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DNA of Vegetative Bacteriophage Lambda, VI.* Electron Microscopic Studies of Replicating Lambda DNA

JOHN A. KIGER, JR.†, AND ROBERT L. SINSHEIMER

Division of Biology, California Institute of Technology, Pasadena, Calif. 91109

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ABSTRACT Electron micrographs of replicating lambda DNA molecules, during the period of progeny-particle DNA formation, show single-tailed circular structures. The tails are, predominantly, shorter than one viral genome.

Previous studies of the biophysical properties of replicating bacteriophage lambda DNA have led us to propose a model for circular DNA replication in which initiation of one daughter single-strand proceeds through covalent addition to the 3′-OH of the open, parental strand of a component II molecule (a ring containing one or more single-strand breaks) (1). Very similar proposals have been made by Gilbert and Dressler (2).

Our model was prompted by two findings. First, a method was developed that allowed the single-strand composition of replicative intermediate molecules of lambda DNA to be analyzed. It appeared that for the more purified preparations of intermediates, each molecule contained a single-strand ring of viral DNA length, one single-strand linear molecule longer (up to twice) than the length of a viral DNA single-strand, and one single-strand linear molecule of less than viral DNA length (1). Second, sedimentation analysis of pulse-labeled replicative intermediates revealed that approximately one-half of the label incorporated during a short period of time was incorporated into single-strands longer than viral DNA single-strands (3).

The proposed model for DNA replication suggests that the native structure of the replicative intermediate is a double-strand ring with a double-strand tail of variable length attached to the ring through one phosphodiester bond. We present here electron micrographs of replicating lambda DNA molecules that we believe fulfill this prediction.

METHODS

The materials, techniques, and bacterial strains employed here have been described (1).

Heavy-light labeling

Heavy TG medium (4), supplemented with 10 μg/ml of [^{14}C]thymine (Schwarz BioResearch, Inc., 3.25 mCi/mg) is inoculated with 159T$^-$(λc_{ts}) adapted to heavy medium, and the cells are grown to a concentration of 3×10^8 cells/ml. The cells are then collected by centrifugation and resuspended in one volume of K medium (5) supplemented with 10 μg/ml of thymine. The culture is induced by incubation at 45°C and simultaneously treated with mitomycin C (20 μg/ml) to inhibit host DNA synthesis. After 10 min of incubation at 45°C, the cells are collected by filtration on Millipore HA filters, washed, and resuspended in one volume of K medium supplemented with 5 μg/ml of thymine, 5 μg/ml of uracil, and 40 μCi/ml [^{3}H]thymine (New England Nuclear Corp., 16.8 Ci/mmol). Incubation at 45°C is continued for 5 min, then the culture is shifted to 37°C. 45 min after induction, shortly after the start of phage production (the culture contained 2 plaque forming units (PFU)/infective center), the culture is brought to 10 mM sodium azide, the cells are collected, and the DNA is extracted from sarcosyl-lysed spheroplasts with phenol (1).

Equilibrium density gradient centrifugation

CsCl (1.31 times the weight of the DNA solution) is added to the DNA and the solution is centrifuged in a Spinco angle 40 rotor (6.4 ml/tube; overlayed with paraffin oil) at 30,000 rpm, 20°C, for 46 hr. Fractions are collected dropwise from the bottom of the tube.

Sucrose gradient centrifugation

DNA is sedimented through 5–20% sucrose gradients in 10 mM Tris–1 mM EDTA (pH 8.1) in a Spinco SW 25.1 rotor at 25,000 rpm and 6°C for 6 hr. 1.0 ml of DNA solution, containing a marker of lambda phage [^{14}C]DNA is layered over a 25-ml gradient. Fractions are collected dropwise from the bottom of the tube.

