THE ROLLING-CIRCLE REPLICATIVE STRUCTURE OF A BACTERIOPHAGE λ DNA

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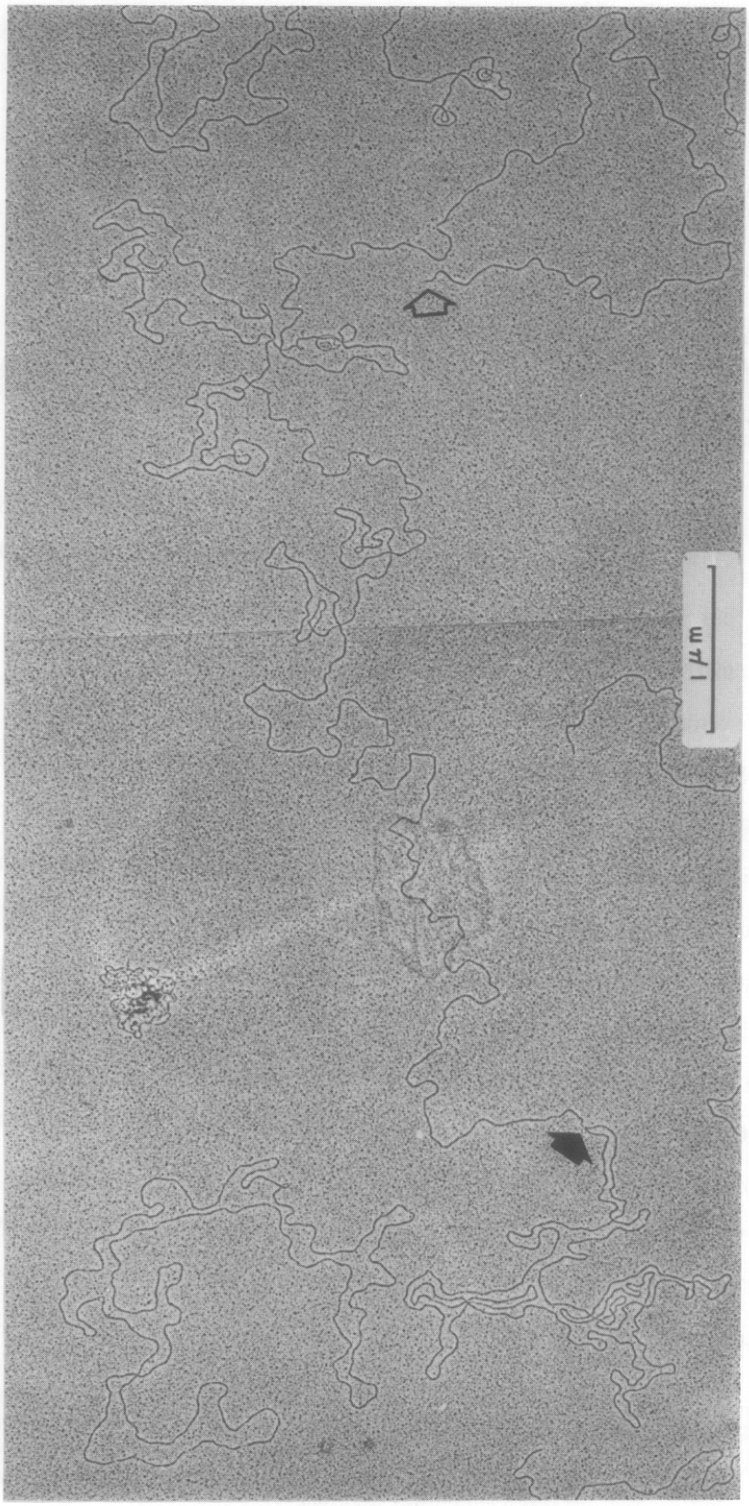
Received September 23,1974

SUMMARY: Intermediates of λ DNA replication in the second half of the latent period have been isolated and investigated in the electron microscope. The isolated replicative structures were predominantly single-branched "rolling-circle" replicative forms. The long linear tails (concatemers) may be the precursor of mature λ DNA.

