Molecular Recombination in T4 Bacteriophage Deoxyribonucleic Acid

II. Single-Strand Breaks and Exposure of Uncomplemented Areas as a Prerequisite for Recombination

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Deoxyribonucleic acid (DNA) from several "DNA-deficient" amber mutants was observed to be either nicked (amber 22, 82, 122, and wild type) or cut (amber 453) after injection into a nonpermissive host. This effect was inhibited by chloramphenicol (CM), indicating that it is due to phage-induced enzymes. Although most of the mutants tested for replication in a density-label system were in fact DNA-deficient (amber 22, 82, 122), one (amber 81) was found to replicate almost identically to the wild type, and another (amber 453) was found to assume a hybrid density only. The hybrid moiety was less than, or equal to, one phage equivalent length, and was more efficiently extracted from infected bacteria than was similarly replicated DNA from wild-type phage. Interparental recombination between heavy and light parental DNA was observed for amber 82, 122, and wild type, but was not observed for amber 453; it was inhibited by CM. In contrast to amber 82 and wild type, the amber 453 intracellular DNA does not have single-strand regions. Because amber 453, unlike amber 82 and wild type T4, does not recombine, nicking and exposure of single-strand regions is postulated to be a prerequisite for recombination.

Kozinski, Kozinski, and Shannon (11) have shown that the molecular exchanges between parental and progeny deoxyribonucleic acid (DNA) are inhibited by chloramphenicol (CM) added 5 min after infection with T4 phage. The injected phage DNA is recovered as either an unreplicated or as a "hybrid" molecule. [The term "hybrid," as well as "heavy," "light," "phage equivalent length (PEL)," "round of replication," "replicative hybrid," and "recombinant molecule," were defined in the first paper of this series (10).] Addition of CM 7 min after infection did not interfere with molecular exchanges. Parental and progeny moieties were distinguished by means of a density label, the progeny being more dense or "heavy." Subsequently, using essentially the same techniques (10), we were able to resolve this process still further, showing that the molecular events corresponding to exchanges or recombination proceed in at least two steps: a nicking, or breaking of phosphodiester bonds of DNA, followed by repair of the covalent structure of the polynucleotide chain in the recombined molecule. It was inferred that these steps are due to enzymes which were designated,

according to their functions, "nickase" and "repairase." Nickase is probably identical to recombinase (11). Weiss and Richardson (17) recently reported the partial purification of an enzyme obtained from T4-infected cells, "ligase," with the same properties as repairase.

Recombination in wild T4 phage always follows replication of parental labeled molecules and thus results in hybrid subunits. To test for recombination in the absence of replication, it is necessary to use DNA-deficient (i.e., deficient in synthesis) amber mutants. [Although 5-fluorodeoxyuridine (FUDR) at high concentrations greatly inhibits DNA replication, residual synthesis exists (1, 6).] Such recombination does, in fact, occur (1, 2). The work described in the present paper confirms these results and demonstrates the necessity of exposed single-strand regions for recombination.

MATERIALS AND METHODS

Media and labeling procedures have been described previously (6).

Procedures for analysis in alkaline sucrose gradients have also been described (10), as have the details of using CM (11). Extraction of native intracellular DNA was by the sodium dodecyl sulfate-Pronase-

phenol (SPP) method (12), which is similar to the method of Berns and Thomas (3). The experiments on interparental recombination are similar to those discussed previously (7). Analysis for single-strand regions, with a methylated albumin-kieselguhr (MAK) column and nitrocellulose filters (B6; Carl Schleicher & Schuell Co., Keene, N.H.) was performed by the methods of Mandel and Hershey (15) and Gillespie and Spiegelman (4).

CsCl density gradient centrifugation was performed with the swinging bucket rotor SW 39L, or with an equivalent rotor (13); a 0.3-ml sample was added to 3.0 ml of 9.3 molal CsCl and centrifuged for 72 hr at 31,000 rev/min at 15°C.

