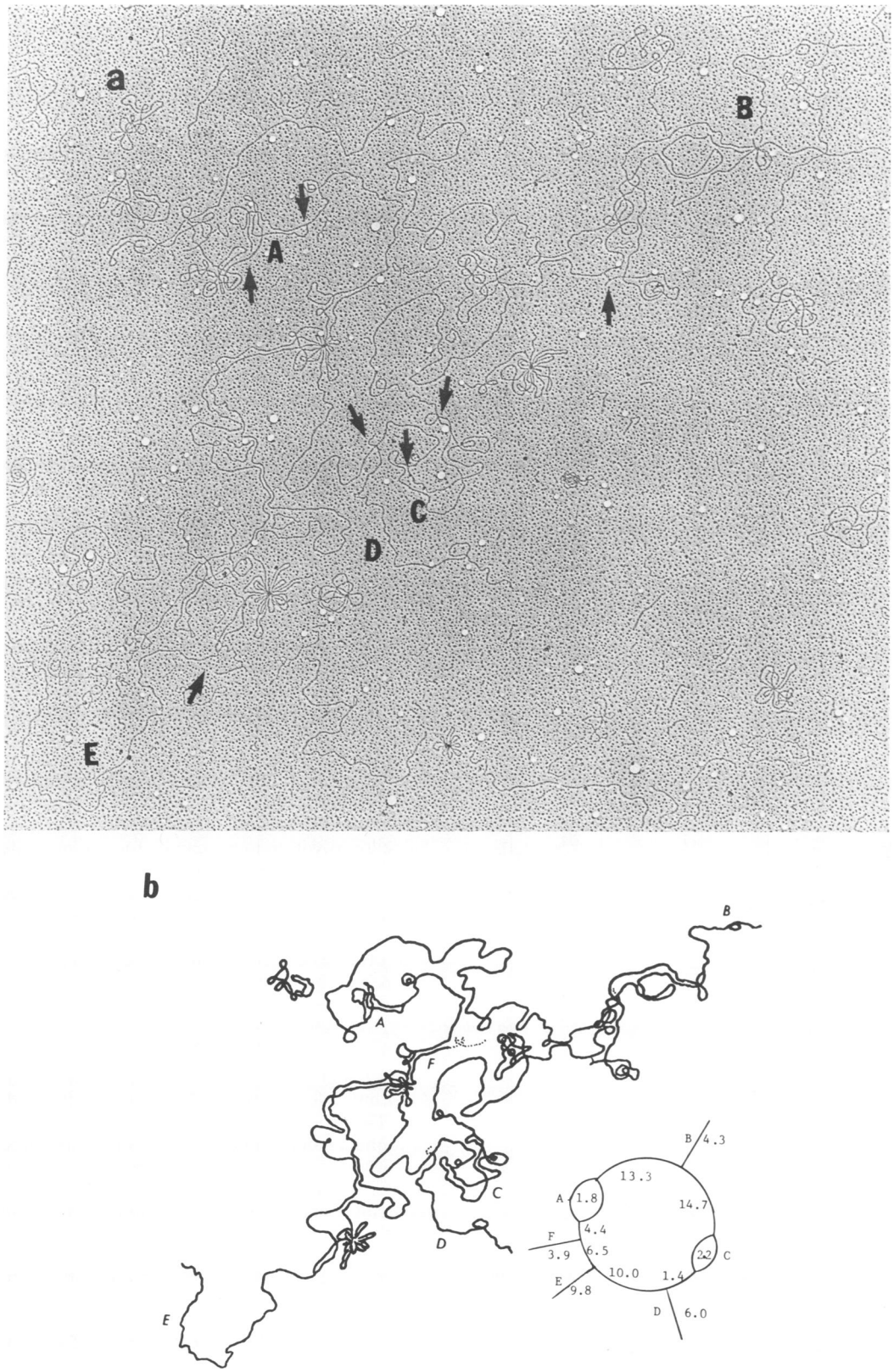

FIG. 8. (a) "Firewheel" DNA. Arrows indicate Branch points. (b) Traced in a certain way (lower right), the contour of an inner circle measures 54 μm (158,800 base pairs). Several branches extend from this circle like a firewheel (85). Solid lines, linear duplex; dotted lines, single strands. A and C, The two loops (whose two branches are of equal length), and B, D, E, and F, the branches of this molecule. Numbers represent micrometers.

infection (Fig. 10 and 11) or in recombination-deficient mutants (18).

On the other hand, as in the coinfection experiment, some of the loops showed long single-stranded whiskers at both branch points (Fig. 14). In some molecules a single-stranded tip was invading ahead of a replication fork (Fig. 15), and 5 of the 210 replicating molecules contained two loops in tandem. In all five of these molecules, one loop was without whiskers and the other loop showed long single-stranded whiskers (Fig. 16, loop D-E). Note that this molecule was considerably longer than unit-length T4 chromosomes; i.e., it must have recombined. In addition to these molecules, highly complex structures resembling that shown in Fig. 4 were found. None of the molecules spread from the parental density fractions showed any secondary structure.