INTRODUCTION: The finding of fast-sedimenting λ DNA in an alkaline sucrose gradient (1,2,3) has suggested that the replicative form in the second half of the latent period should be different from the first round replicative form which generates progeny circles from parental circles.

Fast-sedimenting DNA which is longer than the monomer length (the DNA length found in phage particles) was also found in a recombination deficient system (Rec A$^-$, red$^-$, int$^-$) (4), strengthening the argument that this structure can be produced by replication in the absence of recombination. Sensitivity to physical shearing and binding to benzoylated DEAE cellulose (consistent with the existence of single-stranded regions) have been reported for this replicating structure (5). The recBC-nuclease sensitivity of this replicating structure, which is normally protected by the λ gam gene product suggests that this replicative form contains linear free ends or extended single-stranded regions (6,7). The replicative structure ("rolling-circle") observed in the present study is compatible with these properties. The rolling-circle replicative structure can reproduce more than an entire genome in a catenated form while preserving the original circular template.

MATERIALS AND METHODS: An E. coli preculture was grown in D-medium (9) at 37°C. The preculture was inoculated into 20 ml of fresh D-medium and the cells grown to A_{590} = 0.70. The cells were centrifuged, and resuspended in 1 ml of Tris-$MgSO_4$, 0.01 M each in D_2O. After starving the cells for 15 min at 4°C,


1 μm

[^{3}H]-labelled λ phage (in D_2O) was added at a multiplicity of infection of 10, and incubated at 4°C for 20 min for phage absorption (D_2O freezes at 3.8°C). The infected cells were diluted with 20 ml of prewarmed D-medium, and incubated at 37°C.

DNA replication was terminated by pouring the cells into 20 ml of an ice cold solution containing 0.01 M-KCN, 0.15 M-NaCl and 0.015 M-sodium citrate. Lysozyme-pronase treated lysates were made up to the density of 1.67 with CsCl and centrifuged (Ti-60 rotor, 30,000 rev/min for 3 days at 7°C). The fractions containing DNA of density between half heavy and heavy were used for preparation of electron microscopic specimens. Molecules were photographed on 35 mm film and measured with a curve length integrating device. Data computation was as described previously (9).

RESULTS AND DISCUSSION: Previous experiments have shown that the molecules found in fractions located between light and hybrid density in a CsCl density gradient exhibited a characteristic replicating structure consisting of double-branched theta (θ) type molecules similar to those found in E. coli (8,9,10). This replicating structure is believed to represent the first round of λ DNA replication, which generates a progeny circle from a parental circle. An important question is whether the second round λ DNA replication proceeds in

Table 1. Summary of electron microscopic observation.

DNA solutions in fractions located between light and heavy light or between heavy light and heavy positions in Figure 1 were prepared by standard neutral spreading procedures (9). *Molecules are denatured with high temperature (50°C for 30 min). **RF: Replicative Form.

Time after infection	No. of single-branched (δ) type R F	No. of double-branched (θ) type R F	No. of molecules scanned		δ/θ (%)
15 min	67	163	230		29.1
30 min	126	2	128	181	98.4
	(53)*	(0)*	(53)*		(100.0)*
50 min	168	10	178		94.4

Plate 1. Electron micrograph of a "rolling-circle" λ DNA.

Branch point is shown by a black arrow. Attached tail possesses several single-stranded segments. End of the tail is shown by white arrow. This molecule is 215% replicated (circular length is 17.7 micrometers and tail length is 38.1 micrometers).

the same manner as the first round of replication or whether it involves a different process. To answer this question, we have carried out the following experiments:

Three cultures grown in heavy medium were infected with [^{3}H]-labelled λ phage and incubated in heavy medium for 15 min, 30 min and 50 min, respectively. Fifteen minutes after infection, about 36% of the label in parental DNA was found in a half heavy position, indicating that parental λ DNA had started to replicate [Figure 1-(a)]. Observations on the replicating molecules located between the density of light and half heavy peaks exhibited predominantly (71%)

