*REPLICATION OF BACTERIOPHAGE DNA, II. STRUCTURE OF REPLICATING DNA OF PHAGE LAMBDA**

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A duplex viral DNA molecule has been found to exist in various structures, which may be linear or circular (with or without tertiary turns) and monomer or polymer.[1, 2] Little is known, however, about the structure in which viral DNA replicates. Linear molecules of phage λ DNA become circular shortly after infection[3, 4] and replicate to form circular molecules.[5, 6] This suggests that the phage DNA molecule replicates in a circular structure, but does not at all prove that the molecule replicates while retaining a circular structure. To determine the replicating structure, it is essential to isolate the replicating molecules. When the replicating λ phage DNA molecules were isolated, they were circular.

Materials and Methods.—Bacterial and phage strains: Bacteria used were *E. coli* K12 N3360, a *thy*$^-$ derivative of W3350 (Lederberg), and the phage strain used was λc_1.

Media: DT-medium, which contained 20 mmoles of potassium phosphate buffer, pH 7.0; 1 mmole of $MgSO_4$; 1.6 μmoles of $FeCl_3$; 0.5 gm of $(N^{15}H_4)_2SO_4$ (99.0 atom % of N^{15}; Radioisotope Association, Tokyo); 0.05 gm of D_2O algal hydrolysate (98.0 atom % of D; Merck Sharp and Dohme, Montreal); 2.5 gm of maltose and 5 mg of thymine per liter of D_2O (99.75 atom % of D; Merck, Darmstadt) was used to grow bacteria. The medium used for washing bacteria and for adsorption of phage was 0.01 *M* $MgSO_4$ in D_2O; that (with H_2O) for labeling phage by P^{32} (0.5 c/gm P) or H^3-thymidine (2 c/gm thymidine) was described in a previous paper.[7] Phage particles were suspended in 0.01 *M* $MgSO_4$ in D_2O supplemented with 50 μg/ml of bovine serum albumin. SSC is 0.15 *M* NaCl and 0.015 *M* sodium citrate in H_2O.

Preparation of cell lysate: DT-medium (40 ml) was inoculated with a 1-ml culture of bacteria, previously adapted to grow in the medium, and incubated at 37°C for 12 hr. At that time, the cell concentration reached about 5×10^8/ml. The cells were centrifuged, washed, suspended in 2 ml of 0.01 *M* $MgSO_4$, and further incubated for 30 min. P^{32}-labeled "light" phage was added at the multiplicity of infection of about 6. After 10 min, 20 ml of warm DT-medium was added. The culture was incubated for 12 min at 37°C and cooled in an ice bath, and the cells were harvested by centrifugation. They were then washed with cold SSC with 0.01 *M* KCN and suspended in SSC containing 500 μg/ml of lysozyme and 0.01 *M* ethylenediaminetetraacetate at a cell density of 5×10^9/ml. After being frozen and thawed three times, the cells were incubated at 37°C for 15 min, followed by 60 min incubation with 300 μg/ml of trypsin (Worthington, 2× crystallized). The clear lysate was made 0.1% in sodium N-lauroyl salciocinate (Wako Pure Chemical Ind., Tokyo) and heated at 75°C for 10 min.

CsCl density-gradient centrifugation: The lysate thus prepared was suspended in CsCl solution to give a final density of 1.72 gm/cm^3, and centrifuged in a 40 rotor of a Spinco L2 ultracentrifuge for 40 hr at 36,000 rpm at about 5°C.

Electron microscopy: Samples prepared by CsCl density-gradient centrifugation were dialyzed against SSC and then overnight against 2.0 *M* ammonium acetate. The outside of the dialyzing bag was kept under slightly reduced pressure, and thus the dialysis and threefold concentration of DNA (to about 1 μg/ml) were carried out simultaneously. Samples were prepared for electron microscopy by the protein film technique.[8] The sample, containing 0.25 μg/ml of DNA and 0.01 per cent of cytochrome *c* in 2.0 *M* ammonium acetate, was spread onto the surface of double-distilled water. Portions of the

protein film were transferred onto grids coated with carbon film. The dried samples were shadowed rotationally with platinum-palladium (4:1). They were then examined with a JEOL JEM-7A electron microscope. Photographs were taken at a magnification of 9000. The magnification factor was calibrated by standard latex spheres (Dow Chemical Co., Midland, Mich. soon after the observation of the sample. The length of the DNA strands on enlarged photographs was measured with a map measure (Maruzen Co., Tokyo).

