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Bacteriophage T7 DNA Replication: A Linear Replicating Intermediate*

(gradient centrifugation/electron microscopy/*E. coli*/DNA partial denaturation)

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ABSTRACT **The T7 chromosome in the first round of replication is a Y-shaped DNA rod. Thus, it differs from previously observed bacterial and viral replicating chromosomes that are circular.**

In microorganisms, viruses, and organelles, all actively replicating chromosomes observed are circular. Such circles have been of two types: Cairns circles (1) (Fig. 1*a*) and rolling circles (2) (Fig. 1*b*). The replication of phage T7 DNA appears to be different: it does not involve a circular replicating intermediate. Instead, electron microscopy shows Y-shaped rods, analogous to those proposed 19 years ago by Watson and Crick (4) (Fig. 1*c*).

Experimental design

When ($^{14}N^{1}H$) phage particles infect cells growing in the presence of heavy isotopes ($^{15}N^{2}H$), the density of the viral chromosomes increases from light (LL) toward hybrid (HL) as they begin to replicate. Molecules in their first round of replication contain predominantly light nucleotides (HLL), molecules at the end of the first round of replication are fully hybrid (HL), and molecules engaged in subsequent rounds of replication have densities ranging from hybrid (HL) to heavy (HH). Bacterial DNA remains fully heavy at all times. This protocol offers a rather sensitive way to fractionate candidates for partially replicated viral chromosomes and, most importantly for any electron microscopic study, allows one to obtain these chromosomes free of host-cell DNA.

This extension of the Meselson and Stahl experiment (5) was developed by Ogawa, Tomizawa, and Fuke (6) to obtain partially replicated lambda chromosomes. The technique has been applied by Schnös and Inman to study both lambda and P2 DNA replication(7, 8), and we have used it to study the replication of T7 DNA.

Isolation of actively replicating T7 chromosomes

Escherichia coli growing in a defined medium containing $^{15}NH_4Cl$ and $^{2}H_2O$ were infected at a multiplicity of ten with wild-type T7. The phage life cycle progressed normally, and ended 25 min later with a burst of 150 T7 particles per cell. At 10, 13, and 16 min after infection, aliquots of the culture were harvested as a source of actively replicating T7 DNA. The infected cells were opened with lysozyme and detergent, and the lysates were digested with Pronase. The intracellular DNA forms were purified by CsCl density gradient centrifugation. The material banding between light and hybrid (HLL) was recovered and examined in the electron microscope.

The types of T7 molecules present in the HLL fractions from a typical experiment are shown in Fig. 2.

(*a*) 10% of the molecules were tangled and thus untraceable; these were of about T7 length, and were not long pieces of host-cell DNA.

(*b*) 60% of the molecules were DNA rods of the same contour length as the mature T7 chromosome. Although most of these unit-length rods appeared in the HLL position of the CsCl gradient because of spillover from the LL and HL positions, some of them may have been the products of genetic recombination.

(*c*) 20% of the molecules were Y-shaped rods (Fig. 2*a*); in these, two arms of the Y were of equal length, and the combined length of one of these arms plus the remaining stem was equal to the length of the mature T7 chromosome.

(*d*) 8% of the molecules were linear DNA rods, the length of T7, that contained a laterally duplicated segment (Fig. 2*b*). We call these structures "eye forms". The additional DNA segment was anchored at both of its ends in such a way as to indicate that it was aligned opposite a homologous T7 DNA sequence.

(*e*) 2% of the molecules from the HLL region were more complex, for instance, molecules that contained several forks.

(*f*) No DNA circles were found (<0.1%).

Replication or recombination?

The Y-shaped molecules could be intermediates in either replication or recombination. If they are intermediates in recombination, then one would expect that these molecules would sometimes have the left region in duplicate and sometimes the right. However, if the Y-shaped structures were involved in replication, one would expect that all of the molecules would possess the same end in duplicate.

To determine whether a unique arm was present in duplicate in the Y-shaped molecules, we applied the technique of denaturation mapping of DNA. In this procedure, developed by Inman, double-helical DNA is exposed to an increasingly severe denaturation environment until AT-rich regions of the DNA melt out (11). These AT-rich regions, which serve as landmarks on the DNA, appear in the electron microscope as single-stranded bubbles that interrupt the linear DNA duplex.

