An Electron Microscopic Analysis of Pathways for Bacteriophage T4 DNA Recombination

THOMAS R. BROKER†

Department of Biochemistry
Stanford University School of Medicine
Stanford, Calif. 94305, U.S.A.

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Branched DNA molecules that are intermediates in the process of recombination can be observed by electron microscopy of extracts of bacteriophage T4-infected Escherichia coli (Broker & Lehman, 1971). When the phage were defective in various combinations of eleven genes that affect DNA metabolism, DNA structures characteristic of the particular mutants accumulated. From their physical properties and their frequencies of appearance, the intermediates have been arranged into a pathway for DNA exchange in the absence of replication. Recombination of T4 DNA appears to require single-stranded regions (which generally are shorter than 1250 nucleotides) for pairing. At about one-half of the branch points in the recombinational intermediates, one of the branches was single-stranded for a (median) length of 600 nucleotides. Single-stranded fork structures characteristic of strand displacement, a form of branch migration, were observed at one-half of these branch points and indicated that the heteroduplex pairing region had a minimum median length of 420 nucleotide pairs. Correlated multiple exchanges occurred in about one-third of all branched molecules. Some multiple branches must have arisen in a single pairing event that included separatory migration of the branches by either strand displacement or strand assimilation; others were the consequence of reiterated synapse of chromosomes. The variety of intermediate structures is consistent with alternative enzymatic routes being available to DNA recombination.

During wild-type T4 infection cycles, recombination might occur between single-stranded regions generated by displacement (asymmetric) replication in addition to those formed by degradative processes, as in the studies reported here. A model is presented in which linear concatemers are formed by recombination of newly replicated sibling chromosomes at complementary single-stranded ends which remain when the discontinuous mode of replication fails to initiate synthesis of terminally situated segments.

1. Introduction

DNA structures with branches are produced after infection of Escherichia coli with a high multiplicity of phage T4 defective in DNA polymerase and polynucleotide ligase (Broker & Lehman, 1971). The branches are the result of recombination between parental phage chromosomes, as judged from (1) their appearance in cell lysates only after early phage functions have been expressed, (2) their near absence when cultures

† Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. 91109, U.S.A.
are infected with an average multiplicity of one T4 per bacterium, and (3) the enrichment of branched DNA in the hybrid density region of a Cs₂SO₄ equilibrium density gradient of DNA extracted from cells infected with T4 pol lig T phage of two different densities. Branched recombinational intermediates were observed subsequent to the accumulation of single-strand interruptions (nicks) in the parental DNA, the enlargement of nicks to gaps and the exposure of single-stranded termini, and when the gene 32 protein (Alberts & Frey, 1970) was active. We suggested that the heteroduplex region of pairing between the interacting DNA molecules could be extended by either of two types of branch migration: (1) a displacement of one strand of DNA from its complement by the homologous strand in the other branch; (2) a replacement of an exonucleolytically degraded strand of DNA by the homologous DNA in the other branch. Strand displacement has been studied by Lee et al. (1970); strand assimilation by Cassuto et al. (1971).

The imposition of a series of mutational (or chemical) blocks within a metabolic pathway is a classical way to probe its organization. Intermediates accumulate behind each block and can be identified by their enrichment in cellular extracts. Their structural relationships may then permit a logical reconstruction of the pathway. In this paper, I have examined the fate of parental phage DNA extracted from E. coli infected with T4 phages mutant in various combinations of eleven genes involved in DNA metabolism including, in each case, gene 43 (pol−), to prevent replication. The DNA structures observed by electron microscopy were co-ordinated with the structures deduced from ultracentrifuge studies and from the response to enzymatic modification of similar phage T4 recombinational intermediates (Anraku & Lehman, 1969; Anraku et al., 1969; Prashad & Hosoda, 1973). Comparison of these data with the enzymatic properties of some of the pertinent gene products and with the effects on recombination frequencies caused by limiting (but not excluding) the activity of a number of phage T4 early genes (Bernstein, 1968; Berger et al., 1969) has permitted a partial description at the molecular level of phage T4 recombination in the absence of DNA replication. Additionally, the fate of the infecting DNA in the absence of several gene products, about which little is known, has indicated some general features of the enzymatic roles of those products.

2. Materials and Methods

Most of the experimental procedures have been described previously (Broker & Lehman, 1971).

(a) Bacteriophage T4 mutants

The alleles of phage T4 from which multiple mutants were constructed are listed in Table 1. Dr Kitty Hercules kindly provided T4 das (13) 46 (B14 x 5) 47 (A456 x 5) 43 (B22) and T4 das (13) 46 (B14 x 5) 47 (A456 x 5) 30 (E13) from which I made T4 das− 46 − 47 pol− lig−. Combinations of mutations of many T4 early genes with B22 and/or E605 are available on request.