Under these conditions, molecular fragments of DNA as small as 2.5×10^5 Daltons reached equilibrium in 60 hr. The distance separating light and heavy DNA is small compared with the distance of either from the center of the rotor; thus, the density gradient between these two points is virtually linear, and hybrid DNA is located one-half way between light and heavy DNA. This linearity was shown in a previous paper (5).

The "wild" phage used in these experiments was T4 B₀₁r. The various amber mutants investigated were B22, N81, N82, N122, and A453 (genes 43, 41, 44, 42, and 32, respectively), most of which have been characterized as DNA-deficient (18). These were obtained through the courtesy of W. Ginoza. The permissive host used for production of amber mutants was *Escherichia coli* CR63; the nonpermissive host was *E. coli* B23.

To test interparental recombination, it was necessary to use heavy amber phages. Whereas wild phage prepared on heavy *E. coli* CR63 yielded heavy progeny with viabilities [plaque-forming units (PFU) compared to optical density or phosphorous content] upwards of 90%, ambers grown on samples of the same bacteria were mostly "dead," producing only 2 to 5% of the PFU expected from the optical density. The number of spontaneous revertants which normally plate on B23 was not similarly affected, but remained at the same percentage to be expected from similar optical densities of light phage. However, if these heavy ambers are simultaneously labeled with ^{32}P , it is found that they not only inject their DNA into a host bacterium,

but that mixed infection with viable, light, nonradioactive ambers results in the rescue of the heavy DNA. This is accompanied by normal replication and parent-to-progeny dispersion. We consider this to be adequate justification for the use of these heavy ambers in the interparental recombination experiments described below.

RESULTS

Introduction of single phosphodiester breaks (nicking) in DNA strands of amber 82 and amber 453. Tests for nicking following injection of phage DNA into the nonpermissive host B23 were made by use of ^{32}P -labeled ambers 22, 122, 82, and 453. Because of similarities to be noted, data for only the latter two are presented here (Fig. 1). DNA was extracted from infected bacteria at the times indicated and analyzed in sucrose density gradients. Parallel analyses were made on native DNA and on denatured DNA. ^3H -labeled DNA from wild-type phages was added as an internal reference for 1 PEL of DNA; the reference was equally good for native and denatured DNA. (Similar analyses performed on DNA extracted from whole phage revealed no differences between amber and wild-type DNA. The nicked and fragmented properties which we attribute to the DNA of ambers 82 and 453 must arise after infection, and are most likely due to phage-induced enzymes.)

The difference between the two profiles of native and denatured intracellular DNA for amber 82 is consistent with the presence of nicks in the native molecule. Denaturation resulted in the liberation of single-strand fragments of less than 1 PEL, which sedimented significantly more slowly than did the ^3H -labeled integral strands. The profiles for native and denatured DNA from amber 453 were essentially identical for each of the two times shown. This is indicative of the presence of cuts, as opposed to nicks; cuts actually split the double-strand molecule into fragments.

A very slowly sedimenting moiety containing

FIG. 1. Integrity of native and denatured intracellular DNA of amber 82 and amber 453 in *Escherichia coli* B. *E. coli* B23 was grown to a concentration of 3×10^8 cells per ml in tris(hydroxymethyl)aminomethane-Casamino Acids-glucose medium (TCG; 14). These cells were infected (one phage per bacterium) with amber mutant 82 or 453 which had been labeled with ^{32}P (specific activity, 3 mc of ^{32}P per mg of P). At several times after infection, samples were cooled by mixing with cold ethylenediaminetetraacetic acid (EDTA) (0.015 M in 0.015 M NaCl, pH 8.2), sedimented, and resuspended in a small volume of EDTA. This suspension was then separated into two parts. DNA was extracted from one part with SPP (12). Freshly extracted ^3H -labeled reference DNA was added, and the mixture was diluted to less than 1 μg of DNA/ml; 0.1 ml was analyzed in a 5-ml, 5 to 20% sucrose gradient (pH 7.4). Centrifugation was for 3 hr at 27,000 rev/min at 15°C in a Spinco SW 39 rotor. Light, ^3H -labeled phage was added to the other part, which was then appropriately diluted and lysed in KOH at a final concentration of 0.2 M. Analysis in a 5 to 20% sucrose gradient made up in 0.2 M KOH proceeded as above. The data are presented as percentages of total recovery per fraction on the ordinate versus percentages of the total gradient on the abscissa, with zero corresponding to the bottom of the tube. $E = \text{input } (^3\text{H reference}/^{32}\text{P intracellular}) \text{ divided by the recovery } (^3\text{H reference}/^{32}\text{P intracellular})$. E for alkaline gradients is not presented, since it was mentioned in a previous paper (10) that recovery from this gradient is always 100%.