The T7 Denaturation Map. When unit-length T7 DNA was prepared for the electron microscope under conditions

* This is paper No. I in a series, *T7 DNA Replication.*

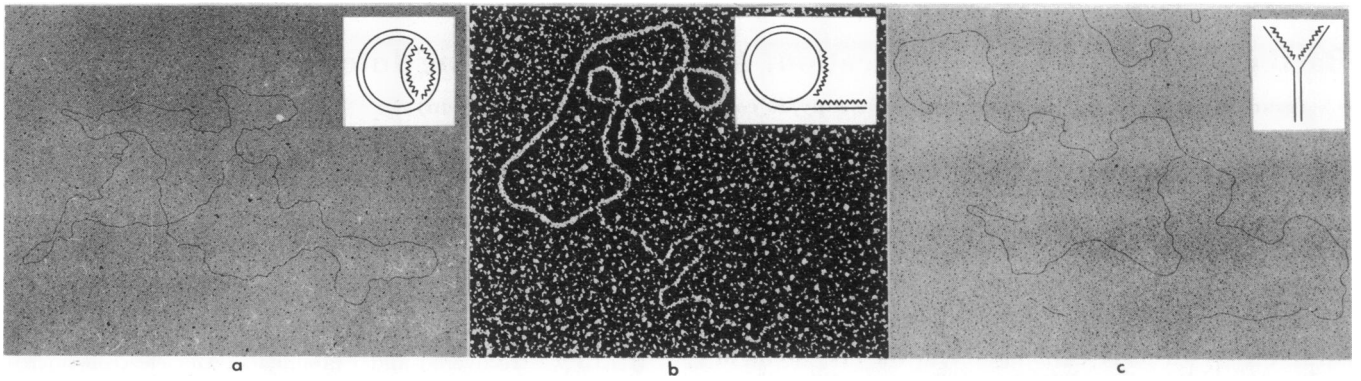


FIG. 1. Three actively replicating chromosomes. (*a*) A partially replicated lambda chromosome illustrates the Cairns configuration for replicating DNA (1). The accompanying line diagram shows a plausible strand substructure for the molecule. Both strands of the parental chromosome may be circular, although at least a temporary nick must be put into one strand to allow strand separation. Both daughter polynucleotide chains (*zig-zag lines*) are shorter than unit length. There are two forks in the partially replicated molecule; one or both forks can be growing points where the parental strands are separating and new DNA is being lain down (7).

(*b*) The rolling-circle configuration for replicating DNA is illustrated by a ϕX-174 duplex ring, which is generating material for a single-stranded circle (2, 3). The line diagram shows the strand substructure of this actively replicating DNA molecule. Synthesis occurs by direct elongation of the open positive strand, using the circular negative strand as an endless template. In systems where double-stranded DNA is the product of replication, the tail becomes duplex. When the tail becomes longer than unit length, and contains homologous base sequences one genome-length apart, a recombination event presumably detaches a progeny chromosome.

(*c*) A partially replicated T7 chromosome illustrates the Y-shaped replicating rod. The accompanying line diagram shows the probable strand substructure, which was proposed by Watson and Crick in 1953 (4).

of partial denaturation, characteristic regions of the double helix preferentially melted out (Fig. 3*a* and *b*). Fig. 3*c* is a histogram of 25 partially denatured T7 molecules, and represents a partial-denaturation map. The map shows that the most prominent denaturation region occurs in the area 15–30% from one end.

Once the T7 denaturation map was constructed, it was necessary to orient it with respect to the T7 genetic map. To do this, heteroduplex molecules (12, 13) were made (Fig. 3) that contained one wild-type strand annealed with a complementary strand carrying a deletion very near the genetic left-end of the T7 chromosome. In the heteroduplex DNA molecules, the unpaired wild-type DNA strand formed a single-stranded loop in the area of the deletion. When the heteroduplexes were prepared for the electron microscope under partially-denaturing conditions, both the prominent denaturation region (15–30% from one end) and the deletion loop appeared in the same end of each molecule, that is, the genetic left-end of the T7 chromosome (Fig. 3*a*).

