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Late Events in T4 Bacteriophage DNA Replication

III. Specificity of DNA Reinitiation as Revealed by Hybridization to Cloned Genetic Fragments

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Through the use of the technique of hybridization to cloned genes, the site specificity of the reinitiation of T4 DNA replication was examined at late times after infection, when a large amount of DNA had accumulated in the infected cell. Replication was examined under two conditions; (i) when there was recombination but the repair of the recombinants was inhibited, and (ii) when recombination was followed by covalent joining. When no covalent repair of recombinant was allowed, reinitiation occurred in the areas known to be also involved in the initiation of replication of the parental molecule: thus late reinitiation, if covalent joining is prevented, is site specific. When there was covalent joining, reinitiation displayed no apparent site specificity. The results are discussed in light of the possibility that at late times after infection recombinant intersections act as primers. The similarity of the model proposed to the "break-and-copy" model for lambda phage and the fitness of the proposed model to the genetic phenomena described by others are emphasized.

The initiation of replication of the parental molecule of T4 phage occurs at numerous specific locations as demonstrated in electron microscopic studies (3, 6). Recently, two initiation areas were identified by hybridization of early radioactive progeny associated with the "initiated" parental molecule to cloned T4 genes (5). These origins are located in the areas of genes 50-5 and uvsW-29. Although due to the difficulty in the cloning of certain genetic areas large regions in the map remain open for possible additional initiation areas, the notion that the predominant area of initiation resides between gene 42 and 43 (21) cannot be sustained by our data (5).

It was observed that early after infection there is amplification of specific areas of the genome (18). These areas correspond to those of initiation (5). The amplifying mode of replication can be "fixed" by the early addition of chloramphenicol (CM), which allows a large number of copies of partial replicas to be accumulated over a long period of incubation with this drug (18). Yet the addition of CM at times when molecular recombination is fully expressed results in the accumulation of progeny DNA which does not display a genetic bias. The genetic representation of the progeny DNA accumulated at later times after infection without CM is also unbiased. What is the mechanism of this equalization? Before the elaboration of a working hypothesis, let us recall still another, possibly relevant, observation. If one superinfects bacteria which had accumulated a large pool of progeny DNA with radioactive phage of different density, one observes the following: very soon after injection the superinfecting DNA recombines with the pool of progeny DNA. This is so since parental label assumes, in a CsCl gradient, a "progeny-like" density. However, sonication of the recombinant reveals that the parental subunit remained nonreplicated. Denaturation of the recombinant released strands of superinfecting phage DNA quantitatively; thus, such recombinants are not repaired. At later times after superinfection, the superinfecting genome becomes replicated, since after sonication it assumes a heavy:light (HL) location. At this time, invariably, one observes an equivalent amount of parental label covalently joined to the progeny molecules (7).

Taken together, the observations discussed above lead to the hypothesis that recombination might provide for initiation at the 3′ end of a recombinational intersection (7). Such initiation should be random with respect to its genetic location. This, in turn, should provide for full genetic representation of so synthesized progeny—an imperative without which maturation of viable phages would be prohibited. This is perhaps why no true Rec$^-$ T4 mutant has yet been isolated.

In the experiments which follow, we will attempt to demonstrate the genetic nature of the pulse-labeled progeny DNA synthesized at late times after the onset of DNA replication under two conditions: when the covalent addition of progeny DNA to recombinant intersections is inhibited, and when there is covalent joining. It should be recalled here that in the past we have demonstrated that timely addition of CM serves as a fine dissecting tool, separating various replicative events (16). When CM is added at 5 to 6 min past infection there is no parent-to-progeny recombination, and the newly synthesized progeny DNA displays a genetic bias, being fixed in the amplifying mode (18). The addition of CM at 6 to 7 min allows recombination, but upon denaturation pure parental strands not associated with the progeny DNA can be separated; i.e., there is no covalent joining. Finally, the addition of CM at 9 to 13 min allows for recombination and covalent joining of the parental segments to the progeny DNA. This sequentiality of events in the phenomenon of parent-to-progeny recombination is very reliably reproducible.

If the working hypothesis of random initiation at recombinant intersections is correct, then one can postulate the following possibilities for the incorporation of a short pulse-label as schematically depicted in Fig. 1.