(b) Infection of E. coli RR with phage T4

Before infection of E. coli with phage T4, the cells were concentrated by centrifugation at 37°C and resuspended at 3 x 10⁹ bacteria/ml in T4 adsorption buffer (Hershey & Chase,

† Abbreviations used: unless mutants are designated otherwise, T4 pol−, T4 amB22 (gene 43, DNA polymerase); T4 lig−, T4 amE605 (gene 30, DNA ligase); T4 32−, T4 amE315; T4 HMChe, T4 amN122 (gene 42, dCMP hydroxymethylase); T4 46−, T4 amN68; T4 47−, T4 amN011; T4 61−, T4 amHL627. The combination T4 das− 46− 47 pol− lig− is described in Materials and Methods.
**PHAGE T4 DNA RECOMBINATION**

**Table 1**

*Bacteriophage T4 mutants used in this study*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function or effect</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Polynucleotide ligase</td>
<td>E605, H39X (Hosoda), E13 (Hercules)</td>
</tr>
<tr>
<td>32</td>
<td>DNA binding protein</td>
<td>E316, A453</td>
</tr>
<tr>
<td>41</td>
<td>(Slow DNA synthesis)</td>
<td>N81</td>
</tr>
<tr>
<td>43</td>
<td>DNA polymerase</td>
<td>B22, 4317</td>
</tr>
<tr>
<td>44</td>
<td>(No DNA synthesis)</td>
<td>N82</td>
</tr>
<tr>
<td>46</td>
<td>Exonuclease?</td>
<td>N68, B14 × 5 (Hercules)</td>
</tr>
<tr>
<td>47</td>
<td>Exonuclease?</td>
<td>N011 (T4B), A466 × 5 (Hercules)</td>
</tr>
<tr>
<td>61(58)</td>
<td>(Delayed DNA synthesis)</td>
<td>HL627, E219</td>
</tr>
<tr>
<td>62</td>
<td>(Slow DNA synthesis)</td>
<td>E1140</td>
</tr>
<tr>
<td>rIIA, B</td>
<td>(Rapid lysis)</td>
<td>del 1589 (T4B) Kaiser via Gellert via Stahl</td>
</tr>
<tr>
<td>das</td>
<td>(Suppresses 46-, 47-)</td>
<td>13 (Hercules)</td>
</tr>
</tbody>
</table>

Unless noted, all alleles are amber mutations in T4D and were obtained from the California Institute of Technology collection administered by Dr William B. Wood.

1952) at 30°C. This procedure may have resulted in some uncontrolled stimulation of DNA recombination due to anaerobiosis analogous to that caused by KCN or NaN₃ and studied by Tomizawa & Anraku (1964a,b). If so, day-to-day variations in the growth and isolation of *E. coli* could have been responsible for the fluctuations in the percentage of branched molecules observed in individual samples of a given mutant.

(c) *Isolation of phage T4 DNA from phage-infected* *E. coli* *BB*

One-fourth to one-half the parental phage DNA was recovered from Cs₂SO₄ equilibrium density gradients of sodium lauryl sulfate-induced lysates of T4-infected *E. coli*. The remainder of the DNA was trapped in the insoluble detergent layer at the top of the gradient. The purified T4 DNA was assumed to be representative of the intracellular pools before lysis.

(d) *Electron microscopy*

The aqueous diffusion method for mounting DNA in protein films has been described (Broker & Lehman, 1971).

Single-stranded DNA was extended during mounting and visualized by the formamide spreading procedure described by Davis et al. (1971).

Platinum–palladium (80:20) shadowing wire (0.008 gauge) was purchased from Baker Platinum Co., 700 Blair Road, Carteret, N.J. 07008, U.S.A.

Siemens I and Siemens IA electron microscopes equipped with sheet film (2.25 in × 3.5 in) and a Philips EM300 equipped with 35-mm roll film were used to analyze the samples.

Measurements of the length of single-stranded DNA are approximate because they were based on the magnification setting of the electron microscope without reference to a standard. Under the conditions used for the preparation of samples described in this paper, double-stranded T4 DNA (1.82 × 10⁶ nucleotide pairs) is 56 nm and single-stranded T4 DNA is 50 nm long, which correspond to 3.25 nucleotides/nm and 3.64 nucleotides/nm,
respectively. Due to the omission of standards and the inherent variability in the length of single-stranded DNA, measurements are ±10%.

Most negatives selected for publication were reversed twice onto high contrast copy film, with the exception of those electron micrographs on which the distinction between single and double-stranded DNA deteriorated unacceptably as a result of this procedure.

3. Results

(a) Genes 43, 30, rII and 32

The general characteristics of parental phage DNA extracted from E. coli infected with T4 pol−, T4 pol−lig−, T4 rII pol−lig−, and T4 32− pol−lig− have already been described (Broker & Lehman, 1971). Additional samples of these mutants have been examined and the combined electron microscopic analyses of the formation of branched recombinational intermediates are presented in Table 2.