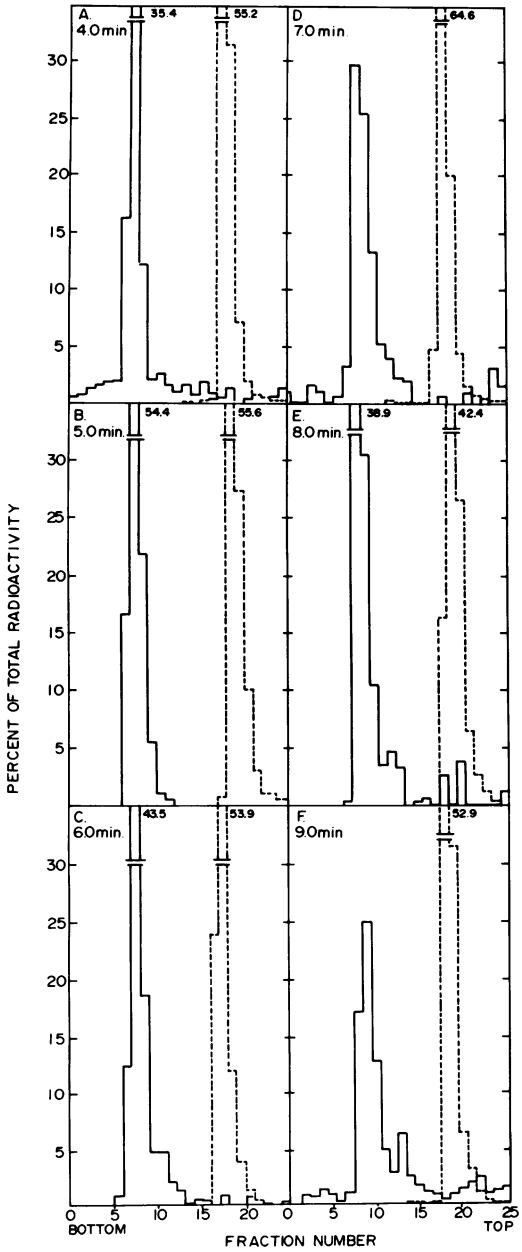

FIG. 9. Distribution in neutral Cs_2SO_4 gradients of unsheared DNA samples isolated at different times after infection of *E. coli* B cells with an average of 0.1 wild-type ^{13}C-, ^{15}N-, ^{32}P-labeled T4D particle per bacterium at 37°C. [^{3}H]thymidine was added at the time of infection. Most of the ^{3}H label (90%) was incorporated into the DNA of uninfected bacteria at the lightest peak, which was used as a density marker. Symbols: ———, ^{32}P; – – – –, ^3H.

A comparison of various intermediates with intermediates obtained under different conditions of infection (Table 1) showed that the early appearance of multiple branches or tandem loops depended on multiple infections and recombination proficiency, i.e., on recombination.

Few secondary loops appeared within primary loops. Of the 110 replicating molecules from single infections and the 210 replicating molecules from multiple infections, 6 and 4, respectively, had a secondary loop(s) inside one (Fig. 17) or both branches of a primary loop. The structures of all 10 loops within loops are shown in Fig. 18. In contrast to replicating λ (72) and T7 (23) chromosomes, such structures were rare in T4. All but two secondary loops appeared off center, and most of the molecules showed a secondary loop in only one of the two branches. Since loops within loops can be formed not only by reinitiation from a primary origin, but also by recombinational invasion (Fig. 1), we concluded that under our conditions of infection reinitiation from the same origin was rare. This is consistent with other evidence that most secondary growing points are initiated from recombinational intermediates (52).

DISCUSSION

Our results can be summarized as follows. (i) After multiple infection of *E. coli* cells with wild-type T4 particles, recombinational intermediates containing label from both parents first appeared as early as 1 min after the apparent onset of DNA replication, long before all parental DNA molecules had finished their first round and before extensive secondary replication was detectable (Fig. 2). (ii) After single infection of *E. coli* cells (i.e., when early intermolecular recombination could not occur), most, if not all, T4 DNA molecules initiated the first round of replication with a single replication loop (Fig. 10). (iii) Molecules in which several loops or branches were arranged in tandem were only found after multiple infection with recombination-proficient T4 (Table 1). (iv) Secondary