In the absence of recombination, a pulse-label will be divided between label which was involved in the process of reinitiation occurring during the time span of the pulse (this should be of pure light density, and likely site specific) and label added to the ends of already initiated, elongating, progeny strands (label thus incorporated would band in CsCl at an intermediate density). In contrast, if recombinational 3′ ends act as randomly located primers, then the distribution of the label in the various density classes is summarized in the lower part of Fig. 1. The isolation of the light strands and the hybridization of these to nitrocellulose filters charged with cloned genes should allow one to discriminate between reinitiation of specific sites versus random initiation.

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MATERIALS AND METHODS

Escherichia coli strain B23 was used in all of the experiments. The phage strain employed was the osmotic-shock-resistant strain $T_4BO_1^r$. The growth medium was TCG (19). The "heavy" medium and phages containing density label 5-bromodeoxyuridine (BUdR) were prepared as previously described (13). All experiments were carried out at 37°C. DNA extraction from infected cells was performed in the following manner. Samples from the infected cell suspensions were diluted twofold in ice-cold LTL-EDTA (0.15 M NaCl, 0.01 M Tris-hydrochloride, 0.015 M EDTA, pH 7.4), and the cells were sedimented and then suspended in 1 ml of LTL-EDTA. For CsCl gradient analysis, the DNA was extracted by the sodium dodecyl sulfate-

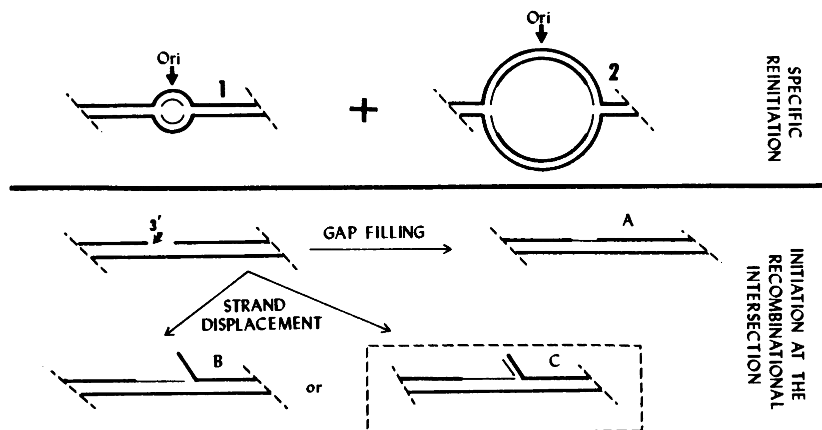

FIG. 1. Schematic diagram of the expected modes of incorporation of short pulse light (and ^3H) label (thin line) when a large pool of heavy cold progeny (thick line) has accumulated. The upper drawings show incorporation when the process of reinitiation occurs at genetically specific sites and proceeds bidirectionally. Note that the radioactive progeny is made up of two distinct classes: (i) radioactive progeny that exists in a replicative loop that was initiated during the pulse (this is of light density upon denaturation) and (ii) radioactive progeny that is attached to elongating heavy progeny strands (this is of an intermediate density upon denaturation). The lower drawings represent events when initiation occurs at the 3′ end of a recombinational intersection. The possibility in the frame is favored. Note that in this case the progeny produced on the displaced strand will be light upon denaturation, but is not site specific.

pronase-phenol method (17). Efficiency of recovery upon extraction is 100%.

When used for labeling progeny DNA, [^{3}H]thymidine ([^{3}H]Tdr) was added as a "package," which upon dilution into the experimental medium resulted in 5 μg of thymidine per ml, an adequate amount of [^{3}H]Tdr to assure the desired specific activity, 5 μg of 5-fluorodeoxyuridine per ml, 5 μg of adenine per ml, and 10 μg of uracil per ml.

The cloning and transfer of cloned T4 DNA segments to nitrocellulose filters have been described previously (28). The hybridization of the experimental DNA to the cloned DNA immobilized on nitrocellulose filters was performed by the method of Denhardt (4). The experimental DNA labeled with [^{3}H]Tdr was combined with ^{32}P-labeled reference mature phage DNA. The mixture was then sonicated, denatured by heat (100°C, 15 min) and hybridized at 65°C for 48 h. For each moiety the hybridization was performed in a single vial containing the nitrocellulose filters charged with the different cloned T4 genes. After hybridization, the filters were extensively washed with 3× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) and dried, and the hybridized radioactivity was counted in a toluene-based scintillation fluid in a liquid scintillation counter. The statistical error did not exceed 3% (see Table 1 of reference 5). The results are expressed as RRx (relative representations of genetic segment x in the progeny DNA hybridized). The ^{3}H/^{32}P ratio observed for a filter charged with a given genomic area x is divided by the ratio of the sums of ^{3}H and ^{32}P hybridized to all the nitrocellulose filters in a set: $RRx = (^3Hx/^{32}Px)/(\Sigma^3H/\Sigma^{32}P)$.