### Table 2

Summary of electron microscopic analysis of branched DNA molecules formed by T4 amber mutants after infection of E. coli BB

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Branched (%)</th>
<th>Branched molecules multiply branched (%)</th>
<th>No. of molecules scanned</th>
</tr>
</thead>
<tbody>
<tr>
<td>pol− lig−</td>
<td>19</td>
<td>39</td>
<td>1950†</td>
</tr>
<tr>
<td>rII− pol− lig−</td>
<td>4</td>
<td>---</td>
<td>1600†</td>
</tr>
<tr>
<td>32−</td>
<td>3</td>
<td>---</td>
<td>700†</td>
</tr>
<tr>
<td>32− pol− lig−</td>
<td>0–1</td>
<td>---</td>
<td>500†</td>
</tr>
<tr>
<td>41− pol− lig−</td>
<td>25</td>
<td>36</td>
<td>600†</td>
</tr>
<tr>
<td>pol−</td>
<td>9</td>
<td>37</td>
<td>1100†</td>
</tr>
<tr>
<td>44− pol−</td>
<td>14</td>
<td>15</td>
<td>300</td>
</tr>
<tr>
<td>62− pol− lig−</td>
<td>27</td>
<td>39</td>
<td>500†</td>
</tr>
<tr>
<td>46− pol− lig−</td>
<td>2</td>
<td>---</td>
<td>500†</td>
</tr>
<tr>
<td>47− pol− lig−</td>
<td>1–2</td>
<td>---</td>
<td>700†</td>
</tr>
<tr>
<td>46− 47− pol− lig−</td>
<td>0</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>61− (HL627) pol− lig−</td>
<td>17</td>
<td>27</td>
<td>970†</td>
</tr>
<tr>
<td>61− (E219) pol− lig−</td>
<td>21</td>
<td>35</td>
<td>200</td>
</tr>
<tr>
<td>46− 61− pol− lig−</td>
<td>1</td>
<td>---</td>
<td>200</td>
</tr>
<tr>
<td>47− 61− pol− lig−</td>
<td>0</td>
<td>---</td>
<td>500†</td>
</tr>
<tr>
<td>das 46− 47− pol− lig−</td>
<td>5–7</td>
<td>---</td>
<td>500</td>
</tr>
</tbody>
</table>

The samples were prepared by the standard procedures described by Broker & Lehman (1971).

† Average of two or more experiments.
‡ E312 pol− lig− and A453 pol− lig− results combined.

The amber peptide made by the amB22 allele of gene 43 possesses the 3′ → 5′ exonuclease activity which is normally associated with the wild-type polymerase (Nossal, 1969). To test the possibility that the formation of branched DNA in our standard mutant, T4 pol−lig− (B22, E605), might require this exonuclease or might otherwise be specific for the E605 mutation in gene 30, phage DNA was purified from E. coli BB infected with T4 pol−lig− (am4317 amE13) or with T4 pol−lig− (am4317 amH39x). Both populations had >10% of their DNA molecule branched (Plate I(a) and (b)). The am4317 peptide fragment is totally lacking in exonuclease activity in vitro (Nossal, 1969; Huang & Lehman, 1972).
(b) Genes 46, 47 and das

T4 46- pol-lig-, T4 47- pol-lig-, and T4 46- 47- pol-lig- phage behaved equivalently in all tests. Defects in genes 46 or 47 reduced recombination as measured in genetic tests (Bernstein, 1968; Berger et al., 1969). This property was reflected in the failure of T4 lacking either the gene 46 or 47 product to form branched DNA (Table 2) or to produce single-strand gaps or termini detectable by electron microscopy. Nevertheless, both the time course and extent of nicking of the infecting parental DNA, as measured by alkaline sucrose gradient centrifugation, were similar to those for T4 pol-lig- DNA (see Fig. 1, Broker & Lehman, 1971).

The products of genes 46 and 47 are necessary to convert the 0.5-μm fragments of E. coli DNA produced after T4 infection to acid-soluble products (Kutter & Wiberg, 1968). DNA of this size is too small to band sharply in Cs2SO4 density gradients run for 48 hours. As a result, such fragments were a contaminant of T4 46- (47-) pol-lig- DNA on electron microscope grids and made interpretation difficult because they were occasionally situated adjacent to long T4 DNA molecules and looked like short branches.

A T4 mutant termed das (DNA arrest suppressor), in combination with conditional lethal mutants of genes 46 or 47, permits some DNA and late protein synthesis, recombination, and progeny phage production under normally restrictive conditions (Hercules & Wiberg, 1971). das maps in a different part of the chromosome from genes 46 and 47 and is not an amber suppressor. T4 das 46- 47- pol-lig- DNA accumulated both single-stranded regions and branches after infection of E. coli BB, although the phenotypic suppression was only partial (Table 2, Plates II(b) and V(b)).

(c) Gene 61 (58)

The mutations defining gene 61 and gene 58 have been found to be allelic variants of a single gene (Yegian et al., 1971). Berger et al. (1969) observed that a gene 61 (58) mutation led to a greater stimulation of genetic recombination and of heterozygote production than any other amber mutant included in their study. A possible basis for the increase was evident in the extensive single-stranded regions of T4 61(58) pol-lig- DNA visible by electron microscopy (Plates II(c), III(g) and VI(h)). Nevertheless, T4 61- pol-lig- DNA was no more branched than T4 pol-lig- DNA (Table 2).