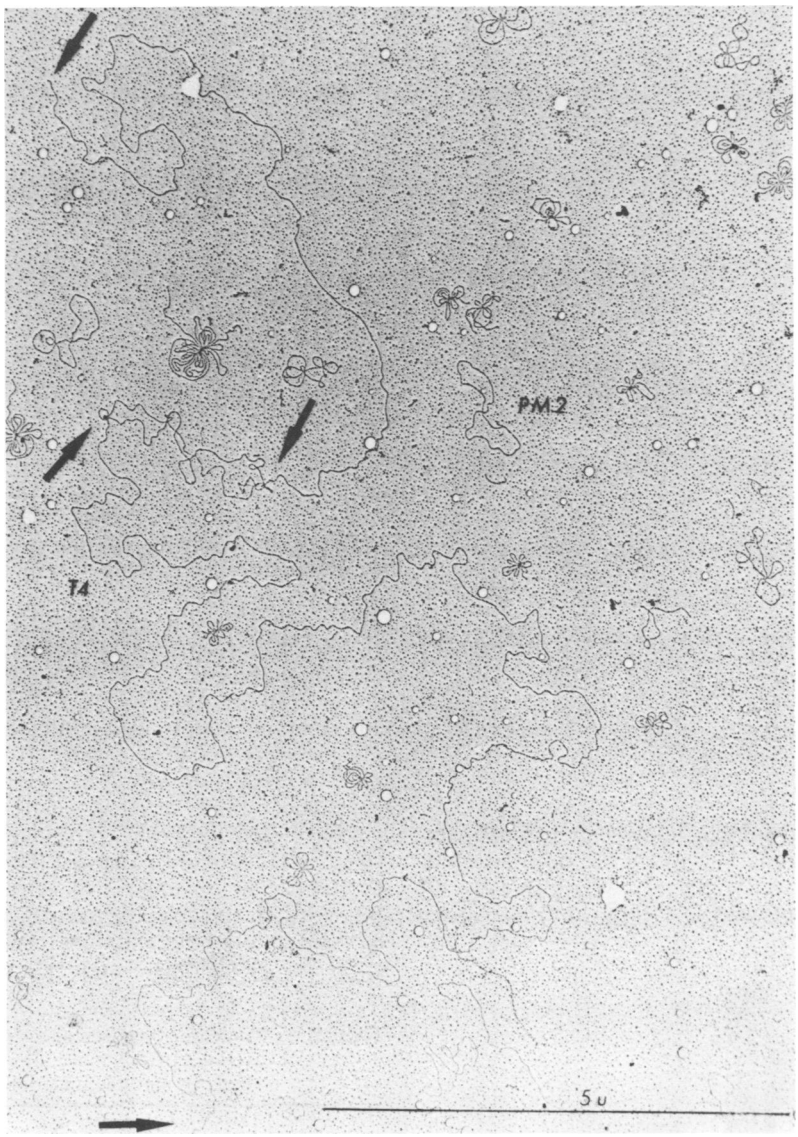

FIG. 10. Replicating T4 DNA molecule isolated from a light shoulder fraction from a low-multiplicity infection. Arrows indicate molecule ends and the replicating loop. Total molecule length was 53.9 μm (158,500 base pairs), i.e., unit length. Margins of the loop measured 3.0 μm (8,800 base pairs).

loops within primary loops were seen after both single and multiple infections, but they were rare and many appeared off center from the primary loop (Fig. 17 and 18). (v) Contrary to common belief (30, 55, 78), concatemers that were complex and highly branched, as late T4 DNA is (3, 17, 31, 39, 41), were found early after infection (Fig. 4, 6, 7, and 8).

Electron micrographs alone cannot answer all arguments for any specific model of DNA replication or recombination. However, together with the density shifts observed and with the results of different experiments (9, 10, 52, 53, 59, 61, 63, 64, 66; A. Luder, Ph.D thesis, Vanderbilt University, Nashville, Tenn., 1981), our electron micrographs are most readily explained as showing intermediates of the model diagrammed in Fig. 1. Although we show here only representative examples, we emphasize that all other replicating DNA molecules (>300) that we found were compatible with this model.

This model incorporates several other previous findings: (i) multiple loops in tandem are not found in recombination-deficient mutants (18);

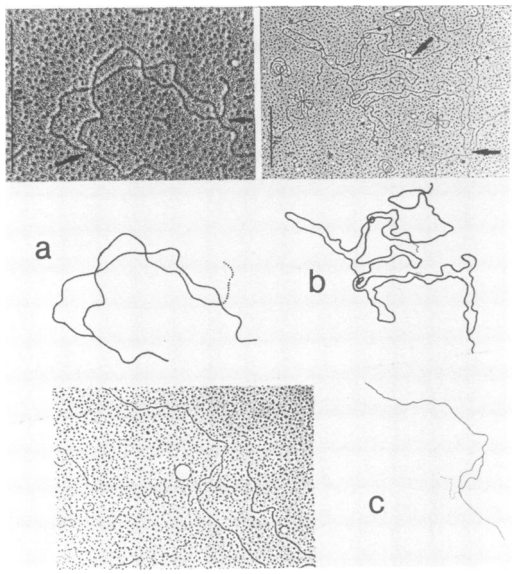

FIG. 11. Electron micrographs and tracings of replicating T4 DNA molecules from light shoulder fractions from low-multiplicity infections. (a) Loop, 0.5 μm, 1,400 base pairs; whisker, 0.25 μm (~800 bases). (b) Loop, 9.0 μm (26,400 base pairs); whisker, 0.24 μm (750 bases). (c) Loop, 0.87 μm (2,600 base pairs), with a 0.16-μm (500 bases) protruding whisker. Arrows indicate branch points of replicating loops.