The expression of the data in this form allows all of the experimental results to be drawn on a common scale depicting RR, the relative representation of each genetic area in the progeny DNA (18, 20). The broken horizontal line (see Fig. 4) at an RR value of 1 corresponds to the RR value which would be expected if all the genetic segments tested were equally represented in the progeny DNA.

RESULTS

Although numerous permutations and repetitions were performed, we document here the results of a single experiment. This allows for the direct comparison of the data obtained in different branches. The principle of the experiment and its rationale are outlined above and in Fig. 1. The goal of this experiment was to obtain homogeneously density-labeled light progeny DNA and to analyze its genetic make-up by hybridization to an assortment of cloned genes.

Heavy cold *E. coli* B23 were grown for two generations in 5-BUdR (heavy) TCG and at 0 min were infected with a multiplicity of infection of 6 of single- or double-stranded DNA substituted with density label BUdR (HH) cold phage. At 6 min and 13 min, samples were transferred to CM and incubated for a total of 25 min. In parallel, part of each suspension was supplemented with ^{32}P, and the net synthesis of DNA was measured. This, at 25 min past infection, represents 50 phage equivalent units per infectious center (IC) for CM added at 6 min and 100 phage equivalent units per IC for CM added at 13 min.

At 25 min after infection, the suspensions were chilled, sedimented and suspended in prewarmed light medium containing a [^{3}H]Tdr package with a specific activity of 40 mCi/mg of Tdr. Ten seconds later, the suspensions were promptly chilled by dilution into 4 volumes of ice-cold LTL. The intracellular DNA was extracted and analyzed in native and denatured form in CsCl gradients. Two types of gradients were run: analytical (Fig. 2 and 3), where ^{32}P reference was added, and preparative (data not shown), where no ^{32}P reference DNA was supplemented. There was no appreciable difference in the pattern or proportions in the distribution of the [^{3}H]DNA between the two types of gradients.

Figure 2 shows the distribution of the pulse-incorporated light label as compared with ^{32}P HH and LL duplex DNA not substituted with density label BUdR references. The pattern of distribution of the ^{3}H label in the native form shows that the 10-s pulse resulted in the incorporation of the label into DNA which does not assume a hybrid density, but bands close to the

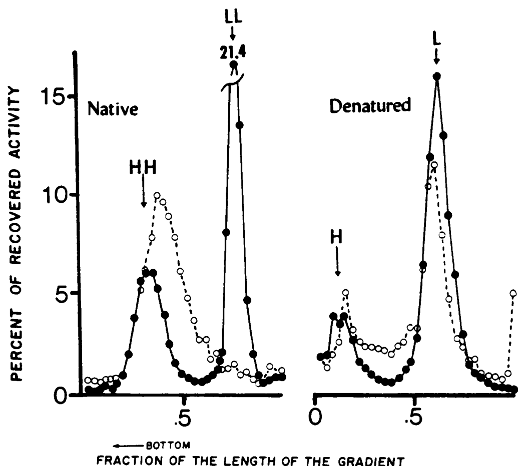

FIG. 2. CsCl fractionation of ^{3}H-labeled progeny DNA produced during a 10-s pulse in the branch of the experiment where CM was added at 6 min past infection. The broken line represents the [^{3}H]Tdr label incorporated into progeny DNA. The solid line represents the ^{32}P heavy and light references added. Note that in native form (left panel), the ^{3}H label bands near the HH location, whereas the denatured (right panel) part, approximately 50% of incorporated label, assumes light density as would be expected for reinitiation during the pulse. The remaining label forms a dispersed pattern as would be expected from the addition of progeny, light DNA to preexisting heavy strands of variable length (elongation). Light moieties from the parallel preparative run were isolated and used for hybridization (see Fig. 4).