Orientation of the Y-shaped Molecules. Preparations containing about 20% Y-shaped molecules were partially denatured and examined by electron microscopy (Fig. 4*a*). The duplicate arms of the Ys contained the prominent denaturation site characteristic of the left end of the chromosome in 46 of 52 molecules (Fig. 4*b* and *c*). Thus, we interpret the Y-shaped molecules to be intermediates in T7 DNA replication rather than intermediates in recombination.

A Y-shaped replicating rod might be generated in two ways. Replication could begin at the left end of the T7 chromosome and proceed inward (4). Alternatively, DNA replication might initiate at an interior point near the left end of the T7 DNA molecule. Bidirectional synthesis (7, 16)† would then generate an eye form (Fig. 2*b*) which, when the leftward growing point reached the left end of the DNA rod, would be converted into a Y-shaped replicating intermediate (Fig. 2*a*). The second initiation pattern predicts that eyes should be located only in the left halves of the T7 chromosomes. This is, in fact, the case when the multiplicity of infection is 1 instead of 10‡. Thus, T7 appears to replicate by the second initiation pattern. At a high multiplicity of infection, however, eyes are found in the center and right arms of the T7 DNA molecules. These multiplicity-dependent, randomly-located eyes are candidates for intermediates in genetic recombination.

DISCUSSION

The chromosome of the lytic coliphage T7 is a double-stranded DNA molecule of molecular weight 25.0×10^6 (17). The studies of Studier, Maizel, and Hausmann (18–21) have divided the genome into 25 complementation groups, 23 of which have been associated with specific proteins. The combined molecular weights of these proteins account for about 87% of the coding capacity of the phage DNA (22).

Six T7 genes appear to be involved in DNA replication (22). They are translated as a block early in the virus life cycle. Two genes specify an endonuclease (23) and an exonuclease (48) that digest the bacterial chromosome, and thus supply 85–90% of the nucleotides for progeny T7 DNA. A third DNA gene directs the synthesis of a Kornberg-type DNA polymerase (49). Ligase is the product of a fourth gene (50). The functions of the two remaining genes are still unknown,

† *Bidirectional DNA synthesis:* in a letter to Cairns (1963), Meselson suggested the possibility that both forks of the Cairns circle might be growing points.

‡ Dressler, D., Wolfson, J. & Magazin, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, in press.

but they too are essential for DNA synthesis in the T7-infected cell.

The experiments in this paper attempt to determine the structure of the partially-replicated T7 chromosome. Our electron microscope analysis of replicating T7 DNA indicates that during the first round of replication, the viral chromosome appears in the configuration of a Y-shaped rod. In each rod, there are two arms of equal length that represent the replicated portion of the chromosome. The third arm of the Y represents parental DNA that has not yet been replicated. The two equal arms in the Y-shaped molecules were shown by denaturation mapping of DNA to correspond to the left end of the genetic map of T7. Thus, replication appears to proceed from left to right.

The physical structure of intracellular T7 DNA has also been studied by Kelly, Thomas, Ihler, Hausmann, Gomez, and Carlson (24–27). They have demonstrated the incorporation of pulse-label into T7 DNA forms that sediment faster