AMBER 82

AMBER 453

parental label was seen 20 min after infection (Fig. 1) and the quantity increased considerably at later times. This was apparently due to a continuing degradation of the molecule, and was confirmed by our observation of a decrease in the amount of trichloroacetic acid-insoluble radioactivity (100% at 3 min, 70% at 20 min, 55% at 60 min). Although the size of the single-strand subunits decreased with time in amber 82, the degradation to the acid-soluble form was not nearly so extensive. No corresponding increase in acid-soluble material was noted.

The extent and rate of nicking were both increased by increasing the multiplicity of infection (MOI) to 13.

The addition of CM at the time of infection stopped all nicking, independently of the MOI. This rules out the possibility that nicking is due to a host enzyme, or to an enzyme injected by the phage. It does not rule out the possibility that nickase is a host enzyme induced by some phage-coded protein, but this seems unlikely.

The behavior of ambers 22 and 55 was similar to that of 82. Amber 122 was similar with regard to the appearance of nicks (as opposed to cuts), but the profile of its denatured DNA was much more highly dispersed.

Test for replication of amber phage DNA in the nonpermissive host. Light, radioactive ambers 22, 81, 82, 122, and 453 were tested for replication in the nonpermissive host B23 by infecting heavy bacteria and analyzing extracts of intracellular DNA, native and sheared, in cesium chloride density gradients. Any replication should result in a shift in the density at which the parental ^{32}P reaches equilibrium; we consider this shift equivalent to replication, and to be a more sensitive indication of replication than tests requiring the determination of net DNA synthesis.

Amber 81 replicated as did wild-type T4, but at a slower rate. This was previously noted by Wiberg et al. (18) on the basis of ^{32}P uptake after infection. Ambers 22, 82, and 122 did not replicate at all.

Amber 453 did replicate (Fig. 2); however, even after 60 min, the replicated moiety was only of hybrid density. [Tomizawa, Anraku, and Iwama (16) believed that amber 453 is nonreplicating, and some of their data are interpreted from this point of view.] Equilibrium was reached between 20 and 40 min after infection. None of the extensive exchanges characteristic of wild-type T4 is visible.

The efficiency of recovery from the normal sucrose gradient (see legend, Fig. 1) of the amber 453 replicative DNA is perfect, in contrast to wild-type T4 replicative moieties (8, 12). Also, sodium dodecyl sulfate (SDS) extraction shows

none of the losses characteristic of wild-type phage, and the CsCl profile of replicative DNA extracted in this manner is no different from DNA extracted by the use of Pronase; i.e., there are none of the preferential losses from the replicative peak which one sees with replicative DNA from wild-type phage (12). The replication of amber 453 is clearly of a different nature than that of wild-type T4.

To examine this in somewhat greater detail, a sufficient amount of hybrid DNA was isolated from a CsCl density gradient for further analyses. The re-run material was, in fact, hybrid (bottom row, Fig. 2). Denaturation made the parental label return to the parental density, indicating that no covalent bonds were present in the hybrid structure; i.e., there was neither repair nor covalent circularization. Shearing to 0.125 PEL leaves the density of the hybrid material unchanged, showing that there are no regions of double strand nonreplicated parental DNA. Finally, analysis of the size of the hybrid molecules in sucrose gradient indicated that they are between 0.5 and 0.75 PEL. Moreover, again in contrast to wild-type T4 (10), this material is quantitatively recoverable from sucrose gradients, as is shown by the efficiency of recovery (lower right-hand panel, Fig. 2).