HH location. Thus, its schematic appearance might conform with the upper drawings in Fig. 1. Upon denaturation, the label becomes multimodal, displaying a "clean" peak at the light location representing approximately 35% of the total recovery, and a "smear" ranging from the nearly heavy to nearly light locations—this last pattern is what one should expect if the label became attached at the end of elongating progeny strands in the replicative loop. From the preparative run of the denatured DNA, a light moiety was isolated for hybridization to cloned genes. Figure 3 shows a similar analysis of the pulse-labeled DNA from the branch where CM was added at 13 min. The profiles of the native DNA assure that all of the incorporated label bands at the nearly HH location (since in this branch we intended to perform integration of the recovery of both light labels, no HH reference was added). Upon denaturation, the ^{3}H label banded as two classes: approximately 40% at the light location and 60% at variable, intermediate densities. This pattern is what one would expect from either expansion of already formed loops, or (more likely in this case) from the addition of radioactive label to the 3' ends of recombinational intersections. From a parallel preparative run of denatured DNA, the light moiety was isolated for hybridization and for measurement of the size of the strands in alkaline sucrose gradients. The results of the alkaline sucrose gradient analysis (data not shown) showed that ^{3}H-labeled progeny light strands sedimented as a broad peak. The calculated sizes ranged between 5×10^6 and 2×10^7 daltons with an average size of 1×10^7 daltons—this being approximately 1/6 the length of the T4 DNA strand. The observed size distributions were similar for moieties isolated from both branches of the experiment. Importantly, no measurable amount of the ^{3}H label of the size of Okazaki fragments could be detected, which is not surprising considering that the 10-s pulse in 37°C is by far a longer incubation time than that used by Okazaki. Nascent fragments would be expected to be some 12 to 20 times shorter than sizes observed in this experiment. To our best knowledge, Okazaki fragments of near 10 μm in length were never observed. During 10 s at 37°C there is an average net synthesis of 1 to 2.5 phage DNA equivalents per infected cell. Thus some 6 to 15 fragments accumulated in the infected cell during the 10-s pulse.

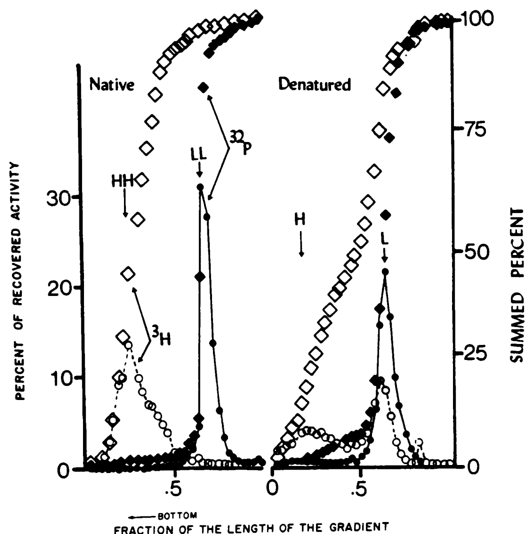

FIG. 3. CsCl fractionation of ^{3}H-labeled progeny DNA produced during a 10-s pulse in the experiment where CM was added at 13 min past infection. The broken line represents the [^{3}H]Tdr label incorporated into progeny DNA. The solid line represents the ^{32}P light reference added. Note that the overall observed distributions are somehow similar to those shown in Fig. 2. Heavy ^{32}P-labeled reference was not added here. This allows one to integrate the reference label along the gradient for a more strict comparison with the integration of the ^{3}H (experimental) label. The integral graph is represented by a sigmoid set of diamonds. Light moieties from the parallel preparative run were isolated and used for hybridization (see Fig. 4).

Hybridization to cloned genes. The light moieties isolated from both branches of the experiment were combined with ^{32}P reference mature phage DNA, sonicated, redenatured, and hybridized to nitrocellulose filters charged with cloned genetic fragments (Fig. 4). The vertical bars depict the representations for moieties obtained from the 6-min branch, and the black dots represent the light moiety from the CM 13-min branch. It is clear that whereas the DNA from the 6-min branch displayed a high preference for the predominant areas of initiation, the DNA from the CM 13-min branch, except for a single "ditch" in the area of tRNA gene, yielded a relatively uniform genetic representation. Thus, the light moiety isolated from the CM 6-min branch represents reinitiation at specific location(s), whereas the light moiety from the CM 13-min branch is not specific.