To determine whether the production of single-stranded DNA and branched molecules by gene 61 mutants was epistatic to their under-production by gene 46, 47 mutants, T4 46- 61- pol-lig- and T4 47- 61- pol-lig- quadruple mutants were constructed and tested. They behaved like the T4 46- pol-lig- and T4 47- pol-lig- controls in that neither single-stranded regions nor branched molecules were observed by electron microscopy of the infecting DNA (Table 2). Fragments of E. coli DNA, 0.5 μm long, contaminated the preparations of T4 46- (47-) 61- pol-lig- DNA as they had in T4 46- (47-) pol-lig- samples.

(d) Genes 41, 62 and 44

Defects in genes 41 and 62 result in a very low rate of T4 DNA synthesis (Warner & Hobbs, 1967). Populations of T4 41- pol-lig- and T4 62- pol-lig- DNA reproducibly accumulated more branched molecules than any other samples examined, including the T4 pol-lig- control (Table 2). By visual inspection, they also appeared to contain longer single-stranded regions than produced by any other mutant, with the exception
of T4 61- pol-lig-. Branched molecules with single-stranded joints, as described in the next section, were particularly evident in preparations of T4 41- pol-lig- and T4 62 pol-lig DNA (see Plate legends for examples).

T4 44- pol- phage generated more branched molecules than the corresponding T4 pol- control (Table 2), but were not studied in detail.

(e) Fine structure of branch points

Single-stranded regions, visualized by the aqueous diffusion method as collapsed "bushes", were occasionally observed at branch points and at termini. When the samples were mounted on electron microscope grids by a formamide technique, the single-stranded regions became extended and measurable and could be distinguished from duplex DNA by their thinner and more irregular coating of cytochrome c (e.g. Plate II(a)).

Table 3

<table>
<thead>
<tr>
<th>Fine structures of branch points</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
</tr>
<tr>
<td>B'</td>
</tr>
</tbody>
</table>

54% 23% 17% 6%

Branch regions exhibit strand displacement
Branch regions exhibit single-stranded segments

Branches appear completely duplex

The approximate relative frequencies of four basic types of branch point observed among more than 200 recombinational joints are presented as percentages. Classification is qualified by the difficulty in detecting single-stranded segments of less than about 180 nucleotides. The Plate legends indicate the types of branch points illustrated in each micrograph.

For some 5% to 20% of the chromosomes in a given T4 DNA sample, if genes 46 and 47 were wild type, one terminus was visibly single-stranded. (For examples, see the cross index at the end of the Plate legends.) The branch points observed were placed into four classifications according to whether single-strandedness occurred at the recombination joint and to whether strand displacement took place, as summarized in Table 3. At about half the branch points, the proximal end of one of the branches was single-stranded. One hundred and six such single-stranded segments were photographed and measured. A histogram of their lengths (Fig. 1) demonstrates that most were shorter than 350 nm (1250 nucleotides). Structures of the type B (or B') (see Table 3 and, for example, Plate IV(c)) with single-stranded segments proximal to
PHAGE T4 DNA RECOMBINATION

FIG. 1. Length distribution of single-stranded segments observed at 106 recombinational branch points of types B, C and D (see Table 3 and the Plates). 1 nm of contour length corresponds to about 3.6 nucleotides. The stippled subset of the histogram represents the combined lengths of the proximal and displaced single-stranded segments at branch points where branch migration was evident.

The branch point are physically able to assume isomeric configurations of the types C and D (or C' and D') by strand displacement (Plates I(b), II(c), III(b) to (f) and (h), IV(b), V(c), and VI(b) to VIII(a)). In this process, the position of the branch point migrates back and forth as two homologous single strands compete with each other for more extensive pairing with a unique complementary strand, as observed in vitro by Lee et al. (1970) and as suggested to be responsible for lengthening the region of heteroduplex pairing in recombinational intermediates (Broker & Lehman, 1971). The length of the single strand displaced from its original complement in structure C and D (or C' and D') is a minimum estimate of the length of the heteroduplex segment established between the recombining molecules. Most displaced strands were shorter than 750 nucleotides (Fig. 2; Table 4, entries 2 and 3).

Branch points at which the single-strand segment(s) was longer than about 500 nucleotides exhibited branch migration more frequently than those with shorter
single-stranded segments. The energy equivalence of all intermediate positions of migrating branches is reflected in the equal number of branch points at which the displaced strand was either shorter or longer than the proximal single strand and in the similar median and average lengths of displaced and proximal single-stranded segments at branch points of types C and C' (Table 4, entries 3 and 4). These observations are consistent with the random distribution of migrated forms into available entropy states once an initial energy barrier to migration is overcome (Lee et al., 1970).

Because short single-stranded regions should in general be more frequent than longer ones, the relatively few single strands observed to be shorter than 50 nm (180 nucleotides) (Figs 1 and 2) can be attributed to an inability to recognize single-stranded branches or gaps of less than that length, which approaches the diameter of the DNA-cytochrome c complex. The data presented in Figures 1 and 2 and in Tables 3 and 4 necessarily reflect this qualification.

