ABSTRACT \(\phi X174\) DNA structures containing two different parental genomes were detected genetically and examined by electron microscopy. These structures consisted of two monomeric double-stranded DNA molecules linked in a figure 8 configuration. Such DNA structures were observed to be formed preferentially in host \(\text{recA}^+\) cells or \(\text{recA}^+\) cell-free systems. Since the host \(\text{recA}^+\) allele is required for most \(\phi X174\) recombinant formation, we conclude that the observed figure 8 molecules are intermediates in, or end products of, a \(\phi X174\) recombination event.

We propose that recombinant figure 8 DNA molecules arise as a result of "single-strand aggression," are stabilized by double-strand "branch migration," and represent a specific example of a common intermediate in genetic recombination.

Genetic recombination in bacteriophage \(\phi X174\) (1, 2) and in the closely related bacteriophage S13 (3) has been analyzed extensively by both genetic (4–9) and physical (10–16) methods. Most \(\phi X174\) recombinants are formed by a major pathway, Tesser's primary mechanism (4, 5), which requires the host \(\text{recA}^+\) allele (6) but apparently does not need any of the nine known \(\phi X174\) gene products (7). Recombinant formation via this major pathway involves two parental replicative form (RF) DNA molecules (5, 7), and occurs very early in the infection process (10). Single recombination events via the major pathway usually generate only one parental genotype and one recombinant (7). The goal of this work was to identify \(\phi X174\) DNA structures which were formed by this major pathway.

To determine whether a particular DNA structure was recombinant, parental replicative form DNA molecules were isolated from cells infected with two parental genotypes (11). After purification and fractionation of the DNA molecules by velocity and equilibrium sedimentation procedures (12, 13), the frequency of recombinants associated with various DNA structures was examined genetically using a spheroplast assay in which further recombination could not occur (10). In addition, we were able to identify putative recombinant DNA molecules formed in mixed infections by using two parental genotypes which could be distinguished by electron microscopy (13).

In this paper we present electron micrographs of \(\phi X174\) DNA molecules which apparently are recombinant. These structures appear to be "figure 8" molecules (15, 17) consisting of linked monomers of two double-stranded parental genomes. The existence of figure 8 molecules containing two parental genotypes supports the proposals of Doniger et al. (15, 18), and Benbow (10) that figure 8 DNA molecules are intermediates in the major pathway of \(\phi X174\) recombinant formation.

We propose a simple mechanism to generate figure 8 DNA molecules that are recombinant. It is based on two molecular processes—"single-strand aggression" (11) and "branch migration" (19–21). These were previously implicated in recombinant formation in \(\phi X174\) (10, 11, 13, 15) and in other organisms (20, 22).

MATERIALS AND METHODS

Recombinant form and multiple length DNA molecules were isolated from cells infected with two genotypes (9, 11, 13, 23; R. M. Benbow, M. Eisenberg, and R. L. Sinsheimer, to be published). The fractionated DNA molecules were examined for recombinants by carrying out spheroplast infections (24), after which the progeny phage were assayed for wild-type recombinants (9). To minimize recombination during the assay, spheroplasts were prepared from NH4547, an \(E. coli\) K12 strain that is \(\text{recA}^-\), \(\text{recB}^-\), \(\text{uvrA}^-\) (7, 10). It is important that the spheroplast assay strain contain both \(\text{recA}^-\) and \(\text{recB}^-\) alleles, and be \(\phi X174\) resistant.

Electron Microscope Assay for Recombinant DNA Molecules. Mixed infections with \(\text{am}3(E)\) and \(\text{delE}15\) (13, 25), two genotypes shown previously (13) to be distinguishable by electron microscopy, were carried out. RF DNA molecules were prepared (11) and viewed by electron microscopy (26).