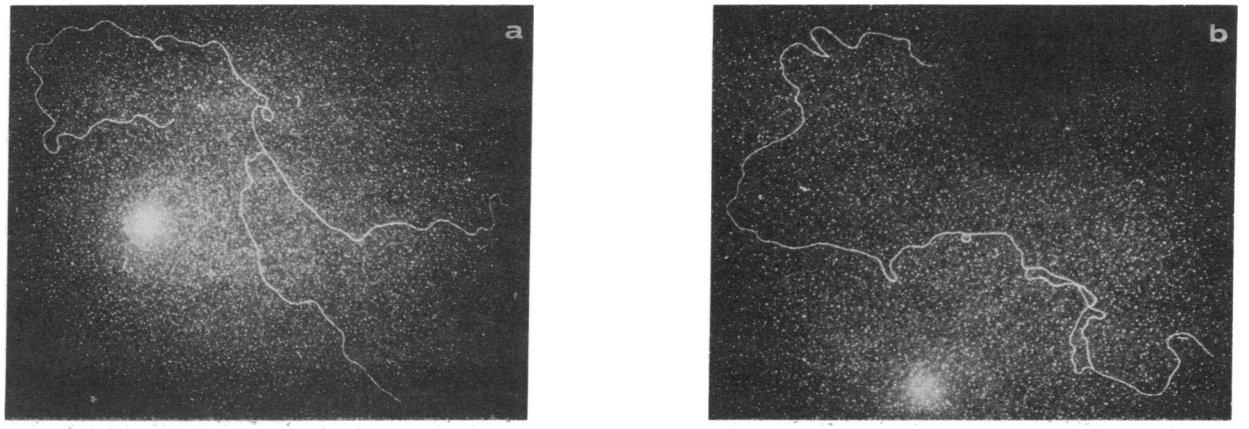


FIG. 2. Preparation of T7 chromosomes in their first round of replication. *E. coli* strain 011′ (*thy*⁻Su⁺) was adapted for growth in heavy medium by serial passage through 20, 40, 60, 75, 90, 95, and 100% substituted medium. The medium contained, per liter of ^2H_2O: 7.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g $^{15}NH_4Cl$ (Merck, Sharpe and Dohme), 0.5 g NaCl, and 0.03 ml of 0.1 M $FeCl_3$. After autoclaving, the following were added: 1.0 ml of 1 M $MgSO_4$, 0.1 ml of 1 M $CaCl_2$, 5 ml of 1 mg/ml thymine in ^2H_2O, 12.5 ml of 20% glucose in ^2H_2O, and 1.12 ml of heavy algal whole hydrolyzate (Merck, Sharpe and Dohme). The adapted cells grew at 37°C with a generation time of about 80 min, as compared to 35 min for growth in light medium.

100 ml of cells were grown in heavy medium supplemented with [^{14}C]thymine (New England Nuclear) so that the bacterial DNA could later be used as a marker for the density of fully heavy (HH) DNA. When the cell titer reached 3×10^8/ml, the cells were infected with wild-type T7 at a multiplicity of 10. A normal one-step growth curve followed, leading to a burst of about 150 phage/cell in 25–30 min. At 10, 13, and 16 min after infection, aliquots of the culture (containing 1×10^{10} cells) were pipetted into equal volumes of acetone (at −70°C) to stop further DNA synthesis (Cairns, J., personal communication). The cells were harvested by centrifugation and resuspended at 2×10^9/ml in a lysis buffer [0.1 M NaCl–0.02 M EDTA–0.01 M KCN–0.01 M iodoacetate–0.1 M Tris (pH 7.4)]. The cells were broken enzymatically (400 μg/ml of lysozyme, 0°C, 45 min) and with detergent (0.1% sodium *N*-lauroyl sarcosinate, from Sigma; 65°C, 20 min). The cell lysates were then deproteinized with self-digested Pronase (1 mg/ml, 37°C, 4 hr).

The lysates were diluted 1:1 with H_2O and brought to a density of 1.70 by the addition of 1.25 g of CsCl per ml of solution. The 10-, 13-, and 16-min lysates were centrifuged to equilibrium in a Beckman 65 rotor (32,000 rpm, 15°C, 72 hr) in polyallomer tubes that had been treated for at least 30 min with a 10% solution of bovine serum albumin.

Each gradient was collected under negative pressure to give about 40 fractions of 300-μl each. These fractions were assayed for ^{14}C (representing primarily fully-heavy (HH) *E. coli* DNA) and for ^{32}P (representing fully-light (LL) lambda DNA that had been added as a marker at the time of centrifugation). The HH and LL peaks are quite sharp, even in the first CsCl gradient of the cell lysate, and are separated by about 30% of the gradient. The regions of the gradient with hybrid densities were pooled and recentrifuged to further segregate out HH and LL species. The fractions of the second CsCl gradient between light and hybrid were pooled and dialyzed into 0.1 M Tris(pH 8.5)–1 mM EDTA–0.1 M NH_4OAc, and 10% formamide, and were used as the source of material for electron microscopy. The infection and analysis were done three times under slightly different conditions.

Electron microscopy of T7 intracellular DNA forms. The material isolated for electron microscopy was prepared for viewing by the Davis, Simon, and Davidson (9) modification of the basic protein technique of Kleinschmidt and Zahn (10). In a typical analysis, the density-shifted T7 DNA molecules appeared in these categories: 60% of the molecules were linear rods of T7 unit length, 20% were Y shaped molecules (*a*) where two arms of the Y were of equal length, and the sum of one of these arms plus the remaining stem was equal to T7 length, 8% were T7-length rods with an internally duplicated region (*b*), 2% were complex molecules, and 10% were tangled molecules of about T7 length. More than 400 Ys (*a*) and 200 "eye forms" (*b*) have been photographed and measured.