Several types of branched molecules are subject to conflicting interpretations. Molecules that appear to illustrate single-strand exchange at internal gaps (Plates II(c) and III(g)) can also be interpreted to result from the chance intersection of a DNA complex and a single-strand gap in a second molecule. Reciprocally recombinant double-stranded cross-over branches generated by double-strand branch migration, as shown diagrammatically by Broker & Lehman (1971), are difficult to distinguish from adventitious overlaps of two duplexes, except that such cross-overs occur with much higher frequency than expected in molecules that additionally contain well-defined recombinational branches (Plate VI(a) and (b)). As discussed in a preceding paper (Broker & Lehman, 1971), technical precautions during the preparation of grids reduced the possibility for adventitious overlaps, as indicated in part by their near absence from DNA samples that lacked other signs of recombination (e.g. T4 pol-lig- samples prepared 15 minutes or less after infection; T4 46- (47-) pol-lig- and T4 32-pol-lig- samples). In any case, most cross-over branches were not included in the data reported in Table 2. Unequivocal experimental evidence for double-strand
All T4 DNA samples were extracted from *E. coli* BB 30 min after infection at 30°C, were purified by Cs₂SO₄ equilibrium density gradient centrifugation, and were prepared for electron microscopy by the formamide spreading procedure (except Plate VI(a), which was prepared for electron microscopy by aqueous diffusion). Arrowheads point to single-stranded regions at branch points. Arrows point to single-stranded termi, gaps, or completely single-stranded branches. The Plate legends are coded to indicate the features illustrated in each micrograph: A, B, C, and D refer to the four types of branch point diagrammed in Table 3. Single-stranded termini (T), gaps (G), single-stranded branches (S), and completely single-stranded interbranch segments (I) are also indicated. A cross-reference of the latter 4 features is presented below.

Cross-index of features,

Single-stranded termini (T): Plates I(a), II(b), IV(a) and (b), VI(b) and VIII(b).

Single-stranded branches (S): Plates I(a), VI(b), VII(b) and VIII(a).

Single-stranded interbranch segments (I): Plates I(a), V(b) and (c), and VIII(b).

Single-stranded gaps (G): Plates II(a), (b) and (c), III(g) and (h), IV(c), V(a), VI(b) and VII(a).

**PLATE I.** (a) T4 *pol− lig−* (4317 − E13); magnification 28,400 ×. B, T, S, I. (b) T4 *pol− lig−* (4317 − E13); magnification 32,800 ×. A, C.

**PLATE II.** (a) T4 *41− pol− lig−*; magnification 55,100 ×. B, G. (b) T4 *46− 47− pol− lig−*; magnification 47,200 ×. B, T, G. (c) T4 *61− pol− lig−* (HL627 − B22 − E605); magnification 29,700 ×. C, G.

**PLATE III.** (a) T4 *41− pol− lig−*; magnification 47,200 ×. B. (b) T4 *41− pol− lig−*; magnification 57,300 ×. C. (c) T4 *41− pol− lig−*; magnification 67,700 ×. C. (d) T4 *pol−* (B22); magnification 49,500 ×. C. (e) T4 *41− pol− lig−*; magnification 85,800 ×. C. (f) T4 *rII pol− lig−*; magnification 84,800 ×. C. (g) T4 *61− pol− lig−* (HL627 − B22 − E605); magnification 47,800 ×. C, G. (h) T4 *pol− lig−*; magnification 48,500 ×. (Compare with Fig. 3.) D, G.

**PLATE IV.** (a) T4 *62− pol− lig−*; magnification 104,000 ×. B, T, G. (b) T4 *pol− lig−*; magnification 67,500 ×. C, T. (c) T4 *62− pol− lig−*; magnification 51,000 ×. B, G.

**PLATE V.** (a) T4 *41− pol− lig−*; magnification 34,600 ×. B, G. (b) T4 *46− 47− pol− lig−*; magnification 57,800 ×. B, I. (c) T4 *41− pol− lig−*; magnification 40,000 ×. B, C, I.

**PLATE VI.** (a) T4 *pol− lig−*; magnification 29,800 × (aqueous diffusion). (b) T4 *61− pol− lig−* (HL627 − B22 − E605); magnification 15,000 ×. D, T, S, G.

**PLATE VII.** (a) T4 *41− pol− lig−*; magnification 79,800 ×. B, G. (b) T4 *41− pol− lig−*; magnification 59,600 ×. C, S.

**PLATE VIII.** (a) T4 *62− pol− lig−*; magnification 75,600 ×. B, C, S. (b) T4 *46− 47− pol− lig−* (13 − B22 − E13); magnification 29,600 ×. B, B, T, I.
Plate V
branch migration in T4 recombinational intermediates is at present lacking. In model systems prepared in vitro, double-strand branch migration has been demonstrated (Kim et al., 1972).