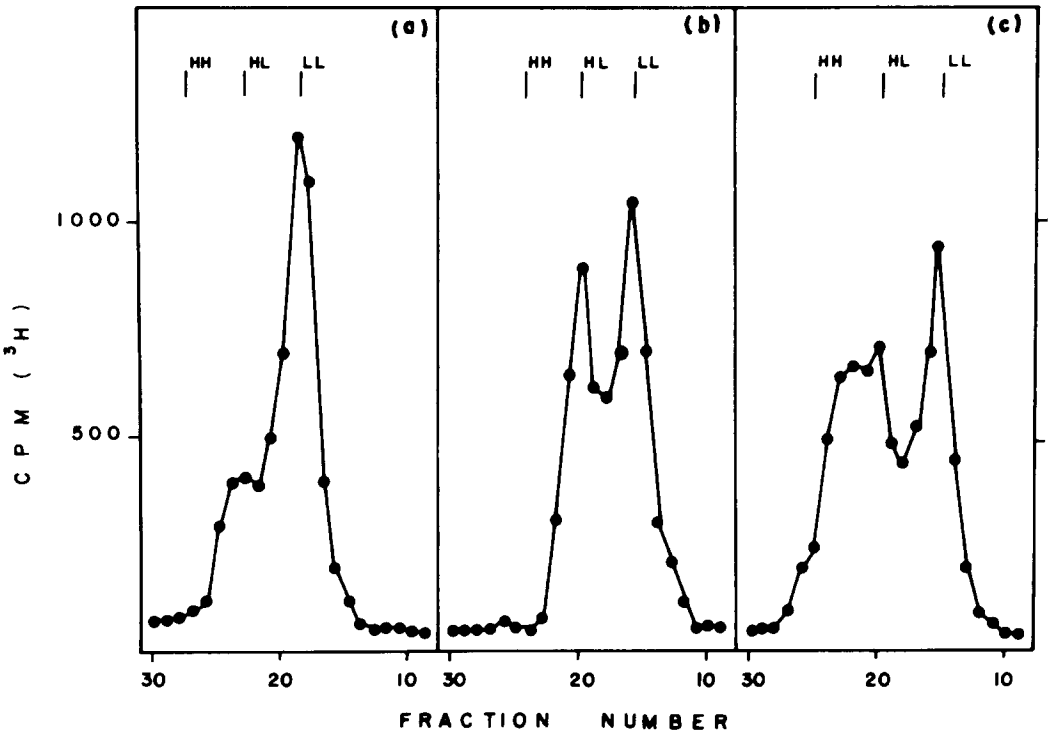


Figure 1. CsCl density-gradient centrifugation of DNA from cells which are grown in a heavy medium, infected with light [^{3}H]-λ.

E. coli W3102 (gal$^-$, su$^-$) was grown in heavy medium at a cell density of 2 x 10^8/ml. [^{3}H]-labelled λ phage were added at a multiplicity of 10. DNA was extracted and subjected to CsCl density-gradient centrifugation as described in the methods. The positions where the heavy, the half heavy and the light DNA are located are indicated with the signs HH, HL and LL respectively (based on the density). 15 minutes after infection at 37°C (a), 30 minutes after infection (b), and 50 minutes after infection (c). The recovery of radioactivity was (a) 97%, (b) 93%, (c) 90%, respectively.

theta (θ) type replicating structures. This result is quite consistent with the previous observation that, after 10 minutes of infection, 85% of the replicating molecules exhibited a double-branched circular structure (9). Thirty minutes after infection [Figure 1-(b)], the progeny circles located at the half heavy position had started to replicate and we have observed replicating molecules in the fractions between the half heavy and heavy positions. Almost all (98%) of these replicating molecules exhibited a single-branched delta (δ) type structure despite the fact that the DNA extraction procedure was identical for the early and late samples. Molecules of this kind could be copied completely by a rolling-circle mechanism even if replication were initiated or terminated imprecisely (11). Fifty minutes after infection [Figure 1-(c)], the parental DNA label was located at a still denser position and replicating molecules were also single-branched circles (94% of 178 replicating molecules).