Electron microscopy

DNA is prepared for electron microscopy essentially as described by Kleinschmidt and Zahn (6). A solution containing 100 μl of DNA solution (approximately 0.2 μg/ml), 30 μl of 6 M ammonium acetate, and 20 μl of 0.1% cytochrome C (Calbiochem) is spread over a 0.25 M ammonium acetate solution dusted with talc. The film is picked up on grids coated with 3% parlodion, and the grids are fixed by submersion in 95% ethanol, followed by submersion in isopentane and air drying. Grids are rotary shadowed with Pt–Pd 80:20 and viewed in a Phillips 200 electron microscope. Magnifications are determined using a carbon replica of a grating containing 54,864 lines/inch (about 22,000 lines/cm). Contour lengths of DNA molecules are measured with a map measurer.

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† Present address: Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oreg. 97331.

RESULTS

Studies of purified replicative intermediates of lambda DNA

Light ^{3}H-labeled intracellular lambda DNA synthesized after induction of heavy ^{14}C-labeled cells was separated from the heavy host DNA by equilibrium centrifugation in CsCl. The separation obtained is shown in Fig. 1. The fractions composing the peak of light DNA were pooled and dialysed against 0.1 × SSC (0.015 M NaCl–0.0015 M sodium citrate) overnight. This DNA was adsorbed to benzoylated–naphthoylated DEAE-cellulose and the column was eluted with 1.0 M NaCl, then with 1.8% caffeine in 1.0 M NaCl as described (1). 64% of the DNA was eluted with caffeine. The DNA eluted by caffeine, which we have shown to contain the replicative intermediate of lambda DNA, was dialysed against 0.1 SSC and examined in the electron microscope.

A heterogeneous population of molecules was observed, consisting of three types: circular molecules; circular molecules with attached linear tails; and linear molecules. Measurement of 52 circular molecules gave an average contour length of 13.2 μm with a standard deviation of 0.97 μm. This value is markedly lower than previous published lengths for intact lambda viral DNA. MacHattie and Thomas (7) and Caro (8) both obtained a value of approximately 17.2 μm for lambda DNA on grids with a carbon film. Our value of 13.2 μm was obtained on parlodion-coated grids and is in agreement with values obtained for lambda DNA by others using parlodion-coated grids (N. Davidson, personal communication).

Of the circular molecules with tails, nineteen were spread well enough to be measured. Of these nineteen molecules, seventeen possessed rings of viral DNA length and two possessed rings of twice this length. Representative molecules are shown in Plate I. A double-length ring with tail is shown in Plate II (*bottom*). All of the molecules with tails (except one) had tails of less than viral length. The molecule shown in Plate II (*top*) has a tail 17% longer than the ring to which it is attached. Fig. 2 is a histogram of measured tail lengths presented in units of viral DNA length. Molecules with two branches and no free ends, similar to those presented by Ogawa, Tomizawa, and Fuke (9) and Schnös and Inman (10), were not observed.

Sedimentation rate of circular molecules possessing tails

While the molecules shown in Plates I and II have the form that we had anticipated, more than circumstantial evidence is necessary to establish that the tailed molecules observed in the electron microscope are the replicative intermediate we have described previously from biophysical evidence (1, 3).

The DNA eluted from benzoylated-naphthoylated DEAE-cellulose by caffeine was sedimented through a neutral sucrose gradient along with a marker consisting of linear phage [^{14}C] DNA and annealed rings. The fractions containing material sedimenting ahead of linear phage DNA were examined in the electron microscope.

The degree of spreading of DNA molecules on individual grids using the technique described in *Methods* appears to to be a poorly controlled variable. Considerable aggregation (exhibited in a mild form in some of the micrographs in Plate I) is often observed, making it impossible to accurately classify many molecules. Thus, a quantitative classification

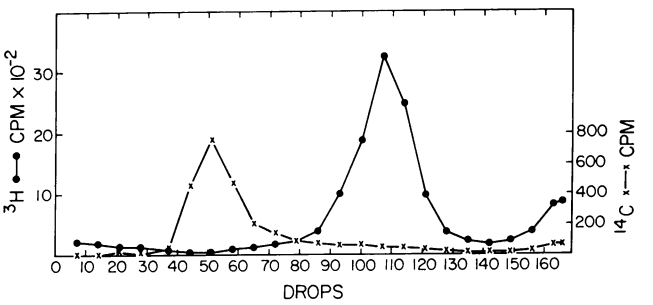


FIG. 1. Separation of light lambda DNA from heavy *E. coli* DNA.