(f) Multiply branched DNA

The correlated origin of branches in multiply branched DNA is far more pronounced than indicated in the original calculation (Broker & Lehman, 1971). Based on the Poisson distribution, multiply branched molecules are four to eight times more frequent than expected from the sizes of the null and singly branched classes, respectively. From the average number of branches per unit length of T4 chromosome (about 1-2), molecules with a simple H-branched structure and an interbranch spacing of less than 3000 bases (about 5% of all molecules) occur over 300 times more frequently than anticipated from a random origin of branches. This calculation argues that clustered exchanges follow a primary pairing event.

The short hybrid branch anticipated to occur by strand displacement at migrating branch points (Fig. 5 in Broker & Lehman, 1971; Plate VI(b)) is absent from most multiply branched molecules. To explain its absence, I suggest that an exonuclease degrades the hybrid branch as migration progresses. The molecule would retain an H-like structure. But such a nucleolytic action coupled to strand displacement is simply a variation on branch migration facilitated by strand assimilation (Broker & Lehman, 1971; Cassuto & Radding, 1971). The recombinant structures observed in this study can not indicate whether the postulated exonucleolytic degradation precedes migration or vice versa. (In either case, however, extension of the heteroduplex would be facilitated by the exonuclease and would be irreversible.) According to this hypothesis, the entire segment of DNA between the two branch points must be double-stranded, an expectation compatible with some (Plates I(b) and IV(a) and (b)) but not all (Plates IV(c) and V(a), (b) and (c)) of the H-branched molecules studied.

The two homologous branches of a single branched molecule are capable of secondary interaction and pairing. Plate VII(a) and (b) depicts closed loops with both interbranch point distances equal. These particular loops are the products of recombination between a fragment of DNA, which is single-stranded at each end, and a larger DNA molecule. Loops occur in roughly 0-5% of the branched molecules, a frequency which might be determined in part by the probability that the molecule will have a second pair of complementary single-stranded regions. Plate VIII(a) and (b) illustrates more complex examples of reiterated cross-over.

4. Discussion

Defects in eleven phage T4 genes known or believed to be required for normal DNA metabolism were linked in various combinations to form a collection of double, triple, quadruple and quintuple mutants. These were compared with respect to the extent of degradation suffered by the parental DNA (as determined quantitatively on sucrose velocity gradients (Broker & Lehman, 1971; unpublished observations) and qualitatively by electron microscopy) and with respect to the accumulation and configuration of branched recombinational intermediates formed after infection of E. coli under non-permissive conditions. The data are interpreted in Figure 3 in the form of a pathway for recombination in the absence of replication, but the Figure might as well
FIG. 3. Pathways for T4 DNA recombination in the absence of replication. Recombination between a terminal and an internal site was chosen arbitrarily for representation. Other recombining structures can be represented by similar pathways. Documentation for each step is presented in Results and in Discussion. T4-specified proteins are referred to by gene name. P-41, P-62 and P-44 may help to constitute a repair pathway alternative to that of P-43 (polymerase). The placement of P-61 is not definite. Mutations in gene 1 (deoxynucleotide kinase) and gene 42 (deoxy- cytidylate hydroxymethylase) and the inhibitor of thymidylate synthetase, fluorodeoxyuridine, impair pyrimidine metabolism and stimulate recombination (Tomizawa, 1967; Berger et al., 1969). P-32 may have additional roles. Branch migration can occur by strand displacement (as diagrammed) or by strand assimilation. The placement of single-strand interruptions during linearization of branched molecules determine whether the DNA flanking the heteroduplex is in the recombinant (as diagrammed) or parental configuration.

The participation of E. coli polymerase and ligase in the covalent repair of linear joint molecules is implicit in the results of Anraku & Lehman (1969).

be considered an organized summary of the effects of individual genetic lesions on parental DNA. This representation of recombination requires: (i) an endonuclease(s) to introduce single-strand nicks; (ii) an exonuclease(s) to convert nicks to gaps and termini to single-stranded ends; (iii) the gene 32 protein to protect single-stranded regions, then to promote their complementary pairing; (iv) additional nucleases to resolve branched molecules to linear forms; (v) polymerase and ligase to repair gaps and nicks separating parental DNA segments, with the generation finally of linear covalent recombinant molecules. (The observed effect of most of the mutants is the result of their influence on the production and maintenance of single-stranded regions at ends and at internal sites of the infecting DNA.)

The increased accumulation of single-strand interruptions that leads to an elevated production of branched molecules when DNA ligase (gene 30) is defective and the reversal of these properties when T4 rII mutations phenotypically suppress phage ligase mutations have been described (see Table 2) (Broker & Lehman, 1971).