Results.—Preparation of replicating phage DNA molecules: The lysate described above was divided into two equal parts and centrifuged in CsCl density gradient. The centrifugation pattern of one of the samples is shown in Figure 1*a*. More than 90 per cent of P^{32}-labeled DNA was recovered, of which more

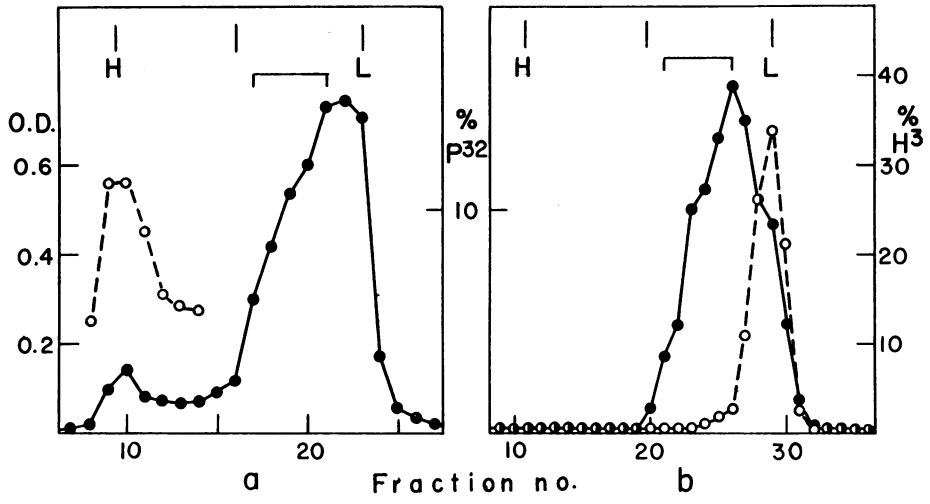

FIG. 1.—(*a*) Density-gradient centrifugation profile of the lysate of N3360 infected with P^{32}-labeled "light" λc_1 and incubated for 12 min in DT-medium: The lysate was divided into two equal parts and centrifuged in CsCl solution for 40 hr at 36,000 rpm. A sample of 5 ml was fractionated in 40 tubes. The radioactivity of 5-μl portions of each fraction was measured. Fractions 8–14 were diluted with 4 vol of SSC, and the optical density at 260 mμ was measured. The centrifugation profile of one of the samples is presented. The other sample gave a similar profile. *Ordinate:* OD 260 mμ (—O—), or per cent recovery of the input P^{32}-DNA (—●—).
(*b*) Recentrifugation profile of the pooled DNA: Fractions 17–21 of the first centrifugation presented above and the corresponding fractions of the other sample were mixed and centrifuged. Four ml of the sample with H^3-labeled reference λ DNA was centrifuged and fractionated in 53 tubes, and the radioactivity was measured. *Ordinate:* per cent recovery of the input P^{32}-DNA (—●—) or H^3-DNA (—O—).

than 60 per cent was found at the fractions with a density heavier than that of the parental phage DNA. Fractions 17–21 of the sample shown in Figure 1*a* and the corresponding fractions of the other gradient were pooled and centrifuged again in CsCl density gradient with H^3-thymidine-labeled phage DNA added as a reference. Figure 1*b* shows the second centrifugation pattern, that of the pooled DNA. Fractions 21–26 were pooled, dialyzed, and examined by electron microscopy. When H^3-thymidine was added well before infection to label only bacterial DNA and the DNA was similarly centrifuged, the H^3-labeled DNA was completely separated from the phage DNA by these successive centrifugations. When H^3-thymidine was added after infection, H^3-labeled phage DNA formed a

band at the heavier side of the P^{32}-labeled phage DNA peak. Thus the DNA molecules to be examined were replicating phage DNA and were almost free from unreplicated phage DNA as well as bacterial DNA.

Structure of replicating molecules: When the DNA molecules as prepared above were examined with an electron microscope, approximately one half of the molecules in the field showed various degrees of clumping. Of 42 molecules, in which entire DNA strands could be traced, 30 were shown to have no ends. Of these 30, 5 had a structure indistinguishable from an unreplicated circular molecule and 25 had two branched points in a molecule as shown in Figure 2. These