DISCUSSION

We summarized above the evidence in favor of the working hypothesis that in the presence of the proper proteins, the recombinant intersections, presumably the ones with 3'-hydroxyl ends, can act as randomly located origins, thus providing for equalization of all the genes. This equalization is important, since at early times after infection there is preferential amplification of the genes located in the vicinity of the origins. Somehow the equalizations have to occur. The discussion will be presented in the following

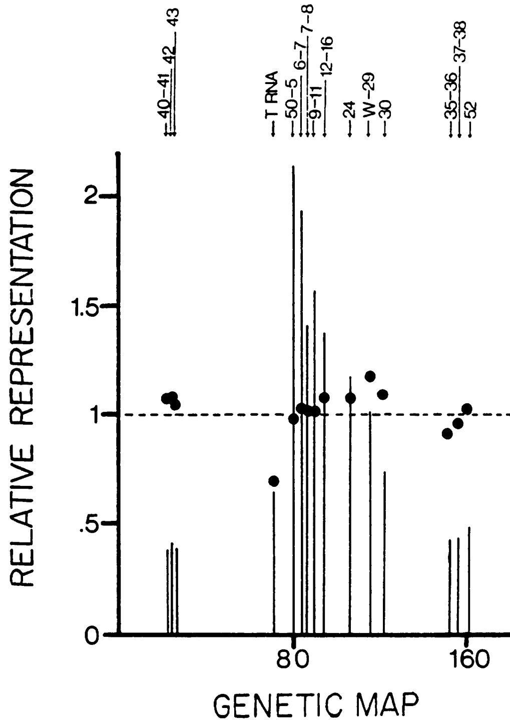

FIG. 4. Hybridization of the light moieties to cloned genes. The thin lines represent the patterns of hybridization of the light moiety from the CM 6-min branch, and the black circles represent the results from the CM 13-min branch. The broken line corresponds to the RR values that would be expected if all of the genetic areas were equally represented.

order. First, the results will be summarized, and our interpretations will be justified. Second, a generalized view of events during T4 development will be provided.

There were two parts to the experiment described: in one, CM was added at a time which allowed abundant DNA replication to occur and also allowed a sizable amount of parent-to-progeny recombination; in the second, such recombination was followed by covalent joining with progeny DNA. We reasoned (Fig. 1) that a short pulse of label can be incorporated either into elongating structures (expanding larger replicative loops, or recombinants), or alternatively into just-initiated strands. In this latter case, one expects that this will occur at a specific location(s). The experiments were performed to isolate newly synthesized fragments of progeny not covalently associated with preexisting strands (thus not participating in elongation) and, upon isolation, to hybridize such to cloned genes.

The branch in which CM was added at 6 min yielded what we believe to be a simple and conclusive result, that the reinitiation of DNA replication occurs at locations which are site specific and correspond well to those described for the initiation of the parental molecule. However, there are subtle differences in the observed patterns, as area uvsW-29 is not as strongly represented as that observed for the initiation of the parental DNA. Otherwise, similar to the initiation of parental molecule, there is a predominant peak of incorporation at the genetic segment 50-5 and minimal hybridization to cloned genes 41, 42, and 43. Thus we can emphatically state that even though there was approximately 50 phage equivalent units of DNA accumulated in the cell, reinitiation occurs at reasonably similar areas to those involved in the parental molecule.

The branch where CM was added at 13 min past infection resulted in data which would best fit to the bracketed mode depicted in Fig. 1. The reasoning for this is as follows: if only elongation and ligation were involved (as in model A in Fig. 1) then CsCl analysis should reveal pulse-label at intermediate locations. A similar expectation should apply to strand displacement (as in model B in Fig. 1). We had, however, observed that 35% of the pulse-label was noncovalently joined to accumulated heavy progeny strands. This would be in conformity with the possibility that the replicative head (3′ end) displaces a strand and, moreover, that the displaced strand becomes replicated (as in model C in Fig. 1). Recently, Nossal and Peterlin (23) demonstrated that if one adds to the reaction mixture gene products 32, 45, 44, and 62, then T4 polymerase can initiate replication at a nick and displace the strand ahead of the 3′ end of the advancing progeny. There is no reason to exclude the good possibility that, in vivo, the displaced strand might be replicated more efficiently than observed in vitro. Such replicated branches would yield, after denaturation, light progeny strands which are random in their genetic representation. This is what was observed when hybridizing the light moiety to cloned genes.

A small ditch in area of gene tRNA needs further explanation. The hypothesis which favors initiation at an intersection and a switch of replication to the displaced strand could be challenged by an alternative hypothesis stating that coincidentally with recombination and covalent joining of recombinants a protein is expressed which acts as a random priming factor. At present, we are not in a position to exclude this possibility. However, due to the good correlation of the covalent joining and semiconservative replication of superinfecting phages (7), we consider the hypothesis of initiation at recombinational intersections to be more plausible.