●——● Intracellular lambda [^{3}H]DNA.
×——× *E. coli* [^{14}C]DNA.

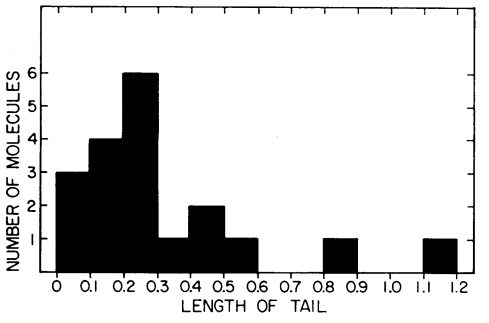
of the molecules on a grid was not possible. All of the fractions examined in the electron microscope from the sucrose gradient shown in Fig. 3 contained circular molecules. We have used the ability to score circular molecules as evidence of good spreading on a particular grid. Thus, the presence of circular molecules with tails in each fraction is presented in Fig. 3 as the ratio of tailed molecules to simple ring molecules. The data from which the points in Fig. 3 are derived is given in Table 1.

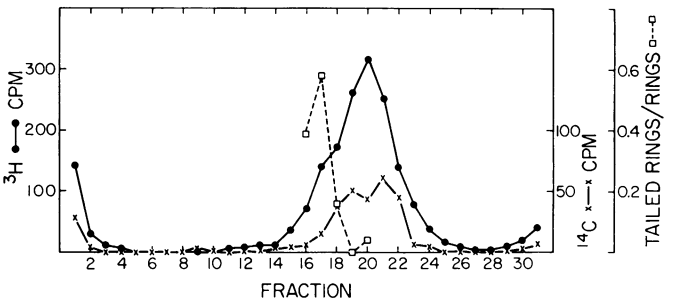
It is noteworthy that all of the sedimentation fractions examined possessed a large percentage of simple circular molecules without tails, whereas the annealed rings of the marker [^{14}C]DNA sedimented principally in their expected position, at 1.2 times the rate of linear phage DNA. This most probably indicates that during the preparation of DNA for electron microscopy, tails are broken from the tailed rings. This explanation helps to explain the presence of linear molecules in all of these fractions. The relative predominance of linear molecules in all of the fractions is most probably due to the fact that linear molecules are the easiest type to classify (it is sufficient to identify two free ends), while many circular molecules or tailed molecules could not be scored as such with certainty and were not recorded.

The data indicate that the tailed molecules sediment faster than the annealed rings present in the marker DNA; they sediment at a rate from 1.4 to 1.6 times the rate of linear phage DNA. This is the rate at which the replicative intermediate previously described is found to sediment (1).

DISCUSSION

It is important to ascertain to what extent the molecules observed in the electron microscope are intact. As mentioned above, there is evidence that tails are broken from rings during preparation of the DNA for electron microscopy. The histogram of tail lengths presented in Fig. 2 might be interpreted as indicating that molecules with long tails are more sensitive to fragmentation than those with short tails. As the cells are not synchronized, one would naively expect a uniform distribution of tail length. While accepting the logic of this argument, we must point out the possibility that the rate of DNA replication may vary, dependent upon the extent of replication, e.g. the first portion of the molecule might be replicated more slowly than the remainder, giving rise to a pool of incomplete intermediates possessing short tails. Pulse-chase experiments have led us to postulate that the replicative intermediates form a pool from which they are matured at





(*Left*) FIG. 2. Distribution of tail lengths. Tail length is presented in units of phage length DNA.
(*Right*) FIG. 3. Sedimentation of caffeine-eluted DNA and tailed molecules. Sedimentation is from right to left.