A protein dependent upon the expression of genes 46 and 47 is the major exonuclease directed against parental T4 DNA after phage infection (Prashad & Hosoda, 1972).
The single-strand interruptions in parental T4 pol-lig DNA are gaps of an average length of 300 to 400 nucleotides which require both DNA polymerase and ligase to be repaired in vitro (Anraku et al., 1969). In contrast, two-thirds of the interruptions in T4 46- (47-) pol-lig DNA can be repaired in vitro with ligase alone, indicating that, in the absence of the gene 46 or gene 47 product, they remain as nicks with 3'-OH and 5'-PO₄ termini (Prashad & Hosoda, 1972). T4 46- (47-) pol-lig DNA did not accumulate either single-stranded regions or branched intermediates of recombination that I could detect by electron microscopy, as shown in Table 2. Furthermore, T4 46- pol-lig phage fail to produce hybrid density recombinational intermediates when phage of two different densities co-infect the same bacterial culture (Prashad & Hosoda, 1972). The behavior of T4 46- (47-) pol-lig phage suggest that single-strand gaps, as well as nicks, are prerequisites for T4 DNA recombination.

The mutation das, in as yet an unknown manner, provides a partial suppression of the gene 46-47 defect. In T4 das 46- 47- pol-lig DNA, both single-stranded segments and recombinational branches were observed by electron microscopy, but much less frequently than when genes 46 and 47 were wild type (Table 2). These observations are in agreement with the characterization of the das phenotype by Hercules & Wiberg (1971), who found some restoration of recombinational ability to T4 46- phage with the introduction of the das marker into the phage genotype.

Mutations in several T4 genes necessary for DNA synthesis (Warner & Hobbs, 1967) stimulate T4 recombination. (1) Defects in genes 43, 41 and 62 favor the accumulation of single-stranded gaps and termini and of branched recombinational intermediates (Table 2). (2) T4 41- HMCa + pol - 44- 45- phage produce both joint and recombinant density hybrids (Anraku & Lehman, 1969). (3) Limiting the activity of gene 43, 41, 62 or 44 products increases genetic exchange (Berger et al., 1969). The gene 44 and gene 62 proteins co-purify as a complex necessary for T4 DNA synthesis (Barry & Alberts, 1972). Chemical inhibitors that impair deoxynucleotide metabolism also stimulate recombination (Tomizawa & Anraku, 1964a). Because the single-stranded regions of T4 41- pol-lig- and T4 62- pol-lig- DNA are, by electron microscopic observation, longer than those of T4 pol-lig- DNA, lesions in genes 41 or 62 may block a repair pathway that supplements or is independent of the pathway in which the gene 43 polymerase functions. That limiting DNA (repair) synthesis promotes recombinational pairing further supports the postulated requirement for single-stranded segments before recombinational pairings.

Amber mutations in gene 61 (58) result in a prolonged lag in the onset of DNA replication (Yegian et al., 1971) which may be a consequence of abnormal deoxyribonucleotidic pools (Gosein & Hall, 1972) and the intracellular ionic environment (Guttman & Begley, 1968). Recombination, including heterozygote formation, is unusually active when the expression of the gene 61 function is limited (Berger et al., 1969). T4 61- pol-lig- DNA observed in the electron microscope had longer single-stranded regions than found in T4 pol-lig- DNA samples, but a similar level of branching (Table 2). The properties of gene 46 and 47 mutants are epistatic to those of gene 61 mutants, as determined by the near absence of detectable single-stranded regions and branches in 46- (47-) 61- pol-lig- DNA (Table 2).

The gene 32 protein may have at least two functions during DNA recombination. First, upon binding to single-stranded termini, it blocks the 3'→5' exonuclease activity of T4 DNA polymerase (Huang & Lehman, 1972); nevertheless, I observed single-stranded termini in parental T4 32- DNA after infection. The total extent of
single-strandedness in T4 32− DNA extracted from infected cells is much less than when 32 protein is active (Kozinski & Felgenhauer, 1967). Second, the 32-protein facilitates trial and error pairing of single-stranded DNA in vitro both by extending the strands and by lowering the effective \( T_m \) (Alberts & Frey, 1970). T4 32− and T4 32−pol−lig− DNAs, which have strand interruptions and at least some single-strandedness, contain about tenfold fewer branched recombinational intermediates than T4 pol−lig− DNA (Table 2).

Strand displacement occurs readily in vitro within molecules prepared in such a way that two homologous strands can compete with each other for a single complementary strand (Lee et al., 1970). The strand displacement observed at one-fourth of the branch points in recombinant DNA molecules (Table 3) may have occurred in vitro during extraction and purification of the DNA. Consequently, the biological relevance of strand displacement to recombination remains unproven.

According to dynamic models (coiled telephone cords), strand exchange could proceed readily if the paired right-handed double helices were rotating around their long axes with a right-handed screw sense (Fig. 4). With such axial rotation, the interacting molecules could maintain a fixed spatial orientation, thereby avoiding the translational rotation otherwise required for branch migration.

Although T4 pol−lig− hybrid molecules cannot be repaired to a covalently recombinant form in vivo, there is little reason to have anticipated that branched molecules would have accumulated in infected cells because the repair steps affected by those lesions presumably follow an (unidentified) endonucleolytic cleavage that converts...
branched molecules to linear forms (Fig. 3, step 5). Branch migration past a predisposed nick would result in the loss of the branch containing the nick. Branched DNA probably represented only a portion of the intermediate and product structures of T4 recombination present in the samples studied.