There is another hypothesis, which we reject, to explain our results. One can assume that light progeny strands are predominantly nascent,

Okazaki fragments. One can then postulate that the early addition of CM results in barriers for the extension of the replicative loops, thus restricting the genetic representation of the Okazaki fragments to the areas adjacent to origin. (Of course, this possibility would by no means weaken the conclusion that, in the absence of recombination, late reinitiation occurs at origins corresponding to those involved in the initiation at earliest times of replication of the parental molecule.) One could then postulate further that if CM is added at 13 min such "barriers" are removed through expression of some putative protein, thereby allowing the extension of the replicative loops throughout the entire length of molecule. In this case, Okazaki fragments residing at the fork should be random in their genetic representation. This interpretation, involving nascent fragments, we consider unlikely since the size of the light moiety is some 12 to 20 times larger than Okazaki fragments and ranges from 5×10^6 to 2×10^7 daltons. No such nascent fragments were ever observed. We wish, however, to emphasize that we do consider the existence of barriers for the early times of CM addition as a very likely possibility (see reference 18). Such barriers would lead to repeated replication and reinitiation of the areas endowed with origins and thus amplification of such areas. Indeed, recently we have demonstrated that one can introduce such termination signals, most likely cuts, by UV irradiation of parental phages. The replication of the irradiated phage DNA results in the amplification of areas corresponding to the areas of initiation (20).

T4 is an organism in which large numbers of recombinational events occur during the history of nearly every molecule (13, 16). Attempts to isolate "healthy," truly Rec^- mutants have, thus far, not been successful. In the previous papers from this series on late events, it was shown that late replication does not differ from the early phases with respect to the basic exponential mode of replication. This was shown by demonstrating that despite numerous events of recombination, clonal distributions of mutants are observed with the same frequency at early and late times of DNA replication (8, 12). Now, we observe a difference in the intricacy of these early versus late replicational events. We postulate, on the basis of the present results, that there are two distinct stages of T4 DNA replication: the first stage occurring early, when replication initiates at specific origins but does not allow the progeny strands to extend all the way. Rather, replicative extension is terminated, yet reinitiation is possible. This leads to gene amplification, where the origin areas are significantly overrepresented. The second stage of replication commences at a time when there is recombination, and a putative protein allowing the covalent addition of progeny to recombinants is expressed. This would be a critical stage in replication providing for the equalization of all the genes—a fact observed in the progeny DNA synthesized late after infection, coincidentally with the onset of molecular recombination and covalent joining of recombinants.

We will now present more specific models of replicative events which follow the joining of recombinational subunits through areas of complementarity.

It should be noted that proposed postrecombinational replication occurs at late times after infection when there is already a large pool of progeny DNA accumulated. It should not be confused with the possibility of interparental recombination before the initiation of the first round of replication, such recombination presumably leading to initiation at recombinational interactions. We are aware of publications where the occurrence of interparental recombination was concluded (22). We do not consider, however, those data as supportive of those conclusions since there were no internal density references provided, and firmly repeatable density alteration, which occurs at early times without application of density label (10), was ignored. In addition, no control experiment was performed without the use of the density labeled phages. We therefore adhere to our previous conclusion indicating extreme low frequency, if any, of interparental recombination among phages productively infecting bacterial cell (10). (We do, of course, support occurrence of interparental recombination at very late times after infection with DNA replication-deficient mutants [11].)

For the purpose of consistency, the possibility of filling in gaps and the excision of single-stranded whiskers will be excluded. We will present the argument that by following the proposed sequence of replicative events, one can accommodate genetic recombination, preferential terminal recombination, as well as the phenomenon of high negative interference (for review, see reference 1). However, it might be reasonable to postulate that some of the infrequently occurring genetic recombinational events might be achieved by molecular mechanisms not representative for the majority of the molecular recombinations.

The basic assumptions of the simplistic model presented are as follows.

(i) The 3' hydroxyl ends of recombinational intersections act as primers initiating replicative elongation.