●——● Lambda [^{3}H]DNA eluted from benzoylated–naphthoylated DEAE-cellulose by caffeine.
×——× Lambda phage [^{14}C]DNA, consisting of linear molecules and annealed rings.
□- - -□ Ratio of tailed molecules to rings.

random to phage DNA. Such a pool might consist of intermediates temporarily arrested at a partially replicated stage. Thus, the data in Fig. 2 may not necessarily indicate selective loss of tails.

The possibility has been proposed that long linear concatenates may be an intermediate in lambda DNA replication (11, 12). We have previously shown that under the conditions employed in our experiments, no more than 10% of the intracellular DNA that is labeled for periods from 10 sec up to 35 min sediments faster than double-phage-length circular molecules would be expected to sediment. [The remaining 10% of the labeled DNA may be a result of incorporation of label into replicating *E. coli* DNA (1).] Thus,

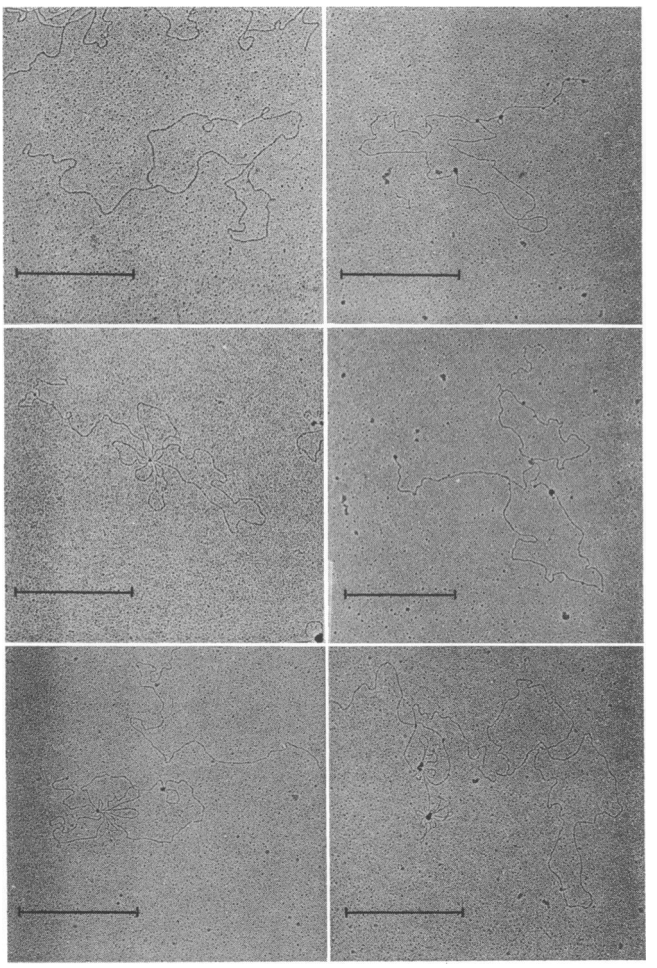


PLATE I. Tailed molecules in DNA eluted by caffeine from benzoylated–naphthoylated DEAE-cellulose. Each line represents 2 μm. Each ring is of lambda DNA length.