(ii) early T4 recombination occurs predominantly in the terminal regions of the infecting chromosomes (21, 22, 57, 61, 87); (iii) early T4 recombination can yield either patch or splice recombinants (78), which we had previously called insertion-type and crossover-type, respectively (61); (iv) initiation of replication forks from recombinational intermediates is essential for normal T4 growth (52); and (v) when an invading chromosome forms a patch recombinant (Fig. 1, panel 2, VIII), its terminal marker is more frequently replicated than is a subterminal marker; on the other hand, when an invading chromosome forms a splice recombinant (Fig. 1, panel 2, VII), its terminal marker appears less frequently among the progeny than does a subterminal marker (61). The latter results were obtained by single-burst analysis of 19-factor crosses between a single complete and a single incomplete chromosome (61). As previously discussed (52), these results are readily explained by join-copy recombination but are difficult to reconcile with join-break recombination (Fig. 1).

We now explain the various DNA structures which we found as DNA intermediates in our model. Replication, initiated at an origin (Fig. 1, panel 1, A) generates first a loop (Fig. 5 and 10), which grows into an open fork (Fig. 1, panel 1, B; Fig. 7). Because of problems in initiating the last Okazaki piece, one of its branches remained single stranded at the 3′ end. This single-stranded terminus could invade a homologous region of another chromosome (Fig. 1, panel 1, C) inside a replication loop (Fig. 6) or in an apparently linear DNA segment which may or may not have replicated (Fig. 7). (Since T4 chromosomes are circularly permuted [80], genetically determined origins appear at various distances from an end, and primary growing points initiated there must require different times to reach an end.) The 3′OH end of an invading strand can

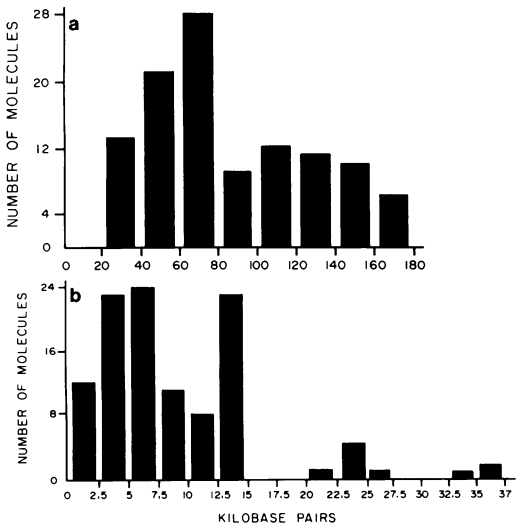

FIG. 12. Frequency distribution of molecule lengths (a) and loops (b) in T4 DNA isolated early after single infection.

TABLE 1. Numbers and percentages of different types of (traceable) DNA molecules found after single or multiple infection[a]

TYPE	high m.o.i. NO.	high m.o.i. %	low m.o.i. NO.	low m.o.i. %	high m.o.i. Gene 32⁻ %
	117	56	97	84	90
	37	18	13	11	9
	4	2	4	3	1
	0	0	2	2	0
	23	11	0	0	0
	5	2	0	0	0
	6	3	0	0	0
	18	8	0	0	0

[a] For easy comparison, the last column shows the percentages of such structures in gene 32 mutants which are recombination deficient (18). In all molecules summarized here (with the sole exception of the sixth molecule shown in Fig. 18), the distances between two corresponding branch points of a loop were measured as equal in both branches of that loop. m.o.i., Multiplicity of infection.