(ii) When the elongating progeny strand encounters a duplex area of the recombinant, displacement of the 5'-ended opposing strand

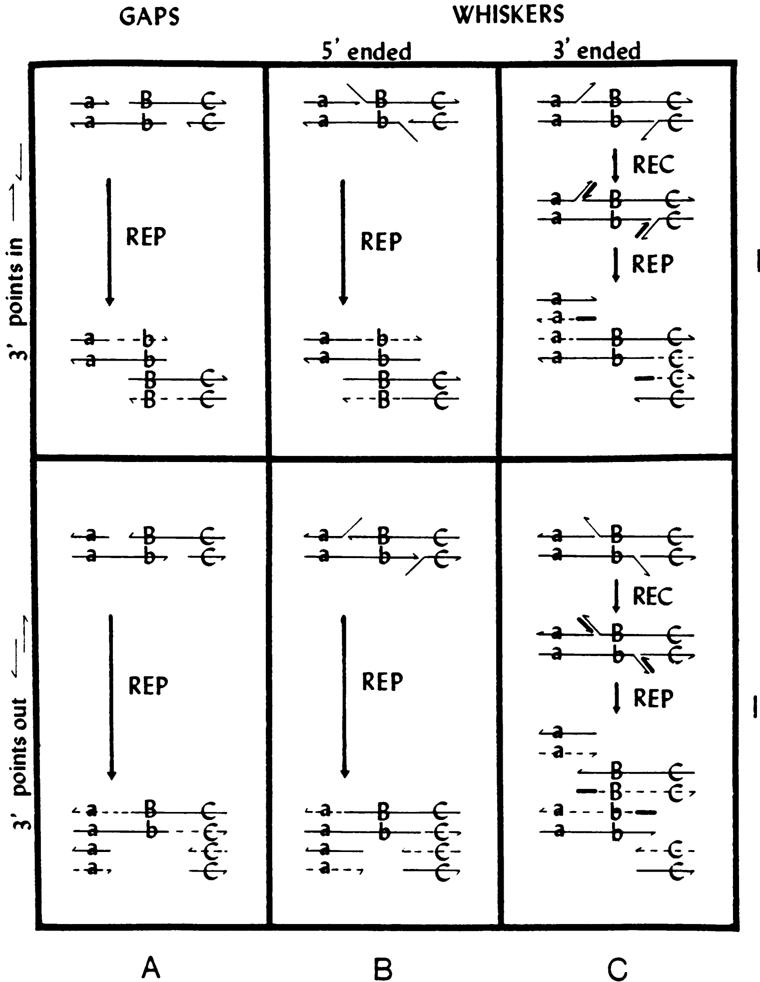

FIG. 5. Schematic representation of initiation and elongation of replication in recombinant molecules. We start with a recombinant between subunits BC and ab. Replication is then initiated at the 3′ termini of the recombinational intersections. The results of this replication are depicted at the bottom of each panel. In panel C, an additional round of recombination is required to provide a primer for the initiation of replication. (The dotted line represents newly synthesized DNA.)

occurs. The displaced strand is replicated by a mechanism resembling, if not identical to, that operating in replicative fork.

(iii) We will assume that branch migration may or may not occur in vivo. Although displaced single-stranded whiskers are observed in the forks of replicative loops (3), it would be naive to assume that this is the way that these whiskers exist in vivo. Similarly, this point might well apply to recombinational intersections. It is quite possible that only after extraction of DNA and spreading for electron microscopy, one observes configurations which are best interpreted as resulting from branch migration (2). If branch migration does occur in vivo, then we have assumed that it simply allows for fluctuation between various configurations, with either the 3′ or the 5′ termini as single-stranded branches. At any point during these interconversions, replicative enzymes could stabilize the recombinant in a particular form conducive for replication (e.g., with the 3′ end of the strand hydrogen-bonded as shown in Fig. 5B) and then replicate it. In Fig. 5, replicative events leading to the segregation of subunits without single-stranded interruptions are presented. We end the diagram at the formation of heterozygotes, since an additional round of replication should result in the segregation of homozygous recombinants. At the top of each panel a recombinant between subunits BC and ab is drawn. The schemes presented are limited to representations of un-

mixed patterns of joining, i.e., either-only gaps, or only whiskers of the same polarity in each recombinant. If this were not done then the abundance of combinatorial possibilities would prohibit us from attempting to graph the schematic events. Upon the conclusion of replication, the obtained subunits might be either complete recombinants (as in panels AII, BII, and CI) or truncated recombinants containing potentially complementary termini (as in panels AI, BI, and CII). Such complementary termini might provide for preferential recombination such as that drawn in Fig. 6A. It should be noted that in the absence of branch migration, the recombinant containing 3′ whiskers represented in panels CI and CII of Fig. 5 cannot replicate until a second recombinational event adds a single-stranded fragment to serve as a primer.