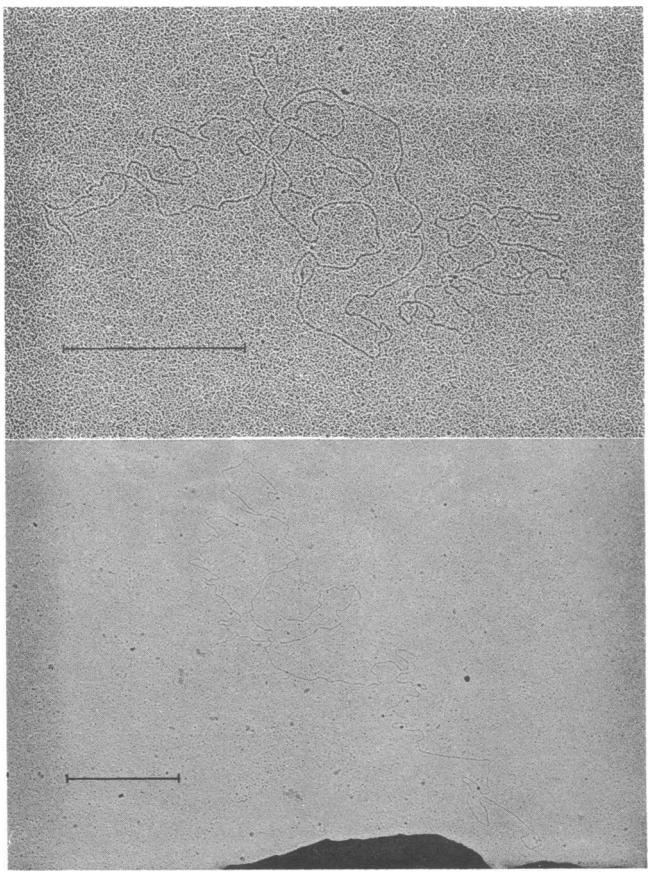


PLATE II. Tailed molecules in DNA eluted by caffeine from benzoylated–naphthoylated DEAE-cellulose. *Top.* A molecule with a tail longer than the ring to which it is attached. *Bottom.* A tailed molecule with a ring twice the length of viral DNA Beside it is a ring of unit phage length. Each line represents 2 μm.

TABLE 1. *Statistical distribution of DNA forms seen in electron micrographs of successive sedimentation fractions*

Fraction	Circles with tails	Circles	Linear molecules	Total number of molecules
16	7 (9.7%)	18 (25.0%)	47 (65.3%)	72
17	21 (15.3%)	36 (26.3%)	80 (58.4%)	137
18	3 (3.3%)	19 (21.1%)	68 (75.6%)	90
19	0	18 (14.9%)	103 (85.1%)	121
20	1 (1.3%)	25 (32.0%)	52 (66.7%)	78

we do not expect to find a large percentage of molecules with tails longer than phage DNA. In fact, we have found one molecule out of nineteen that possesses a tail longer than phage DNA.

On the other hand, it is possible that if maturation of phage DNA and DNA replication are not coordinated, conditions may exist under which DNA replication could occur at a greater rate than maturation. Replication might then continue past the point of initiation on the ring and generate a tail longer than one phage DNA length (1, 2, 13). Thus, while we have no evidence for a major role for long concatenates during lambda DNA replication under our conditions, it is possible that they do form a major DNA species under some conditions (the finding of a molecule with a tail longer than one viral DNA length suggests that this may not be idle speculation). It would be expedient to investigate the course of DNA synthesis in capsid mutants of lambda, and under conditions where protein synthesis is inhibited, to determine what role the capsid plays in maturation and regulation of the length of tails.

The existence of double-length rings with tails is an interesting finding. The production of double-length rings during infection by ϕX174 has been noted already (14, 15). As pointed out previously, double-length rings may be a consequence of a mode of replication that involves the production of single-strands longer than viral DNA single-strands (1, 2). The electron micrographs would indicate that double-length rings are also capable of replication. This point is supported also by the work of Weissbach, Bartl, and Salzman (16).

We have observed no molecules in which the tail appears to be attached to the ring at more than one point, to give circular structures with two forks and no free ends, as have been observed by Ogawa, Tomizawa, and Fuke (9) and Schnös and Inman (10). Possibly these structures are identical to those presented here except that the end of the tail is held to the ring at the point of initiation by noncovalent means, e.g. protein–nucleic acid interactions, and that this bonding, while not broken by the preparative method employed by Ogawa *et al.* (9) and Schnös and Inman (10), is broken by the phenol extraction employed here.

Alternatively, the molecules pictured by Ogawa *et al.* (9) and Schnös and Inman (10) may represent only the early replication of parental molecules. Young and Sinsheimer (17) did not find component X (our replicative intermediate) during the period of RF replication preceding viral DNA synthesis. Thus, it is possible that more than one mode of DNA replication exists: one involving the generation of single-strands longer than viral DNA and the other not, leading to different structures for the respective intermediates at different stages during infection. We feel that the latter explanation for the diversity of replicating structures is more probable than the former.

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