Electron microscopy was as follows: the DNA solution contained 10 μl H_2O, 10 μl of 0.5 mg/ml cytochrome *c* (Calbiochem) in 0.5 M Tris (pH 8.5)–0.05 M EDTA (pH 8.5), 5 μl of DNA sample, and 25 μl of formamide (redistilled Matheson, Coleman, and Bell, a gift of Dr. Hajo Delius). This solution was immediately spread onto a fresh hypophase of 20% formamide (Matheson, Coleman, and Bell) in 0.01 M Tris (pH 8.5)–1 mM EDTA.

A part of the cytochrome film containing the DNA was transferred to a Parlodian film supported on a 200-mesh copper grid. The sample was then stained for 30 sec in a solution of 50 μM uranyl acetate–50 μM HCl in 90% ethanol. The staining solution was made by a 1:1000 dilution of a stock of 0.05 M UrO_2Ac–0.05 M HCl in H_2O, and used within 15 min. The grid was dried by immersion in 2-methylbutane (Eastman) for 10 sec. Then the grid was examined in either light-field (Fig. 1*a*) or dark-field (Fig. 2) in the electron microscope (Philips 300, 60 kV, 50-μm aperture). The required time for preparation of a sample to view in the electron microscope was about 10 min.

Negatives from the electron microscope were printed on no. 5 Kodabromide paper, but not otherwise processed.

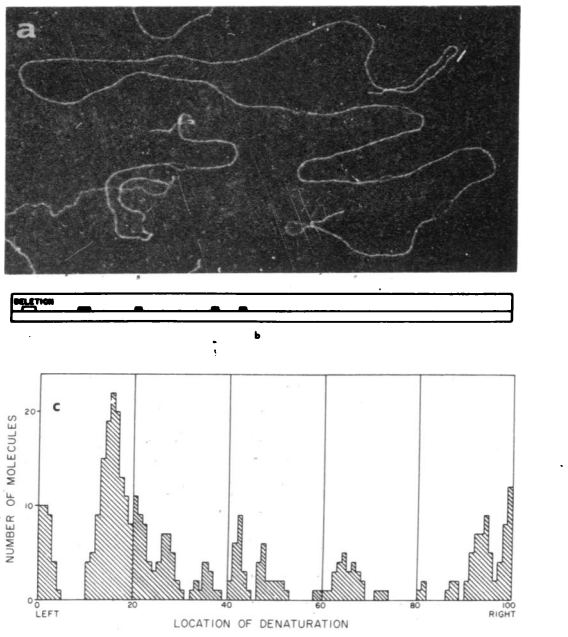


FIG. 3. T7 denaturation map and its orientation with respect to the T7 genetic map. When unit-size T7 DNA is prepared for the electron microscope under conditions of partial denaturation (11), characteristic regions of the double helix melt out. The denatured regions, when observed in the electron microscope (Fig. 3*a*), appear as extended (9, 12) single-stranded blisters interrupting the linear DNA duplex. T7 DNA may be partially denatured by spreading the sample in the presence of a high concentration of formamide. The spreading solution consists of 3 μl of H_2O, 3 μl of 0.5 mg/ml cytochrome *c* (Calbiochem) in 0.5 M Tris (pH 8.5)–0.05 M EDTA, 3 μl of intracellular T7 DNA in 0.01 M Tris (pH 8.5)–1 mM EDTA, and 41 μl of redistilled formamide (added just before the solution is spread). The final concentration of formamide in the solution is 82%; the DNA would be completely denatured by 90% formamide. The spreading solution has a volume of 50 μl, all of which is spread onto a hypophase of 50% formamide. The hypophase contains 1 mM Tris (pH 8.5)–0.1 mM EDTA.

Spreadings are done at 23°C. Picking up of the protein–DNA film and uranyl acetate staining of the DNA are as described in Fig. 2.

This use of formamide to partially denature DNA is an extension of the technique used by Davis, Simon, Hyman, and Davidson to resolve areas of partial homology in heteroduplexes formed from the DNAs of closely-related phages (14, 15).