Recombinant Formation in a Cell-Free System. To assay for recombinant formation in a cell-free system, it was first necessary to prepare replicative-form DNA molecules that lacked recombinant DNA structures. One-liter cultures of HF4712 \(\text{recA}^-\) were infected mixedly with \(\text{am}3(E)\) and \(\text{delE}15\) (multiplicity of infection \(\cong 5\) for each genotype), and replicative-form DNA molecules were prepared as described by Benbow et al. (11). Regions containing RF II DNA molecules (16 S) and some RF I DNA molecules (up to one fraction before the peak of RF I) were pooled from preformed CsCl gradients, concentrated, and dialyzed.

To carry out the assays, cell-free sonicates of \(\text{recA}^+\) cells and of \(\text{recA}^-\) cells were prepared. One-liter cultures of HF714 (\(\text{recA}^+\)) or HF4712 (\(\text{recA}^-\)) were grown in KC broth (9) at \(37^\circ\) with aeration to a concentration of \(5 \times 10^6\) cells per ml. Cells were concentrated 20-fold by pelleting and resuspending in fresh KC broth containing 400 \(\mu g/ml\) of lysozyme, 0.05 M EDTA. After 20 min at \(37^\circ\) these were sonicated six times for 30 sec with a Branson sonicator (large tip). Cell breakage was
TABLE 1. Genetic assay of recombinant DNA molecules

<table>
<thead>
<tr>
<th>Host</th>
<th>Structure assayed</th>
<th>Spheroplast assay</th>
<th>Recombination frequency (wild type/total phase) × 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF4714</td>
<td>Direct burst of bacteriophage</td>
<td>None</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>HF4704</td>
<td>Direct burst of bacteriophage1</td>
<td>None</td>
<td>9.7 ± 1.2</td>
</tr>
<tr>
<td>HF4704</td>
<td>RF I</td>
<td>NH4547</td>
<td>15.5 ± 1.7</td>
</tr>
<tr>
<td>HF4704</td>
<td>&quot;26S&quot;</td>
<td>NH4547</td>
<td>29.6 ± 2.3</td>
</tr>
<tr>
<td>HF4704</td>
<td>&quot;Catenanes&quot;</td>
<td>NH4547</td>
<td>40.3 ± 2.1</td>
</tr>
<tr>
<td>HF4704</td>
<td>&quot;Interband Region&quot;</td>
<td>NH4547</td>
<td>90.7 ± 10.3</td>
</tr>
<tr>
<td>HF4704</td>
<td>&quot;Circular&quot;</td>
<td>NH4547</td>
<td>27.2 ± 2.1</td>
</tr>
<tr>
<td>HF4714</td>
<td>Direct burst of bacteriophage</td>
<td>None</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>HF4712</td>
<td>recA</td>
<td>Direct burst</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>HF4714</td>
<td>RF I</td>
<td>NH4547</td>
<td>11.4 ± 1.3</td>
</tr>
<tr>
<td>HF4714</td>
<td>&quot;26S&quot;</td>
<td>NH4547</td>
<td>19.8 ± 2.1</td>
</tr>
<tr>
<td>HF4712</td>
<td>recA</td>
<td>RF I</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>HF4714</td>
<td>RF I, no chloramphenicol</td>
<td>NH4547</td>
<td>5.8 ± 2.7</td>
</tr>
<tr>
<td>HF4714</td>
<td>&quot;26S&quot;, no chloramphenicol</td>
<td>NH4547</td>
<td>7.1 ± 0.6</td>
</tr>
</tbody>
</table>

1 Although HF4704 is nonpermissive, am3(E) is complemented by am86(A), which allows lysis; am3(E) grows efficiently even in nonpermissive cells, and the yield of progeny phage is normal.

2 "26S" is the total population of multiple-length DNA molecules; it includes circular, catenated, and figure 8 DNA molecules.

3 The middle band of a propidium bromide-CsCl gradient (Benbow, Eisenberg, and Sinsheimer, to be published); contains less than 10% circular dimers, 70% catenanes, and 20% figure 8's.

4 The region between the middle and upper bands of a propidium bromide-CsCl gradient; contains 44% circular, 24% catenated, and 32% figure 8 molecules.