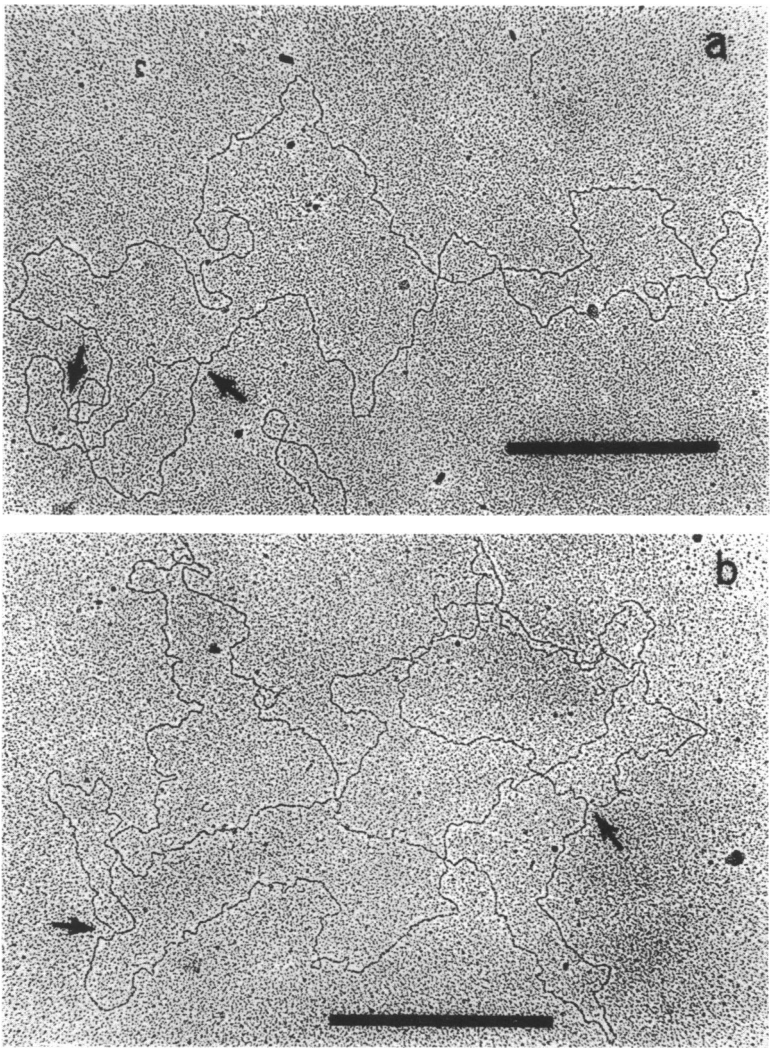

FIG. 2.—Electron micrographs of two replicating molecules: The arrows indicate the branched points, and the scale equals 1 η. (*a*) Molecule no. 2 of Fig. 3; (*b*) molecule no. 9 of Fig. 3.

25 molecules had a total length greater than a whole molecule of the parental phage DNA, that is, about 17 μ, showing that they were replicating. This is confirmed by analyzing the length of the three regions separated by the branched points. The result of such an analysis with ten representative molecules is presented in Figure 3. It is shown that these molecules carry two regions of an identical length and that the sum of the length of one of these regions plus that of the third region makes a length of about 17 μ. These results indicate that these circular molecules are replicating molecules, and that the two regions of an identical length are replicated regions. If these molecules replicate from a fixed point and unidirectionally,[9] one of the branched points is the growing point and the other is the initiation point of replication. No significant disorder in the double-

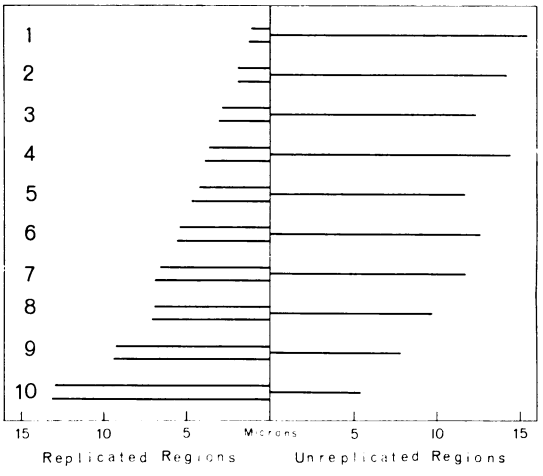

FIG. 3.—Electron micrographic lengths of ten replicating molecules.

stranded structure at either branched point was observed. These molecules were not twisted, indicating the presence of a single-strand break(s), which may be present in the native replicating molecule or may be produced during the course of examination.

The remaining 12 molecules had various shapes. Five had a circular structure with the unit contour length from which a strand with one end extended. Two contained small circles to which two linear strands were attached. Five were linear, of which three had one branched point and three ends, and two had no branched point. No regularity was observed in the length of the regions with ends. They may be produced by breakage of the strands somewhere in circular molecules.

The replicating molecules retained their structure and did not expose cohesive sites by being heated at 75°C.[9] Thus the circularity of these molecules is not maintained by hydrogen bonds at the cohesive ends of linear molecules.

Discussion and Summary.—It was shown that replicating λ phage DNA molecules isolated early after infection are circular. Since λ DNA itself can form

a circular structure,[10] a circular replicating molecule can be free from cellular components other than DNA. A bacterial chromosome may well be composed only of DNA. The replicating bacterial chromosome has been shown to be circular.[11, 12]

Although the present results do not provide direct information on a single-strand break in the native replicating molecule, the presence of circular replicating DNA leads to the following important prediction on the presence of a single-strand break in the molecule. For a duplex DNA molecule to replicate, the parental helix must rotate as the strands separate. If both of the parental single strands are covalently closed circles, the rotation creates right-handed tertiary turns of the molecule. Such a molecule twists more and more while in the process of replication, making it difficult to complete a cycle of replication. Therefore, at least one single-strand break must be present in the unreplicated region of the replicating molecule. The single-strand break may persist during a cycle of replication or breakage and repair may occur continuously during replication. The simplest model is to assume a persistent single-strand break at or near the initiation point or a temporary break at or near the growing point. In both cases a mechanism has to be assumed by which the parental helix and both replicated helices are kept together, forming two branched points stable to various treatments such as were performed here.

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