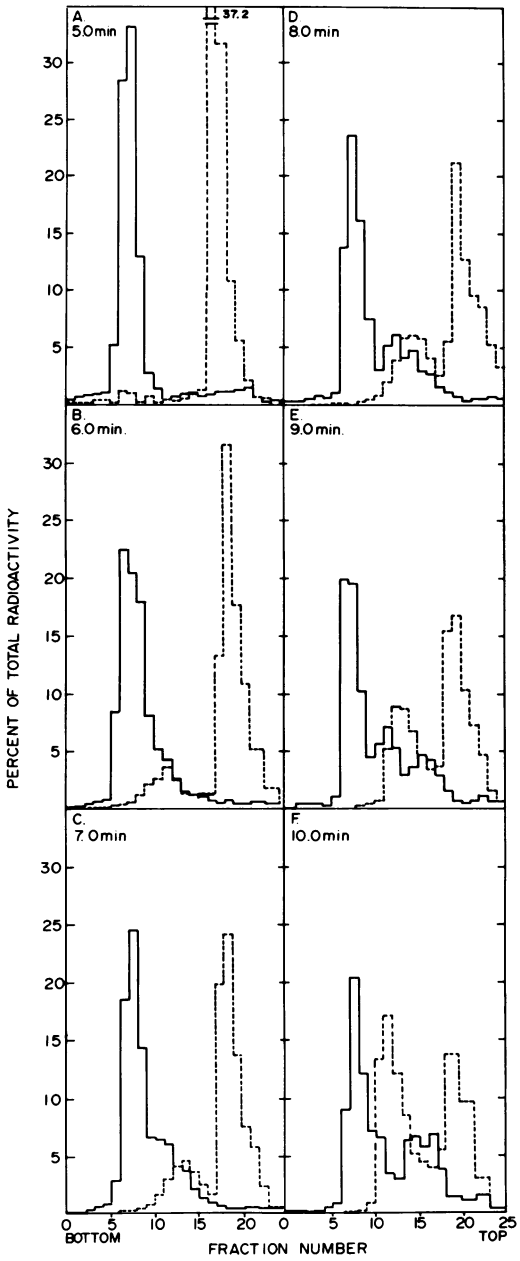

FIG. 13. Density distribution in neutral Cs_2SO_4 gradients of unsheared DNA samples from different times after infection of *E. coli* B cells with an average of 10 wild-type T4D particles per bacterium at 37°C. [^{3}H]thymidine was added at the time of infection. Symbols: ———, ^{32}P; – – – –, ^{3}H.

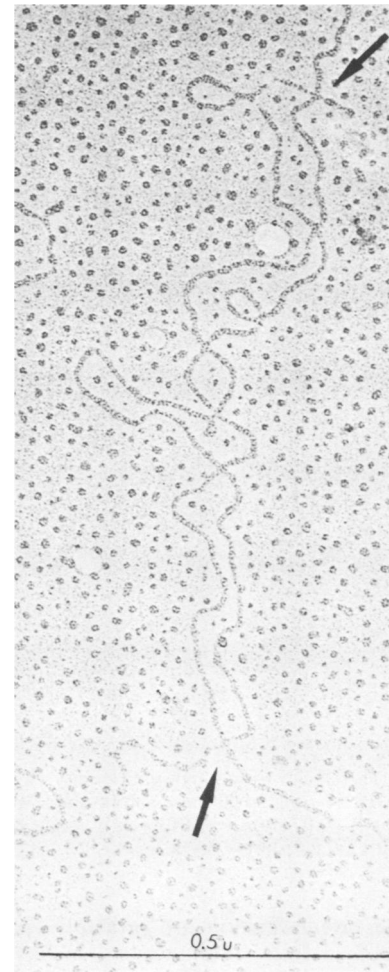

FIG. 14. T4 DNA molecule taken from light shoulder fractions of high-multiplicity infections. Branch points with protruding single strands are indicated by arrows. Loop margins, 1.7 μm (5,000 base pairs); top single strand, 0.074 μm (230 bases); bottom single strand, 0.24 μm (750 bases). Total molecule length was 21.9 μm.

serve as a DNA primer for leading strand synthesis of a replication fork initiated from the recombinational intermediate (Fig. 1, panel 1, D, D'). Lagging strand synthesis can then be initiated on the displaced strand by priming proteins (gp41 and gp61) (2, 74) or from a nick in the recombinational fork (A. Luder, Ph.D. thesis). Reiteration of these processes will rapidly generate highly complex structures (Fig. 4) (39).

Occasional recombination between terminal redundancies of the same (partially replicated) molecule was expected to generate a ring from which one branch of the replicated fork protrudes (Fig. 1, panel 1, C'). Recombinational invasions by other molecules of such a ring should generate firewheel structures. The circumference of the central ring was expected to be of T4 chromosomal length minus the terminal redundancy, i.e., 54 μm (Fig. 8) (6, 85). Chances

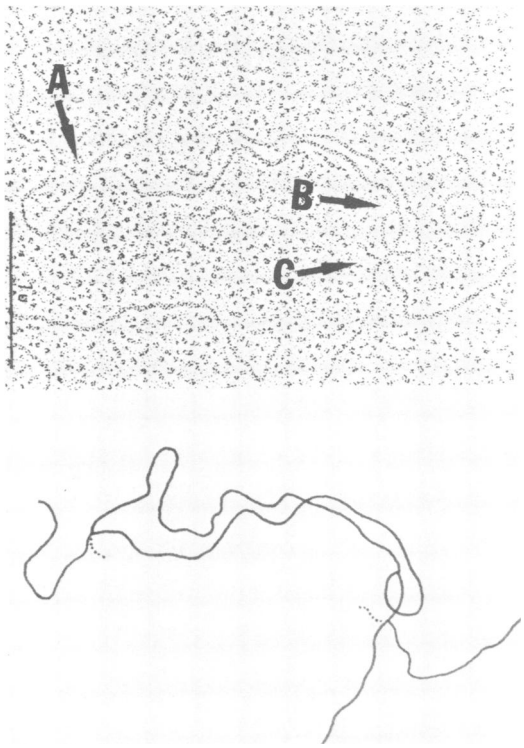

FIG. 15. DNA from high-multiplicity infection. Loop A-B, 17 μm (5,000 base pairs); single-stranded region at A, 0.074 μm (~230 bases); single-stranded region at C, 0.24 μm (~750 bases).

for true circularization are probably rare; because of circular permutation, most single-stranded termini will invade complementary sequences of other chromosomes (Fig. 4). In single infections, however, such recombination between replicated or unreplicated terminal redundancies permits initiation of replication forks via recombination without requiring earlier breakage of the DNA. Obviously, the resulting structures are entangled and extremely fragile and therefore hard to spread intact.