5 The upper band of a propidium bromide-CsCl gradient; contains 61% circular, 27% catenated, and 12% figure 8 molecules.

6 Estimated to be over 99% complete, and survivors were not detected in a 10^-4 dilution.

To the 50 ml of sonicate was added the recombinant-free replicative-form DNA at a multiplicity of infection (based on As20) of 10 RF of each genotype per initial cell. The cell-free mixture was incubated 30 min at 37°C, shaken, centrifuged 6000 × g for 20 min to remove debris, and extracted directly with two volumes phenol (saturated with 0.05 M sodium tetraborate). RF DNA molecules were prepared as described (4) and were examined by electron microscopy. DNA structures were classified according to the criteria of Benbow, Eisenberg, and Sinsheimer (to be published).

RESULTS

Rush and Warner have reported that circular multiple length DNA molecules of bacteriophage S13 were enriched 10- to 15-fold for recombinants relative to monomeric RF DNA molecules isolated from the same infection (12, 14). We were unable to confirm this result in φX174. Instead, as shown in Table 1, we have observed at most a 2-fold increase in recombinants in the total population of multiple length molecules. Furthermore, these recombinants did not segregate with circular dimers during equilibrium sedimentation in propidium bromide-CsCl (Table 1). Instead, a higher recombination frequency was observed in the band containing predominantly "catenanes"; a similar result has been obtained by Doniger et al. for S13 (15). Finally, we also have observed a high frequency of recombinants in regions of propidium bromide-CsCl gradients that do not correspond to any major band (Table 1).

These data support our previous conclusion (13) that most circular dimers are not recombinant, and that some molecules classified as "catenanes" contain two genotypes. These data also establish, as we emphasized previously, that not all catenanes are recombinants. (If all catenanes were recombinants, the catenane band would contain many fold more recombinants than the circular band; it does not, so they were not.)

We now propose that most, if not all, of the observed φX174 recombinants arise from fused dimers (27)—so-called figure 8 molecules (19). In our earlier work (13) using the electron microscope we classified figure 8 molecules with catenanes. However, true catenanes are topologically interlocked rings which usually show two crossover points under our spreading conditions. Figure 8 molecules show only one crossover point. Thus, our earlier report implicating catenanes in genetic recombination did not distinguish true from apparent (figure 8) catenanes (13).

One additional reason for our proposal was that figure 8 molecules were expected to sediment anomalously (in between bands) in propidium diiodide-CsCl gradients, i.e., their rotational constraints would not allow free and rapid conversion from a supercoiled to a fully relaxed figure 8 after nicking, under the conditions used (J. Vinograd, personal communication).

An Electron Microscope Assay for Recombinant DNA Molecules. A φX174 DNA structure that is recombinant is shown in Fig. 1a. This structure appears to contain two parental genomes which are linked in a figure 8 configuration. This structure was isolated from a mixed infection with am3(E) and delE8, two genotypes that can be distinguished by their unequal contour lengths (1.70 μm and 1.55 μm, respectively). Figure 8 structures also can contain two am3(E) genomes (Fig. 1b) or two delE8 genomes (Fig. 1c). The proposed structure of one type of figure 8 molecule is drawn in Fig. 1d, and examples of this structure are shown in Fig. 1e and f.

Figure 8 structures were observed to contain two parental genotypes in over 60% of the molecules measured, as shown in Fig. 2. Therefore, we conclude that figure 8 structures can and often do contain two parental genotypes. The apparent excess of recombinant figure 8's containing two genotypes is unresolved, but may arise from the fact that the two genomes were not completely homologous. Thus recombinant figure 8's containing two genotypes might have been preferentially "trapped" or stabilized.