The pathway outlined in Figure 3 describes T4 recombination in the absence of replication, but it probably is indicative of the structural requirements for T4 recombination under any conditions. Although most of the branched molecules observed after 30 minutes of infection were of about one-fourth to one-half unit length, their size does not imply that T4 DNA normally recombines by "fragmentation and reassembly" but rather that the single-stranded regions which accumulate in the absence of repair can lead to either breakage or recombination. The observation of DNA recombination when replication is prevented by mutational or chemical interference does not necessarily mean that recombination proceeds independently of replication, repair, or transcription in wild-type infection cycles.

In fact, the interdependence of T4 DNA metabolic pathways is emphasized by the requirement of recombination for normal replication. Organized replication complexes (Frankel, 1968; Huberman, 1968) lose their structural integrity when concatemerization of progeny DNA molecules is blocked by inactivation of the gene 46-47 product (Shah & Berger, 1971; Shalitan & Naot, 1971; Hosoda et al., 1971). Arrest of further DNA synthesis accompanies this disorganization. The putative exonuclease(s) specified by genes 46 and 47 apparently is not necessary for the initial association of monomers into concatemers via hydrogen bonds, but only to allow their covalent joining.

The remainder of this discussion is devoted to considerations, as yet untested, of how T4 replication and recombination might be co-ordinated, as illustrated in Figure 5†. Concatemer production via recombination at terminal redundancies was first proposed by Streisinger et al. (1964). DNA synthesis via asymmetric replication is known to displace single-stranded segments (Delius et al., 1971) (Fig. 5(b) and (c)). At each end of any linear duplex, DNA replication would appear to be unable to proceed completely to the end of the 3'-terminated template, a result of the discontinuous mode of synthesis of the unfavorably oriented daughter strand (Fig. 5(d) and (e)). Whatever signal is necessary to initiate a discontinuous segment when some 1000 to 2000 bases of the template have been displaced (Sugino et al., 1972) it should be lacking for the terminal segment. Therefore, each newly synthesized strand will be shortened on its 5' end and, without proper filling of these single-stranded termini, each successive round of replication would result in further losses. One function of terminal redundancy may be to duplicate the genetic information which would be vulnerable to deletion by such shortening. That repeated rounds of replication do not result in progressive chromosome losses attests to the ability of each DNA molecule to restore its complete redundancy on both strands. If this hypothesis is correct, it may do so by recombination. Each progeny chromosome is expected to have only one single-stranded end, the 3'-hydroxyl terminus of the parental strand at which the would-be ultimate discontinuous segment was unable to initiate. The single-stranded ends of sibling chromosomes should in part be complementary, as they are derived from opposite strands, one from each terminal redundancy. Pairing of these ends and covalent recombination thereafter would generate a linear dimer (Fig. 5(f)). Reiteration of this process at the

† After this model appeared in its initial form (Broker, 1972), an analogous scheme for phage T7 concatemer formation was published (Watson, 1972).
(a) A terminally redundant chromosome. Large letters refer to loci on parental strands; small letters to loci on progeny strands.

(b) A replication loop, expanding bidirectionally, with displacement of parental strands by the 3'-primer termini of the progeny strands. ((●) gene 32 protein, after Delius et al., 1971).

(c) Continued replication, with displaced parental strands serving as templates for 10 S discontinuous segments. (—–—–).

(d) Replication to an end, without synthesis of the final 10 S segment at the 3'-parental terminus.

(e) Completion of replication, with the production of two partially complementary parental single-stranded termini.

(f) Pairing of the terminal complements, with spurs of DNA unaccommodated into the pairing region.

(g) Covalent recombination to form a concatemer, subsequent to nucleolytic removal of the spurs.

Fig. 5. Recombination of single-stranded termini produced during DNA replication.

end of additional rounds of replication could produce higher oligomers. If the terminal redundancy (3200 base pairs in T4 (Kim, 1972)) were longer than the sum of the two single-stranded ends left by incomplete replication (1000 to 2000 bases in replicating T4 DNA (Hosoda & Broker, unpublished observations)), then the termini would have no complementary sequences available for pairing. If a single-stranded end were longer than the redundancy, that end would entirely accommodate the sibling complement in a linear hybrid structure. But if the single-stranded ends were longer than
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half and shorter than the full redundancy, then short branches of 1000 to 2000 bases would appear at the recombinational overlaps (Fig. 5(f)). Covalent joining of the dimer could happen only after the removal of the branches (Fig. 5(g)). This, I postulate, is a plausible role for the gene 46–47 exonuclease during phage T4 DNA replication. If so, should it fail to act, a second round of replication would dissociate the dimer, disorganize the replication complex, and lead to arrest of further replication.

Single-stranded ends on newly replicated T4 DNA might also be available for general recombination by invasion of replicating forks and other nicked or gapped regions. The genetic sites that were represented near the ends of the original parental DNA molecules are those that would tend to be retained at ends and amplified in a single-stranded state if replication produces linear progeny from linear parental molecules. These are the very properties that would confer the observed recombinogenic favor on the genetic regions at the original ends (Doermann & Parma, 1967; Mosig et al., 1972).

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