The appearance of single-stranded whiskers at DNA branch points requires some additional comments. Such whiskers can be generated in vitro by (i) branch migration (49) of a leading DNA strand in a replication fork or (ii) displacement of an RNA primer at an origin of replication (52), or in vivo by (iii) recombinational invasion of a double-stranded recipient molecule by a single-stranded donor segment (13, 25) (Fig. 1, panel 2, I) or (iv) displacement synthesis. When the lagging strand initiated from a recombinational intermediate reaches the recombinational branch point, synthesis in that direction may stop (panel 2, IV), or such synthesis may actively promote further branch migration of the invading strand in the direction opposite to the direction of the new replication fork (panel 2, VI), thereby displacing the complementary strand as a whisker (panel 2, VI). There is no unambiguous criterion by which to distinguish among these possible causes, and probably all can contribute to whisker formation. The following considerations argue, however, that under our experimental conditions a large proportion of the whiskers were generated in vivo by recombination through mechanisms iii or iv mentioned above. A total of 31.2% of the 432 forks found under recombination-proficient conditions, i.e., after multiple infection, showed whiskers (Table 1). In contrast, after single infection only 5.2% of the 248 forks identified showed whiskers. Similarly, under recombination-deficient conditions only 4.5% of the 291 forks identified had whiskers (18). The lengths of the whiskers ranged from 200 to 2,200 bases, which is similar to reported whisker lengths of 530 to 1,500 bases in recombinational intermediates formed under replication-deficient conditions (13).

We found recombinational intermediates containing label from both parents much earlier (5 to 6.5 min after infection) than did Broker and Lehman (13), who investigated replication-deficient mutants (15 to 20 min), or Kozinski et al. (44, 45) (11 min). The difference in the onset of recombination between our and Broker and Lehman's experiments suggested that unreplicated DNA is a poor substrate for recombination. This was confirmed by the absence of any recombining molecules among the more than 1,000 unreplicated T4 chromosomes which we inspected from the parental density position of the Cs_2SO_4 gradients. Our results clearly showed that limited replication was sufficient to permit early recombination. One major effect of replication was to generate single-stranded termini that were likely to invade homologous duplex molecules. It is plausible that, in addition, single-stranded regions or simple interruptions in replicated "recipient" DNA and active unwinding (Fig. 15) enhance recombination (for a review, see reference 70). In the absence of replication, nucleases can generate competent substrates for recombination, particularly when ligase is limiting (11), but obviously this requires more time. It also appears that such conditions enhance the formation of H structures by branch migration more than do our conditions.

The different results of Kozinski et al. (44, 45) were, however, obtained under replication-proficient conditions. Several conditions in their experiments were different from ours and could have affected the results. (i) Their use of bromouracil as a density label could have affected recombination and the recognition of origin sequences by certain proteins (13, 14, 51); (ii) their