Interparental recombination in ambers 82, 122, 453, and wild-type T4. An experiment designed to test for interparental recombination was performed with each of the phages, amber 82, 122, 453, and wild-type T4. Light B23 was mixedly infected with a minority of light, radioactive (^{32}P -labeled) phages and a majority of heavy phages. Recombination between parental types is revealed by an increase in the density of the ^{32}P label. DNA was extracted by the SPP method, supplemented with ^3H -labeled light DNA, and analyzed in CsCl.

The general profiles obtained (Fig. 3) were repeatable and typical for each phage used. Amber 82 shows a distinct secondary peak; amber 122 shows a smeared region denser than the light reference; the wild-type displays a very slight but uniform displacement of the parental label away from the reference, and amber 453 overlaps the reference identically. This means that the first three phages mentioned show recombination to different extents, whereas amber 453 does not recombine at all. Addition of CM at the time of infection inhibits all recombination.

Comparing the results of amber 82 and T4^{++} , it is clear that the average densities of the recombined molecules differ, the T4^{++} recombinants being lighter. This is most likely due to the fact that, even in the presence of FUDR, there is some residual synthesis of T4^{++} DNA; hence,

FIG. 2. Replication of ^{32}P -labeled amber 453 in a heavy host. *Escherichia coli* B23 was grown for two generations in heavy TCG to a final concentration of 3×10^8 per ml. These cells were infected (MOI of 3) with light, ^{32}P -labeled amber 453. At intervals, samples were removed, placed in cold EDTA, sedimented, resuspended, and either (I) lysed with SDS (final concentration, 0.5%) or (II) lysed with SDS and subjected to Pronase and phenol for extraction of DNA. In both cases, ^3H -labeled light DNA was added; 0.3 ml was mixed with 3.0 ml of 9.3 molar CsCl and centrifuged for 3 days at 31,000 rev/min. Samples were collected and counted. Despite the formation of a proteinaceous precipitate upon the addition of SDS (I), the efficiencies of recovery and patterns of distribution were identical in both cases. The top row gives the results for method II at the times indicated. Hybrid DNA was isolated from a preparative CsCl run (40 min after infection), dialyzed against 0.15 M NaCl-0.015 M sodium citrate, pH 8.2 (CS), and supplemented with ^3H -labeled light, reference DNA. The bottom row shows the results of rerunning this material in CsCl (left) with no further treatment; (second from left) after alkaline denaturation (0.2 M KOH, 15 min at 37 C) followed by dialysis against CSF; (second from right) after shearing to 0.125 PEL. (The size was confirmed in an independent analysis in sucrose.) The lower right panel shows the results of analyzing the dialyzed hybrid DNA in a 5 to 20% sucrose gradient.

FIG. 3. Interparental recombination between light ^{32}P -labeled and heavy cold phages. *Escherichia coli* B23 was grown to 3×10^8 cells per ml in TCG; 100 μg of FUDR per ml was added to inhibit synthesis of DNA in the wild-infected system. The bacteria were divided into four flasks and infected with light, radioactive (^{32}P -labeled, 3 mc/mg of P, MOI of 2) and heavy, nonradioactive (MOI of 9) phage. MOI estimates were made on the basis of DNA content. DNA synthesis in T4 was equal to 0.7 phage equivalents/min. The bacteria were sedimented 45 min after infection (14 min in the case of T4) and resuspended in EDTA. DNA was extracted by the SPP method, supplemented with light ^3H -labeled DNA, and analyzed in CsCl. Displacement of ^{32}P away from ^3H reference is caused by interparental recombination. Similar experiments in which the MOI was two light and two heavy showed no displacement between the ^{32}P -peak and the reference ^3H peak.

the relative amounts of the light and heavy DNA in the "recombining pool" do not correspond identically to the ratios of the simultaneously infecting parental phage. No such synthesis exists with the ambers; the recombining pools in these cases thus reflect the relative MOI. Because of this, one should not expect the curves in Fig. 3 to be comparable. The important observation to be made is that amber 82, 122, and T4^{++} do exhibit some degree of recombination; amber 453 does not.