Frequency of Occurrence of Figure 8 Structures. Since formation of φX174 recombinants by the major pathway requires
Fig. 1. Electron micrographs of ϕX174 DNA structures that are recombinant, spread by the aqueous Kleinschmidt procedure of Davis et al. (30). (a) A figure 8 structure containing one am3(E) and one delE36 genome. The ratio of the two contour lengths is 1.08. (b) A figure 8 structure containing two am3(E) genomes; the contour lengths are 1.69 µm. (c) A figure 8 structure containing two delE36 genomes, formed in a cell-free system. The contour lengths are 1.56 µm. (d) Proposed structure of a figure 8 molecule. (e) A figure 8 structure formed in vivo. (f) A figure 8 structure formed artificially from a dimeric (−) strand and 2 wild-type (+) strands. Note that only (e) and (f) correspond to the structures observed by Gordon et al. (17). Our classification includes three types of figure 8 as illustrated above; approximately 40% are the type seen in (a), 20% the type seen in (b) and (c), and 40% the type seen in (e) and (f).

The host recA+ allele (6, 7) (Table 1), we examined the frequency at which figure 8 structures could be detected in replicative form preparations grown in recA+ and recA− cells. As shown in Table 2, the frequency of observed figure 8 structures was at least 10 fold greater in each of two different recA+ strains, than in the recA− strain. This suggested that the formation of figure 8 molecules—and of genetic recombinants—were both controlled by the host recA+ allele.

Figure 2. The ratio of contour length of the longer of two monomer rings in a figure 8 to the contour length of the shorter monomer. Figure 8 molecules were defined as molecules which appeared to be 1:1 catenanes in the electron microscope, exhibited one-point attachments, and whose ratio of contour lengths did not exceed 1.15.

It is of considerable interest that figure 8 structures were observed to be formed in sonicates of recA+ cells at a much higher frequency than in sonicates of recA− cells (Table 2). This suggests that figure 8 formation can be used as an in vitro assay for the recA+ protein.

How Are Recombinant Figure 8 Structures Formed? We propose that the observed recombinant figure 8 structures were formed by the mechanism outlined in Fig. 3.

The postulated sequence of events is as follows: two single-stranded parental genomes (Fig. 3a) infect and enter the cell, forming double-stranded parental RF DNA molecules (Fig. 3b) (28), which are attached to the host cell membrane at an essential site (29). At some time early in infection (7), a single-strand break occurs or is introduced into one of the two parental genomes (11) (Fig. 3c). This break may be random or specific, natural or artificial, a few or many nucleotides long (11).

We now propose that “single-strand aggression” (11) results in the formation of figure 8 structures as shown in Fig. 3d and e. Formally, a figure 8 structure is an inescapable consequence of two circular genomes that undergo a re-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Figure 8 molecules</th>
<th>Circular multiple length</th>
<th>Catenated multiple length</th>
<th>Total number molecules examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Frequency* × 10^4</td>
<td>Number</td>
<td>Frequency* × 10^4</td>
<td>Number</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>---------</td>
<td>-----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>HF4714 recA+</td>
<td>49</td>
<td>9.8</td>
<td>119</td>
<td>24</td>
</tr>
<tr>
<td>HF4712 recA−</td>
<td>3</td>
<td>0.6</td>
<td>137</td>
<td>27</td>
</tr>
<tr>
<td>HF704 recA+</td>
<td>16</td>
<td>16</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Starting material</td>
<td>0</td>
<td>&lt;1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>HF4714 recA+ sonicate</td>
<td>26</td>
<td>6.5</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>HF4712 recA− sonicate</td>
<td>1(?)</td>
<td>0.25 × 10^-4</td>
<td>36</td>
<td>9</td>
</tr>
</tbody>
</table>

* Number of molecules found per total number of molecules examined.
combination event involving a single strand. However, if we assume, as seems likely, that the number of base pairs participating in “single-strand aggression” is small (perhaps as few as 3), then such figure 8 structures would be rather unstable. Therefore we also invoke double-strand “branch migration” to stabilize the figure 8 structures (Fig. 3f).

No further assumptions are made. It has been shown previously by Weisbeck and van de Pol (30) and by Baas and Jansz (31, 32) that repair of mismatched base pairs occurs in \( \phi \)X174 heteroduplexes as shown in Fig. 3g. It has been shown previously by Doniger et al. (15) that recombinant multiple length molecules replicate and usually generate nonreciprocal recombinants as shown in Fig. 3h and i.