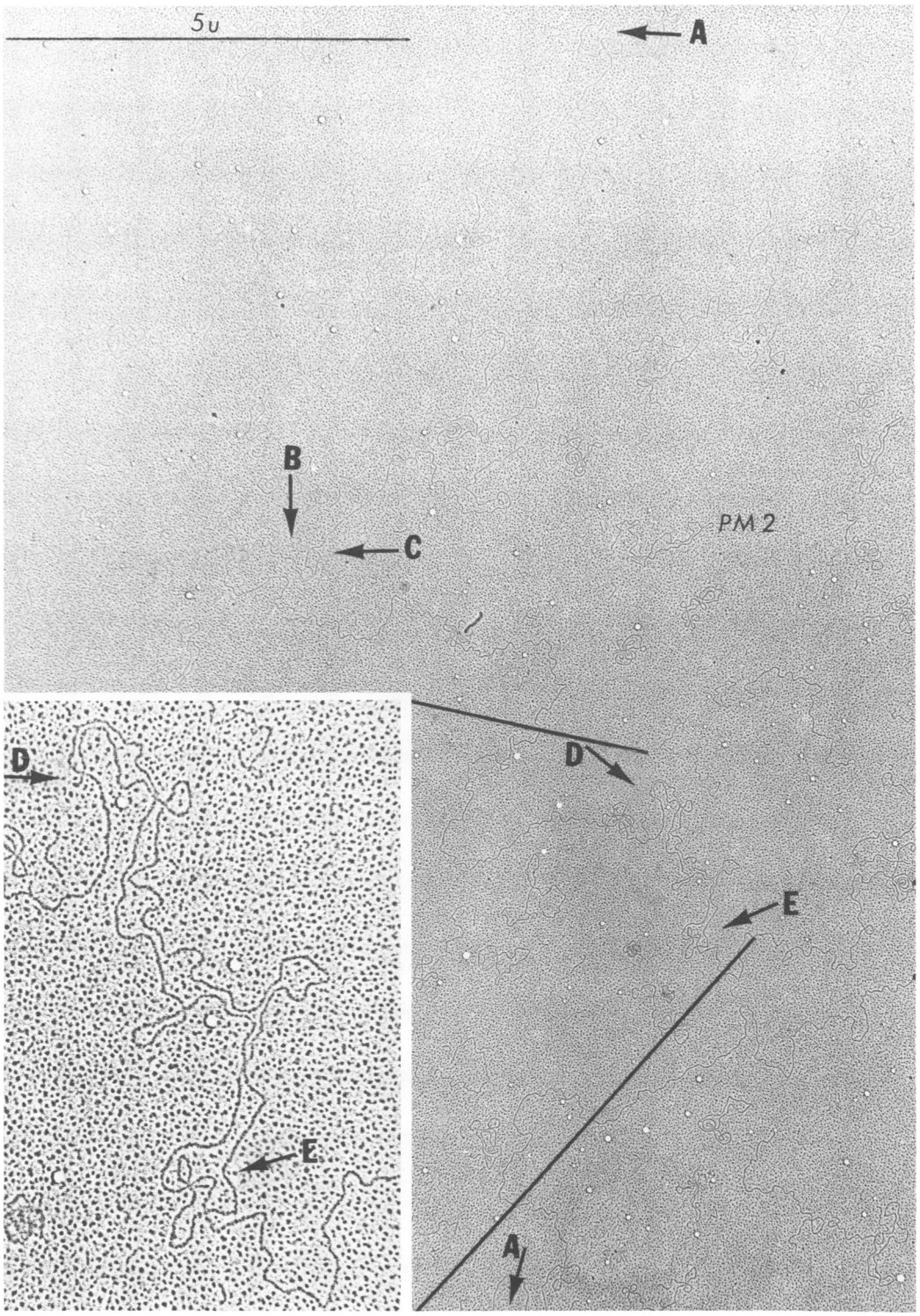

FIG. 16. T4 DNA taken from light shoulder fractions of high-multiplicity infections, with two loops in one molecule. Arrows indicate molecule ends (A) and the branch points of loops B-C and D-E. Inset: Blow-up of loop D-E with single-stranded whiskers. Overall molecule length, 69 μm (202,900 base pairs); loops, 1.2 μm (3,500 base pairs) and 4.6 μm (13,500 base pairs), respectively; single-strand segments, 0.1 μm (300 bases). A small segment of the molecule (lower arrow A) is not shown in this picture. This molecule was isolated 6 min after infection.

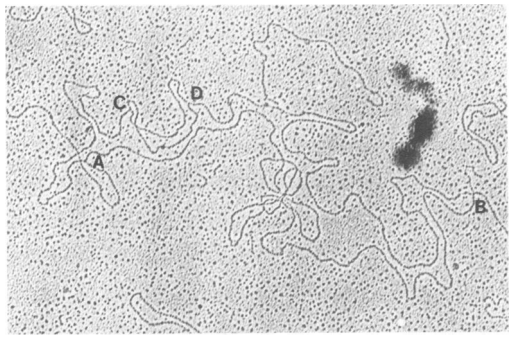

FIG. 17. Loop within a loop (DNA from high-multiplicity infection). A and B, Forks of the primary loop (B with a whisker); C and D, forks of the secondary loop.

use of unequal proportions of differentially labeled parental particles could have lowered the chance of detecting early recombinational intermediates, since some molecules were probably excluded from recombination (76); (iii) the use of chloramphenicol in several of their experiments must have prevented the modification of RNA polymerase and therefore prolonged origin initiation and delayed recombinational initiation (52); and (iv) recombinational intermediates which are joined only by base pairing in heteroduplex regions are not detected in denaturing gradients. The neutral density gradients (44, 45) showed some early shift of light DNA towards heavier densities. Kozinski et al. argued that this density shift was due to single strandedness or association of the DNA with RNA. From our results we concluded that single-stranded regions are generated mainly by recombination. We found the relatively short gaps and whiskers at forks of partially replicated and recombining molecules only in the light shoulder fractions of parental DNA. Apparently these regions were too short to noticeably increase the density in our experiments. (In contrast to Kozinski et al., who used CsCl gradients, we centrifuged in Cs_2SO_4 gradients.)

We found that only 1% of all replicating molecules (5 of 500) contained two loops in tandem arrangement, and none had more than two loops. We do not know whether our results are really different from those of Delius et al. (19), who did not report the proportions of molecules with multiple loops. We conclude, however, that under our conditions few T4 chromosomes first initiated DNA replication de novo from more than one origin. This does not exclude the possibility that T4, like phage T7 (82) or R factors (79), has several origins, but it argues strongly that, as in the other systems mentioned, most T4 chromosomes start replication from one site.