More detailed analysis of interparental recombination of amber 82 will be described in a subsequent paper. It should be mentioned, however, that analysis in sucrose gradients showed that this recombined peak was composed of molecules equal to or less than 1PEL.

Uniqueness of amber 453. Amber 453 differs from other DNA-deficient mutants in several

ways, the two most relevant being its inability to recombine and the fact that its DNA is presumably cut (not nicked) after infecting a nonpermissive host. It is tempting to postulate that lack of recombination is due to a lack of single-strand regions at the end of DNA fragments; these regions may prove to be necessary for recombination. The data below indicate that there are, in fact, no single-strand ends on the fragments which appear after infection of the nonpermissive host with amber 453.

Mandel and Hershey (15) observed that denatured DNA is retained on a MAK column at the salt concentrations used to elute non-denatured DNA. Presumably, then, DNA with "loose ends" should likewise be more difficult to remove from a column than is DNA without single strands at the ends. Similarly, it has been reported (4) that nitrocellulose filters adsorb

FIG. 4. Analysis in MAK, $CsCl$, and alkaline sucrose gradient of intracellular DNA of ambers 82, 453, and wild-type T4. Bacteria prepared as described in Fig. 3 were infected with light, ^{32}P -labeled phages (amber 82, amber 453, and wild-type) at an MOI of 12. At 25 and 45 min after infection with both amber mutants, and 10 and 14 min after infection with the wild type, DNA was extracted by the SPP method and supplemented with 3H -labeled reference DNA. Less than 10 μg of phage DNA was applied to MAK column. This was eluted with a 0.4 to 1.2 M NaCl gradient and collected in 36 7-ml fractions. These were precipitated with 0.3 M trichloroacetic acid and dissolved in NH_4OH ; a fraction of this was transferred to glass-fiber filters for counting. Efficiency of recovery was calculated as described in Fig. 1. Samples of the extracted DNA were analyzed in $CsCl$ in the usual way, except that the number of fractions collected was increased to 100 in an attempt to improve the resolution. The same material was analyzed in a 5 to 20% alkaline sucrose gradient.

TABLE 1. Adsorption of intracellular DNA of phages amber 82, amber 453, and wild-type T4 on nitrocellulose filters

Determination	Amber phage 82		Amber phage 453		T ₄ BO ₁ ^a		Control ^a
	25 ^b	45	25	45	10	14	
³ H (input)/ ³² P (input) = A	0.678	0.913	0.671	0.738	0.779	0.907	0.791
³ H (adsorbed)/ ³² P (adsorbed) = B	0.049	0.037	0.210	0.457	0.041	0.038	0.017
Enrichment = A/B	13.690	24.670	3.197	1.615	19.012	23.880	46.550

^a Control: normal ³H-labeled DNA + ³²P-labeled heated DNA.

^b Minutes after infection.

single-strand DNA preferentially with respect to double-strand DNA. Also, since single-strand DNA has a slightly greater buoyant density than does double-strand DNA, one might hope to see a slight shift in density in CsCl if a fragment has a significantly large single-strand moiety.

We have performed experiments using these three techniques, and, for reasons which will be clear shortly, sucrose gradient centrifugations. Intracellular DNA was extracted (SPP method) from samples of *E. coli* B23 infected with ³²P-labeled amber 82, amber 453, and wild-type phage. The extraction of injected DNA from the amber-infected bacteria was performed 45 min after infection. Although FUDR was added to the bacterial suspension, wild-type phage still matured. For this reason, the DNA was extracted 14 min after infection, before any maturation occurred. ³H-labeled reference DNA was added, and samples were analyzed.