To map the regions of T7 DNA that preferentially melt-out in the partially-denatured molecules, the molecules were photographed, projected, traced, and measured (with a K and E map measure, 620300). The projected T7 molecules ranged in length from 60 to 65 cm. The measurement of each molecule was normalized to an arbitrary length, which was defined as 100% of the length of the T7 chromosome. Each molecule was schematically displayed as a linear rod in which the denatured regions were indicated as black bars along a horizontal line (3*b*). When 15 molecules were analyzed in this way, it was possible to see a pattern in the denatured regions. This pattern is shown in the histogram (3*c*), which represents the ease of melting out of each segment along the T7 chromosome. The histogram may be thought of as a partial denaturation map of the T7 chromosome. It is useful to note that the most prominent denaturation sites occur 15–30% from one end of the T7 chromosome, while the region 15–30% from the other end is almost-never partially denatured. Furthermore the regions 5–15% from the ends of the T7 chromosome differ in their ease of partial denaturation. An essentially identical pattern has been observed with dimethylsulfoxide as a denaturant (Lang, D., personal communication).

The T7 partial-denaturation map, a physical map, was then oriented with respect to the T7 genetic map in the following way: DNA from a T7 mutant containing a deletion very near the genetic left end (a gift of Dr. F. William Studier) and wild-type T7 DNA were denatured and allowed to reanneal (12, 13). 5 μg of DNA from each of the two phages were denatured in 0.5 ml of 0.1 M NaOH. After 10 min at 23°C, the solution was neutralized with 0.1 ml of 1 M Tris (pH 8.5)–0.01 M EDTA. Finally, 0.55 ml of redistilled formamide was added and the solution was incubated for 2 hr at 23°C, during which time annealing occurred.

A common product of reannealing was a "heteroduplex" DNA molecule (12, 13) that contained one strand derived from the DNA containing the deletion. In the electron microscope, this molecule appeared as a duplex rod with a single-stranded loop in the region of the deletion. When these heteroduplexes were prepared for the electron microscope under partial-denaturation conditions (Fig. 3*a*), the prominent denaturation sites (15–20% from one end) and the deletion loop were in the same end, that is, the genetic left end of the T7 chromosome.

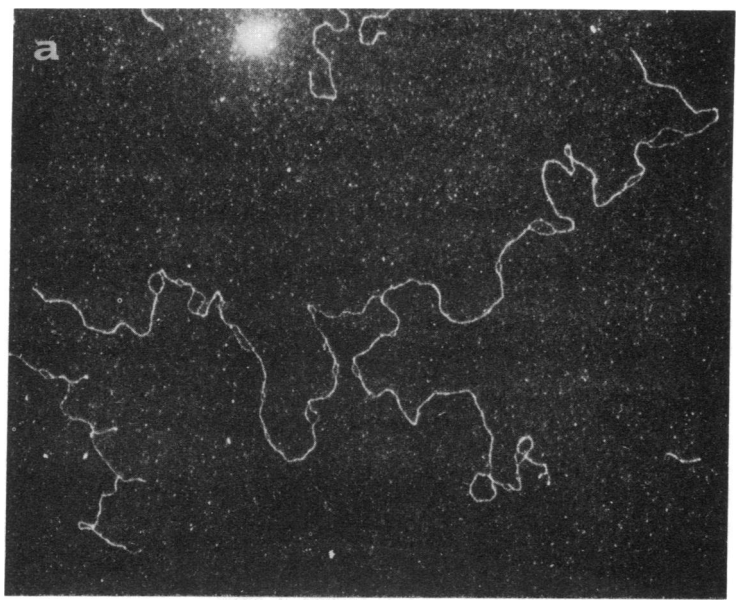
than unit-length T7 rods. When prepared in the presence of chloramphenicol, this material, upon denaturation, releases unit-length and some longer DNA chains. These findings, combined with electron microscopy of the same material, have been interpreted as evidence for the existence of T7 concatemeters (linear duplex DNA molecules longer than mature T7 chromosomes). We have not seen concatemeters during our study of the first round of T7 replication, but the possibility remains that these structures are the products of later rounds of replication.