The replicating molecules which possess a variable length of linear branched portion have been partially denatured to determine whether they are a λ DNA or not (E. coli DNA might be involved). Linear λ DNA derived from a mature λ phage, for example, exhibited a characteristic denaturation pattern (9). This distinctive denaturation map of λ DNA is possible to recognize as λ DNA in a DNA preparation purified from infected cells. Using as a gauge the denaturation map derived from mature linear λ DNA, we have estimated the position of the cohesive sites on the circle, and the extra tail segments attached to their circles are disposed according to the correspondence of the denatured sites to the circles. Twenty replicative molecules which have longer tail segments than circular λ DNA are listed in Figure 2. Out of 20 molecules, eight molecules (40%) had their free end (shown by vertical arrow) located at a region close to the postulated origin of the first round of λ DNA replication (about 18% from the right end) suggesting that these molecules started replication from a region close to this point. The fact that not all tails ended in this position could be due to breakage during preparation rather than that they

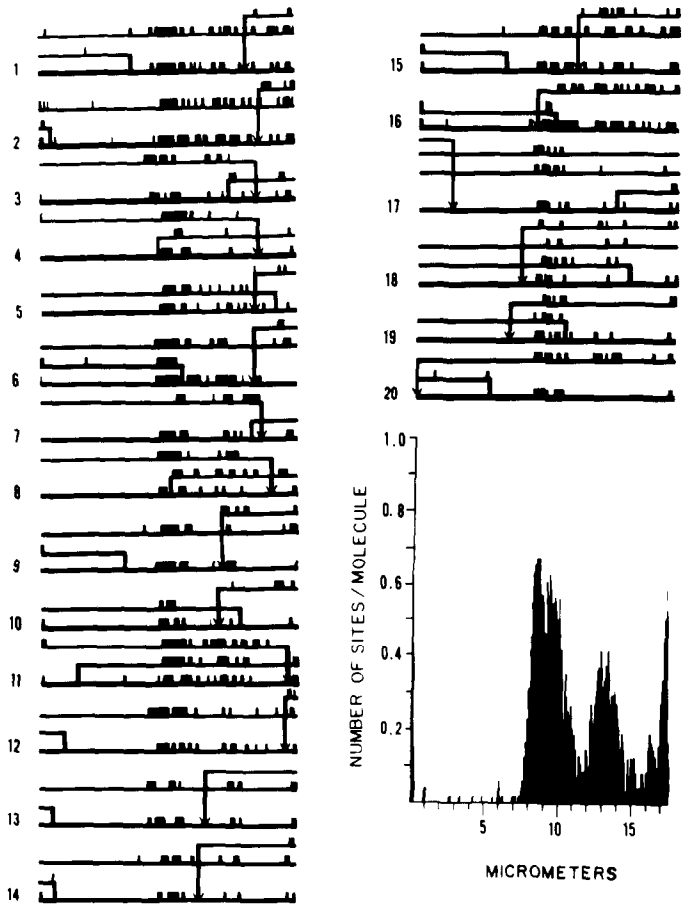


Figure 2. Electron microscopic denaturation maps of the 20 λ replicative structures with a long tail.

The circles are artificially broken at the cohesive sites based on the denaturation maps and are normalized to 17.5 μm, the average length of mature phage λ DNA. The attached tail segments have also been normalized. The denatured sites of the tail were then aligned with those on the circle. Branch point and free end are shown by vertical line and arrow respectively. Histogram shows the weight average of the circle with tail segment. The number of molecules used for this histogram was 53.

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had started in other regions. After the extraction of late stage of λ DNA replication, we observed predominantly a single-branched molecule. This would be explained either by a shift to a different replication process or as a result of recombination between circular and linear molecules. However, we have observed rolling-circle molecules even in a recombination deficient system (RecA$^-$B$^-$ x int$^-$,red$^-$,gam$^-$). (Data will be published elsewhere.) These

results indicate that the molecules which have a longer then genome length λ DNA would be generated if replication proceeded as in the rolling-circle model (12). Rolling-circle molecules observed in the present study rolled in either direction. This bidirectionality could be related to the mechanism of bidirectional replication of the first round of λ DNA replication.

ACKNOWLEDGMENT: We acknowledge Dr. R. Inman for his encouragement. The critical reading and suggestions on this manuscript contributed by Drs. R. Baldwin, W. Dove and A. Skalka are gratefully noted.

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