The profiles of the material eluted from the MAK columns are indeed similar in all three cases, but the efficiencies of recovery were considerably different (Fig. 4). Although DNA from both of the recombinant phages (amber 82 and wild type) was retained in amounts ranging from two-thirds to three-fourths of the input, the DNA from non-recombining amber 453 was quantitatively recovered, compared with the reference. It should be noted that, because the radioactivity estimates are based on acid-precipitable material, there is no meaningful base line.

The results of the filter-retention experiments on the same DNA samples are presented in Table 1. The only valid means of comparing the results is by considering the row of the table labeled "Enrichment," which corresponds algebraically to what has been called efficiency of recovery above.

In a control experiment, heat-denatured phage DNA was treated similarly to the experimental material. It can be seen in Table 1 that the A/B ratio indicates a large difference in the retention of the two labels for this control. Smaller, but still large, numbers were obtained for amber 82

and wild type. The relative retention for amber 453 was significantly less. With increasing time, the enrichment of amber 82 and wild type increased, whereas that of amber 453 decreased.

The CsCl analyses (100 fractions collected) do not reveal (Fig. 4) any tailing which can be attributed to single-strand regions. However, if, in addition to the conventional differential plot of percentage recovery per fraction, one also considers the integral curve of summed percentage from the bottom of the gradient, one sees immediately that the ³²P component of the amber 453 curve rises linearly throughout the gradient until the peak in the differential plot occurs. At this point, when virtually none of the reference ³H-DNA has been collected, almost 50% of the ³²P has been accounted for. Such a distribution is probably due, not to an increase in density of this material, but to its very small size.

This interpretation is further supported by the results of sucrose gradient analysis of this same DNA (Fig. 4). Again, it is the integral curves which are of most interest; when these have plateaued near 100% for both amber 82 and wild type, the corresponding curve for amber 453 has just left a 50% plateau and is increasing to the top of the gradient. This is what one would expect of very small molecules.

DISCUSSION

As with the wild-type phage (10), the integrity of the strands of DNA from DNA-deficient amber mutants is affected after infection of a nonpermissive host. This breakage of polynucleotide chains can be inhibited by CM, which implies that a new enzyme is required for this breakage. This breaking can occur at several places in the molecule, which, if undenatured, maintains its length owing to the persistence of hydrogen bonding. Thus, single-strand breaks (nicks) do not cause double-strand scissions. With a very high number of nicks, of course, the double-strand structure becomes very sensitive to shear. [Therefore, it is not excluded that molecular sizes of native intra-

cellular DNA, in molecules having a large number of nicks, might have been actually higher than estimated, possibly by a factor of two. Although there is somehow more extensive nicking of amber DNA inside the nonpermissive host, at a high MOI, the fact that nicking occurs even at a low MOI contradicts the remote possibility that nicking occurs "*peri-passu*" with recombination as a result of "collision" and exchange.] Such nicks can be inferred to have occurred in a molecule if, upon denaturation, the resulting single-strand fragments are smaller than single-strand reference DNA, or, more conclusively, if the fractional molecular weight of native and denatured intracellular DNA reveals that, after denaturation, the resulting fragments are of smaller fractional size than the undenatured sample (Fig. 1, phage 82).