An interesting corollary of our proposal is that nonreciprocal recombinants must predominate by at least a 2:1 ratio if recombination proceeds through these figure 8 intermediates (Benbow and Sinzheimer, to be published).

**DISCUSSION**

The purpose of this paper is 2-fold: to establish that figure 8 structures are intermediates in, or end products of, \( \phi \)X174 recombinant formation, and to propose a mechanism by which recombinant figure 8 structures can be formed.

(i) **Figure 8 structures are recombinant DNA molecules of bacteriophage \( \phi \)X174.**

A role for figure 8 structures in \( \phi \)X174 recombinant formation was first proposed by Doniger et al. (15, 18) and by Benbow (10). Several direct and indirect lines of evidence support this conclusion.

(i) Figure 8 structures often contain two genotypes (Fig. 2). Since recombinant DNA molecules by definition contain genetic information from each of the parental genotypes, figure 8 structures must be considered “recombinant” in a formal sense. However, by itself this does not necessarily mean that they are relevant to \( \phi \)X174 recombinant formation.

(ii) Figure 8 molecules are formed in recA+ cells or cell lysates at least 10 times more frequently than in recA- cells (Table 2). The host recA+ allele is required for \( \phi \)X174 recombinant formation by the major pathway (6, 7) (Table 1), which indirectly suggests that the observed figure 8 structures are relevant to recombination.

(iii) Sedimentation procedures that enrich for recombinants also enrich for figure 8 structures (Table 1). We suggest that figure 8 structures are responsible for most of the 2- (Table 1) or 3- (15, 23) fold increases in recombinants found in multiple length DNA molecules.

(iv) Figure 8 structures are compatible with nonreciprocal recombination. We thus suggest that the recombinants observed in the single burst experiments of Doniger et al. (15) arise predominantly if not entirely from the 7% or more of figure 8 DNA molecules in their infecting material.
June 1973. This combination
(2) Figure 8 structures that are recombinant are generated by “single-strand aggregation” and are stabilized by “branch migration.”

“Single-strand aggregation” is a process catalyzed by the host recA + protein in which an RF DNA molecule containing a single strand region interacts with another RF DNA molecule, ultimately leading to a recombination event (11). The point we wish to emphasize here is that aggregation by a single strand inevitably leads to a structure which, if stable, looks like a figure 8 in the electron microscope. In contrast, a circular double length recombinant molecule absolutely requires scissions in both strands. Conversely, double-strand breaks do not necessarily generate figure 8 molecules.

Since single- and double-strand “branch migration” occurs in φX174 molecules in vitro (10, 13, 21), we attribute our failure to detect figure 8 structures in recA - cells to a failure to form the nascent figure 8 molecules, rather than to a failure to “branch migrate” in these cells.

Our mechanism of figure 8 recombinant formation seems relevant not only to φX174 recombination but also to prokaryote recombination (φX recombination uses the host recA + protein), and to analogous processes in eukaryote recombination. However, most other organisms do not have small circular genomes; we, therefore, point out that our model in Fig. 3 applies to linear genomes (to draw, cut 180° from the b/b allele), resulting in chiasma (x-) and H-branched structures (20). In addition, in linear or long circular genomes an alternative way to proceed from single-strand invasion to a double-strand exchange (i.e., from 3d to e) exists. This alternative, which is forbidden to φX174 because of its very short circular genome, is shown in Fig. 4.

The consequences of this alternative are striking. Reciprocal recombinants are generated for external markers with non-reciprocal (gene-convertible) regions of hybrid DNA in between.

An oral account of this work was given to the Genetic Recombination Symposium, Bethesda, Md., 6–9 June, 1972, to the British Genetical Society, Kent, April 1973, and to the European Molecular Biology Organization meeting at Aviemore, England, June 1973. This research was supported in part by Grant GM 13554 from the U.S. Public Health Service.