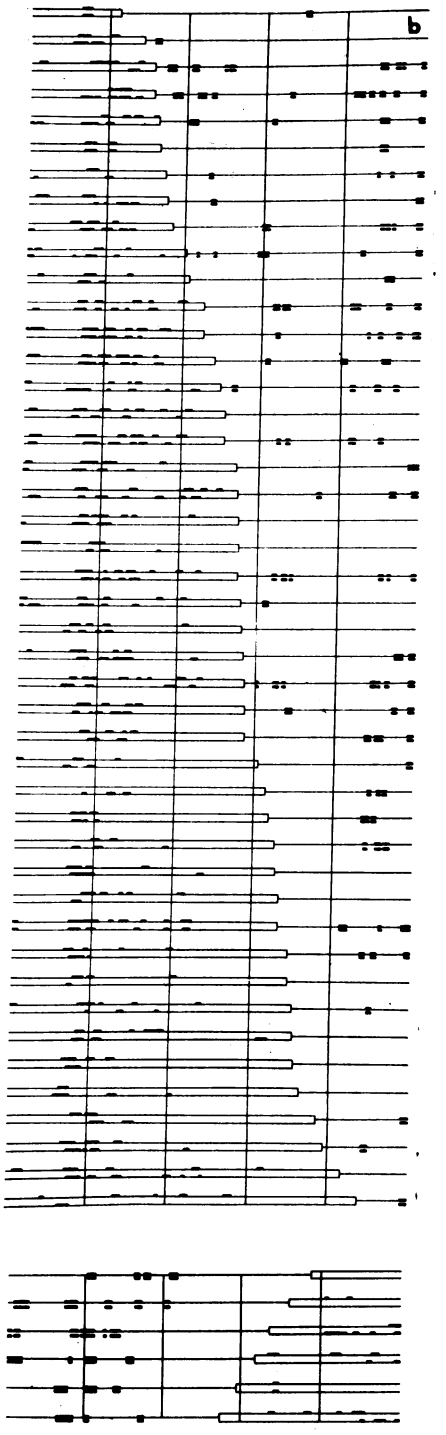
In our studies, well over a thousand Y-shaped molecules were seen, but there were only two molecules that were candidates for circles. Whereas the Y-shaped molecules accounted for 20% of the structures observed in the electron microscope, the circles accounted for less than 0.1%. Moreover, a study by Kelly and Thomas (24) has led to the conclusion that supercoiled DNA circles do not exist in T7-infected cells. Thus, the data appear to indicate that circularity is not involved in the T7 life cycle. This is a conclusion that might be considered surprising for two reasons.

First, Ritchie, Thomas, MacHattie, and Wensink (28) have concluded from their experiments that the T7 DNA molecule is terminally repetitious, and therefore should be able to form circles *in vivo*.

Second, in other microbial, organelle, and viral systems studied thus far, all of the actively-replicating chromosomes that have been analyzed are circular rather than linear. For instance, *E. coli* (1), mycoplasma (30), phage lambda early in the life cycle (7), colicin factor E (29), the tumor-viruses polyoma (31) and SV-40 (32), and the mitochondrial (33) chromosome all appear to replicate in the Cairns pattern (Fig. 1*a*). Phages ϕX- 174 (3, 34, 35) and M13 (36), lambda during its late life cycle (37, 38), P2 (8), PM2 (39), T4 (40–42), P22 (43), the plasmid DNA of *E. coli* 15T$^-$ (44), and *E. coli* during mating (45, 46) all appear to replicate in the rolling circle pattern (Fig. 1*b*).

The reason for the circularity of these replicating chromosomes is unclear. In the case of the Cairns form, the circularity leads to a swivelling problem (1). Because of the closed nature of the circular intermediate, it is necessary to imagine a single-strand nick in the structure that would allow rotation and continued strand separation. Both the rolling circle and the