An exception exists with amber 453. In this phage, intracellular molecules acquire breaks, reducing the fractional molecular size of both the native and denatured DNA to the same extent. This implies the existence, inside infected bacteria, of small double-strand fragments composed of two uninterrupted polynucleotide chains which, if separated by denaturation, remain of a length similar to the native double-strand fragment (Fig. 1, amber 453). The mechanism by which this population of small, double-strand fragments arises is not clear. It is possible that they result from double-strand cuts, or by two trans-situated nicks accompanied by the excision of the DNA situated between the two nicks. The existence of very small breakdown products (very slowly sedimenting moiety, extreme right of the 60-min sample in Fig. 1) and the increase, as a function of time after infection, in acid-soluble label from amber 453 DNA argue in favor of the second alternative. Note, also, that the fragments created in this fashion will not have single-strand regions at either end. There is another difference between intracellular DNA from amber 453 and the DNA of amber 82 and wild type phage. This regards the relative adsorption to nitrocellulose filters and MAK columns, (Table 1, Fig. 4). The adsorption of DNA from amber 82 and wild phage is strong evidence, if not proof, of the presence of single-strand regions within this DNA. These single-strand regions might be of two types. One would be due to partial opening (puffing) of the molecule. This might result from, for example, the replication of one strand, an association of one strand with ribonucleic acid, or interweaving of proteins. The second possibility, which we feel is more likely, is that the open regions are due to the loss of complementary nucleotides. This could be caused by enzymatic removal of a fraction of the strand, or diagonal breakage in which the molecule first acquires two single-strand breaks

FIG. 5. Possible modes of elimination of single-strand ends in amber 453 intracellular DNA fragments.

and is then pulled apart, exposing the single-strand terminal areas. The terminal exposure of the molecules offers the greatest flexibility in reconstructing a large recombinant molecule which is linear. Moreover, even in a recombined molecule, single-strand regions might arise from incomplete overlapping of terminal, complementary single-strand regions of different lengths. This is, in fact, exactly what one should expect and is predicted for random matching of double-strand fragments with exposed single-strand ends. This will be discussed in great detail in a subsequent paper (Litwin and Kozinski, *in preparation*).

Our discussion of Fig. 1 led to the tentative conclusion that amber 453 does not have single-strand regions. This conclusion was strengthened by the adsorption experiments (Table 1, Fig. 4) for the DNA from this phage. As mentioned above, the behavior of this DNA is considerably different from that of both wild-type and amber 82 phage, in a way that can only be interpreted as being due to the absence of single-strand regions in the intracellular DNA in restrictive bacteria infected with amber 453.

The picture which emerges is summarized in Fig. 5. An injected DNA molecule for any of the phages considered is shown on the top. Nicks introducing single-strand regions of undetermined length occur in molecules coming from ambers 22, 82, 122, and wild type, but not amber 453. This accounts for the behavior described as nicking, and the retention on MAK columns and nitrocellulose filters. By contrast, amber 453 is fragmented by some endonuclease as shown in either of the next two rows of Fig. 5, that is, either with clean cuts or with overlapping single-strand regions. However, by the demonstrated uniqueness of amber 453, we know that these are

not significant. Thus, if the breaks occurred so as to leave single-strand ends, they must either be removed by some exonuclease, or their complements must be synthesized by some polymerase. [This possibility should be considered in view of the fact that amber 453 is not completely inhibited in DNA synthetic capabilities, as was thought (16), but undergoes residual replication leading to the formation of a hybrid molecule (when a density label is utilized).] These possibilities are shown in the lower right of Fig. 5. On the lower left are shown two other alternatives, selective partial degradation or synthesis. Although these would remove complementary regions, and consequently abolish the possibility of recombination, the results of the "uniqueness" experiment would seem to eliminate them.

Of the three alternatives (clean cuts, single-strand ends that are removed by exonuclease, and ends that are evened up by synthesis), we prefer the second, a variant with both ends removed, mainly because it is most consistent with the observations in CsCl and sucrose gradients (Fig. 4) and the partial solubilization of parental label (nicking experiment), from which we infer the existence of an increasing amount of very low-molecular-weight fragments. It is also consistent with the decreased adsorption to nitrocellulose filters between 25 and 45 min and with complete lack of retention on MAK columns.

These results, distinguishing between amber 453, which has no single-strand regions, and amber 82 and wild-type phage, which do have single-strand regions, should now be compared with the results of the experiments on interparental recombination (Fig. 3). Here, too, amber 453 was seen to be unique in that it was the only phage tested that exhibited no recombination. We conclude that single-strand regions are necessary for recombination, and suggest that the mechanism whereby this occurs is a random matching of homologous regions at the single-strand ends of small subunits of the DNA molecule.

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