The paucity of molecules containing loops within loops (10 of 500) agreed with other results (19) and confirmed that reinitiation from an

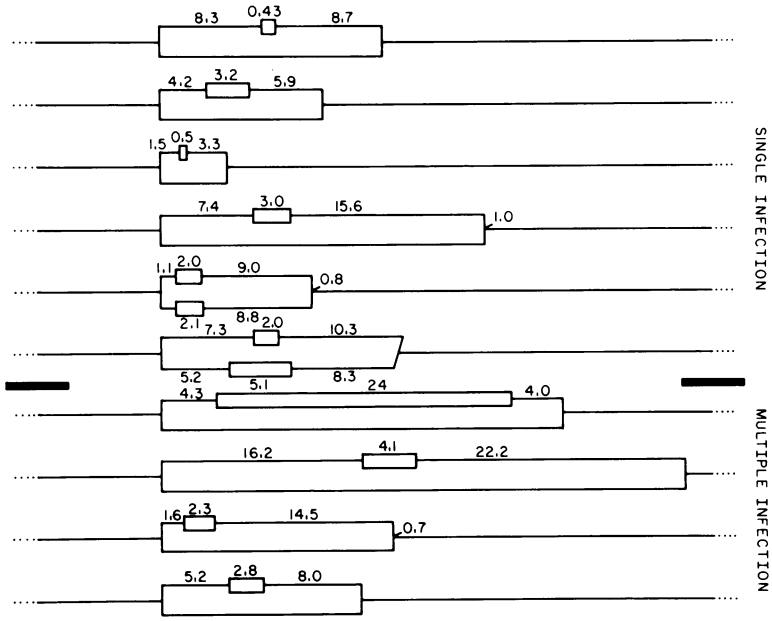

FIG. 18. Diagram of all loops within loops seen after single and multiple infections. The numbers represent lengths in micrometers. With the exception of the sixth molecule from the top, distances between two corresponding branch points were measured as equal in both branches.

origin is rare (64) unless the sequential modification of *E. coli* RNA polymerase during T4 development is prevented by the use of chloramphenicol or by certain mutations (52). In fact, not all loops within loops were necessarily generated by reinitiation from an origin. Although the asymmetric position of secondary loops may be explained by unequal growing point speeds or by slight variations of initiation in both directions within a larger origin sequence, it should be kept in mind that the recombinational invasion of a single-stranded terminus into a replication loop (Fig. 6) could also initiate a secondary loop inside a primary loop.

It has been postulated that T4 DNA replication occurs in two different modes: an early mode, which generates many unit-length daughter molecules, and a late mode, which coincides with the formation of long concatemers (12, 78). Our results and previous observations argue against a defined switch from an early to a late mode and against the idea (12, 20) that multiple replication of linear T4 chromosomes is required before recombination can occur. Our results provide direct visual evidence for the close relationship between DNA replication and recombination in phage T4. Since recombinational forks can be converted to replication forks, no clear-cut distinction between the two could be made. Thus, some of the multiple loops in replicated T4 DNA (19) were undoubtedly initiated by recombination. We have shown elsewhere that the interconversion of recombinational and replication forks is facilitated by the multiple roles and competitive interactions of several proteins with gene 32 protein in replication and recombination (9, 10, 60, 63). The relative proportions of these early proteins must influence the relative contributions of origin versus recombinational initiation to overall T4 DNA replication and, in addition, the choice between join-break and join-copy recombination (Fig. 1, panel 2).

This choice must also depend on the structure of the recombinational forks. The single strands displaced by the invading strands can form a displacement loop or, alternatively, a protruding single-stranded whisker when a proper nick is introduced. Our electron micrographs showed only whiskers, but we consider it likely that some of these whiskers were generated from loops by our lysis procedure at positions where T4 topoisomerase was bound. T4 topoisomerase mutants are defective in initiation of replication forks (54), specifically in initiation from recombinational intermediates (64), without appearing to be recombination defective (16, 32, 50, 67). We used sodium dodecyl sulfate and proteases to deproteinize the DNA, conditions which generate breaks in DNA at positions where topoisomerases are bound (15). We consider it likely that concerted cutting and joining by topoisomerases would facilitate the conversion of recombinational intermediates to replication forks.

Recombinational initiation of DNA replication is probably not unique to phage T4; initiation of DNA synthesis during recombination was first proposed for phage f1 (7), although the underlying results were later explained by a different model. Phage P22 arrests DNA replication when both host and phage recombination systems are inactivated (8). Phages T7 (69) and lambda (75, 78) initiate some DNA synthesis from recombinational intermediates, although their growth does not depend on it. Introducing recombinational hot spots (chi sites, [77]) into lambda results in marked stimulation of DNA synthesis (34). Transposition of transposable elements, including phage Mu, is thought to involve DNA synthesis initiated from an intermediate in this illegitimate recombination (26, 28, 33, 73) and is, in turn, stimulated by replication of the chromosome (83). The recently described DNA intermediates in retrovirus replication (40) resemble the T4 DNA intermediates described here. The *recA*-dependent SOS replication of *E. coli* (43, 48) may depend on initiation from recombinational intermediates (43). Similarly, integration of donor DNA during transformation in *Bacillus subtilis* has been proposed to generate new replication forks (C. D. Laird, Ph.D. thesis, Stanford University, Stanford, Calif., 1967).

Recombinational initiation of replication forks is ideally suited for rapid acceleration of overall DNA synthesis in virus-infected cells and escaping the cellular control mechanisms designed to coordinate cell division and DNA replication. Phage T4 depends on this initiation mode, because the RNA polymerase-dependent priming from origin promoters is turned off as a consequence of the general development program (52).

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