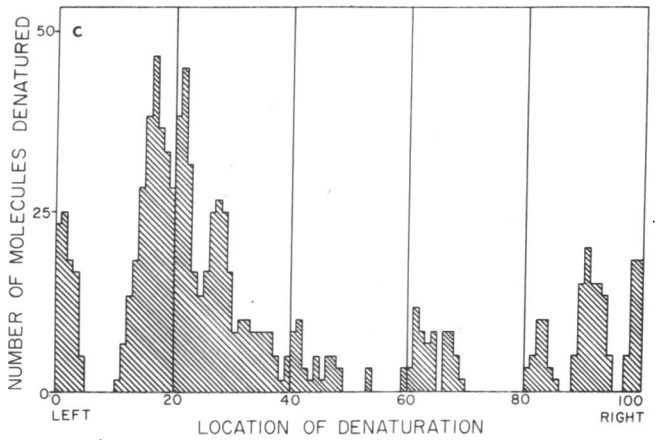


FIG. 4: Partial denaturation of Y-shaped molecules. A sample containing about 20% Y-shaped molecules was prepared for the electron microscope under conditions where the DNA was partially denatured (see Fig. 3). Fig. 4*a* shows a partially denatured Y. This molecule and 51 others are diagrammed with their denaturation bubbles in Fig. 4*b*. Since some regions in each molecule are present in duplicate, the denatured bubbles in the nonduplicated regions were counted twice when the histogram was constructed (4*c*).

Partial denaturation revealed that it was the left end of the T7 chromosome that was present in duplicate in 46 of the 52 Y-shaped molecules, indicating an overall direction of replication from *left* to *right*.

The data were obtained and processed as follows: about 25% of the Y-shaped molecules could be seen in the electron microscope to have more than three denaturation bubbles. All of these were photographed and measured, and the locations of the denatured regions were compared to the map obtained by the denaturation of mature T7 rods. 32% of the photographed molecules had to be eliminated because upon careful analysis they proved either to have an untraceable region, to be shorter than T7 length, or to have no two of the three arms that were within 5% of the same length. The denaturation maps of the 68% remaining Y-shaped molecules are shown in Fig. 5*b*. These data are a composite of three separate experiments, in which the breakdown in the direction of forking of Ys was 15 and 1, 12 and 3, and 17 and 2.

Y-shaped replicating rod avoid the swivelling problem by preserving double-helical segments with free ends. These segments can rotate freely within the replicating molecule, and thus dissipate the twisting that results from strand separation in the growing points.

The Y-shaped replicating rod avoids a second problem that both the rolling circle and the Cairns form must contend with: termination. The rolling circle requires, in addition to the synthesis of new DNA, an event to cut the progeny chromosome free from the tail of the replicating circle (2). A termination mechanism for the Cairns form is also required, and not easily imagined. Unless there is a nick near the termination region, the two daughter chromosomes would remain topologically intertwined. The presence of a nick, on the other hand, is expected to prove lethal to the first growing point that reaches it. In the case of the Y-shaped replicating rod, however, DNA synthesis is itself self-terminating. The product of DNA synthesis is directly the product of replication.

The meaning of a Y-shaped replicating rod to organisms other than T7 is not clear. However, there are experimental results in one other system that might be relevant to a Y-shaped intermediate.

The adenoviruses, a group of more than forty animal viruses (all of which can be oncogenic), contain their genetic information in duplex DNA rods with molecular weights in the range of 23×10^6. The DNA rods from six types of adenoviruses have been tested and are incapable of forming DNA circles *in vitro* (47). Thus, the adenovirus DNAs appear to lack the terminal repetitions in their chromosomes considered necessary for circularization. All other bacteriophages, animal viruses, and organelles whose chromosomes have been characterized are, in fact, either circles or terminally repetitious rods. Because DNA molecules isolated from adenoviruses do not appear capable of circle formation *in vitro*, the adenoviruses might be expected to replicate their chromosomes as Y-shaped rods, in a way similar to T7.

We are greatly indebted to Drs. Ron Davis, Hajo Delius, Ross Inman, and Maria Schnös for their advice and encouragement with the electron microscopy. We thank Dr. F. W. Studier for discussions about T7. Drs. John Cairns, James Watson, and Walter Gilbert provided thoughtful readings of the manuscript. We thank Michael Farber for the gift of a high-titer T7 stock. The Cairns form shown in Fig. 1*a* was found among replicating lambda DNA, which was a gift of Nancy Maizels. This work was supported by the National Institutes of Health (GM 17088) and the American Cancer Society (E-592). J. W. is a trainee under the National Institute of General Medical Sciences Training Grant GM 138-14 and is a medical student on leave from The Johns Hopkins Medical School. D. D. is a fellow of the Helen Hay Whitney Foundation.

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