Figure 6 presents two possible mechanisms which lead to the formation of recombinants containing two terminal genetic markers of one parental subunit, when the centrally located marker originates from a different parent. Such recombinant forms have been attributed to double-switch or double-preferential recombinational events (1). Figure 6A does indeed involve multiple recombinational events, the first of which occurs between subunits originating from the same parent. The probability of these multiple recombinational events is enhanced by a 3′-ending single-stranded branch which cannot initiate replication without prior recombination. Then, this probability is further enhanced by the terminal complementarity of the segregated subunits (Fig. 6A). This model employs terminal joining of complementary areas in such a way as to result in a "replicative loop." In contrast to a true replicative loop, the 3′ ends within the forks are not in the usual transposition, but rather in a *cis* position. However, there does not seem to be any reason for this type of a loop not to successfully replicate. The schematic events presented

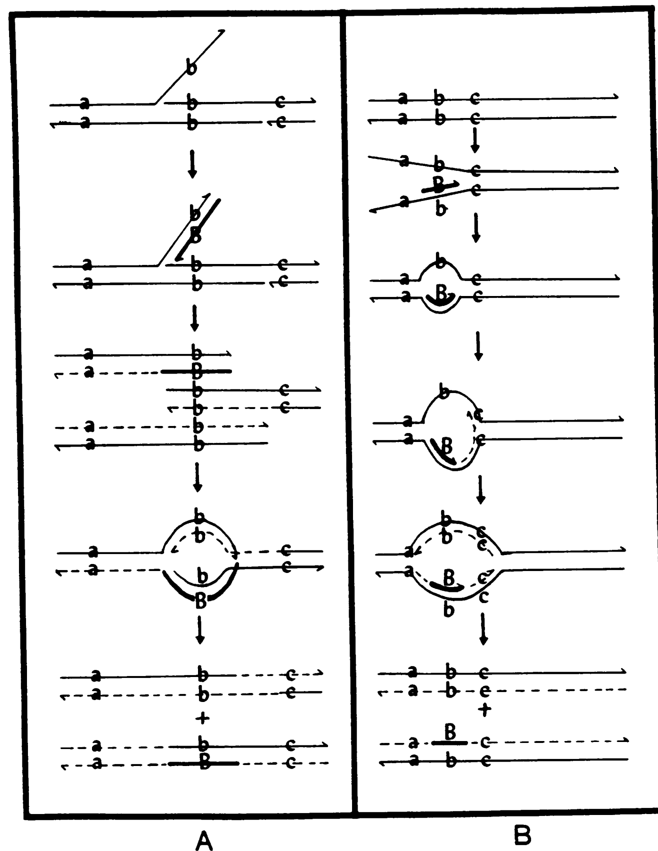

FIG. 6. A schematic representation of possible models resulting in a double crossover event (the phenomenon of high negative interference). The panels present two possible ways of forming recombinants which contain terminal markers of one parental subunit and the central marker from a different parent. Panel A involves multiple recombinational events, whereas panel B involves the invasion of a double-stranded molecule by a single-stranded fragment.

in Fig. 6B involve the invasion of a double-stranded molecule by a single-stranded fragment. We assume that this type of recombination as postulated on the basis of purely genetic experiments (for review, see reference 1) might preferentially occur at the termini of molecules due to the "breathing" effect. However, internal invasion should yield identical results. In summary, we propose mechanisms for the establishment of covalently joined recombinants in which repair involves mostly replication rather than the filling in of gaps, ligation, the excision of single-stranded branches, or a combination of these. It has been observed that contrary to expectation there is efficient covalent repair of recombinant molecules during infection with ligase-deficient mutant phages (15). Furthermore, although both the replicative parental DNA and the progeny DNA are of a very small size, they replicate autonomously as if most of such very short fragments contained origins of replication or, as we consider now more likely, initiation of replication of such fragments could be accomplished by other means (14). At first glance, one would be at a loss to explain the covalent repair and replication of such short recombinant subunits. However, according to the model presented here, the replication of the recombinants could proceed quite efficiently in the absence of ligase simply by the proposed mechanism involving initiation at the 3′ termini of recombinational intersections as presented in Fig. 5 and 6. In experiments of completely different design, in lambda phage, Stahl's group arrived at the conclusion that recombination is achieved by "break and copy" (24–27). There is a similarity of Stahl's model to presently proposed events in T4 phage late replication.

It might be proper now to reflect on the observation that the proposed union of replicative and recombinational events carries the flavor of the theory of "copy choice"—a mechanism of recombination which, in the distant past, was first contradicted in this laboratory (9) while demonstrating the breakage and rejoining of DNA molecules.

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