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The Rolling Circle for ϕX DNA Replication, II. Synthesis of Single-Stranded Circles*

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Abstract. ϕX-infected cells have been allowed to incorporate tritiated thymidine late in the phage life cycle when single-stranded circles are the product of DNA synthesis. Virtually all of the radioactivity is recovered in a continuum of actively replicating viral DNA molecules. These molecules are termed rolling circle intermediates because they are characterized by three structural properties. They possess positive strands that are longer than the length of a mature viral genome, and negative strands that are covalently closed single-stranded circles. The 3′ termini of the long positive strands lie upon the template rings, while the 5′ ends are free in solution.

From these experimental data, the basic mode of synthesis is deduced to involve the continuous elongation of the open positive strand by endless copying around the circular negative strand template. As new bases are added to the template-bound (3′) end of the positive strand, the distal (5′) end is displaced from the template ring as a single-stranded tail of increasing length. It is the tail which serves as the source of material for progeny chromosomes.

These data confirm our characterization of this ϕX intermediate, which initially was based only on the possession of long positive strands, and extend this characterization to include experimental statements about the circular nature of the template DNA strand, and the 5′ to 3′ direction of polynucleotide chain growth within the intermediate. Moreover, the description can now be applied to all of the molecules which acquire label during a pulse.

The replication of ϕX DNA involves a period of double-stranded circle synthesis followed by a period of single-stranded circle synthesis.[1] In the rolling circle model[2] (Fig. 1) an attempt has been made to explain both types of ϕX-circle synthesis under one unified mechanism. For each synthesis, the replicating DNA molecule is characterized by three properties: (1) the possession of one copy of the genetic information (+) in the form of a longer-than-unit-length polynucleotide strand, (2) the maintenance of the other copy of the genetic information (−) in the form of a covalently closed single-stranded circle, to be used as an endless template, and (3) the positioning of the long DNA strand so that its 3′-OH terminus lies upon tne template ring where it may be endlessly elongated by the Kornberg polymerase, or another enzyme with analogous properties.

This paper presents a set of experiments which show that ϕX single-stranded circles are in fact made by a replicating DNA molecule which has the three

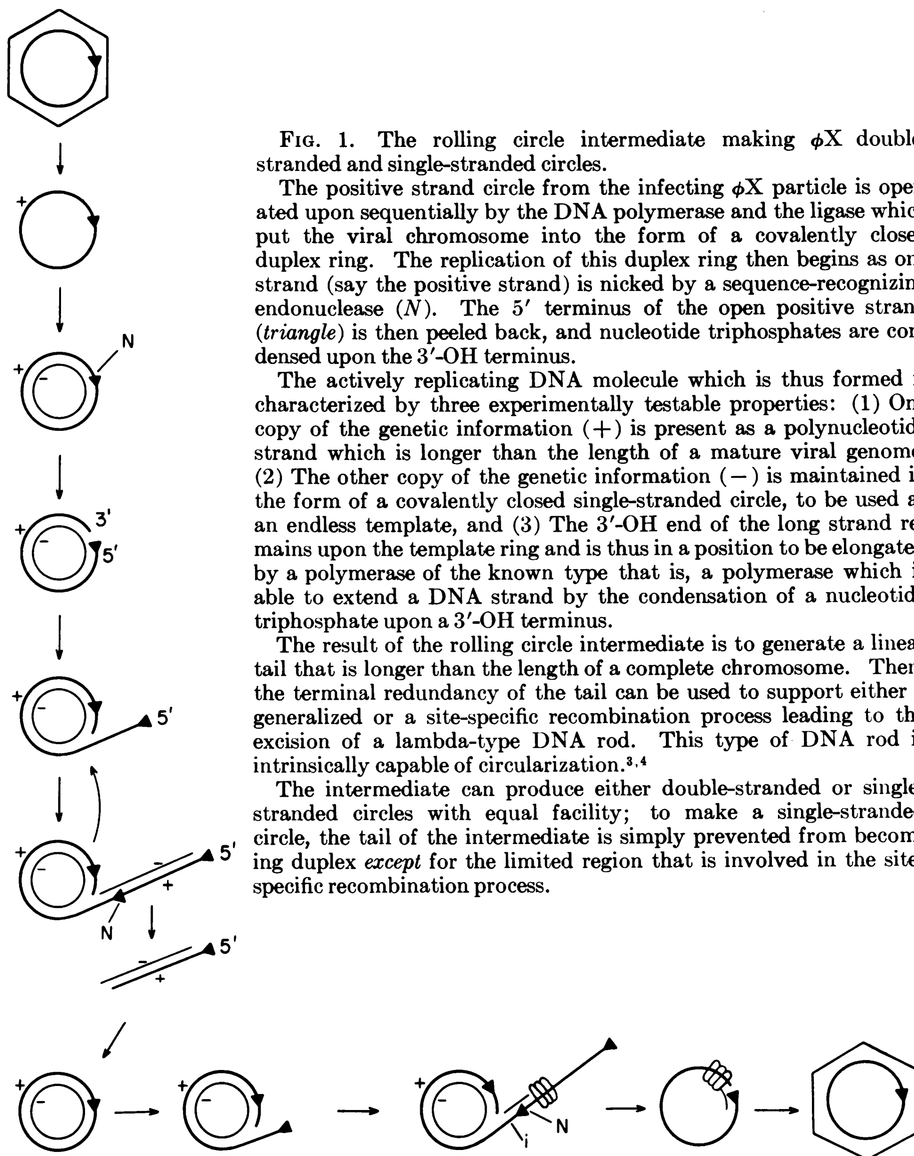

FIG. 1. The rolling circle intermediate making ϕX double-stranded and single-stranded circles.

The positive strand circle from the infecting ϕX particle is operated upon sequentially by the DNA polymerase and the ligase which put the viral chromosome into the form of a covalently closed duplex ring. The replication of this duplex ring then begins as one strand (say the positive strand) is nicked by a sequence-recognizing endonuclease (N). The 5′ terminus of the open positive strand (*triangle*) is then peeled back, and nucleotide triphosphates are condensed upon the 3′-OH terminus.

The actively replicating DNA molecule which is thus formed is characterized by three experimentally testable properties: (1) One copy of the genetic information (+) is present as a polynucleotide strand which is longer than the length of a mature viral genome, (2) The other copy of the genetic information (−) is maintained in the form of a covalently closed single-stranded circle, to be used as an endless template, and (3) The 3′-OH end of the long strand remains upon the template ring and is thus in a position to be elongated by a polymerase of the known type that is, a polymerase which is able to extend a DNA strand by the condensation of a nucleotide triphosphate upon a 3′-OH terminus.

The result of the rolling circle intermediate is to generate a linear tail that is longer than the length of a complete chromosome. Then, the terminal redundancy of the tail can be used to support either a generalized or a site-specific recombination process leading to the excision of a lambda-type DNA rod. This type of DNA rod is intrinsically capable of circularization.[3,4]

The intermediate can produce either double-stranded or single-stranded circles with equal facility; to make a single-stranded circle, the tail of the intermediate is simply prevented from becoming duplex *except* for the limited region that is involved in the site-specific recombination process.

structural properties expected for a rolling circle intermediate. These data extend our characterization of this replicating intermediate, which initially was based on its possession of long polynucleotide strands, to include statements about the nature of the template strand, and the direction of polynucleotide chain growth.

Labeling of ϕX Replicating Intermediates. The replication of ϕX DNA involves a period of double-stranded circle synthesis followed by a period of single-stranded circle synthesis.[1] We have studied the way in which ϕX single-stranded circles are made.

Cells that were accumulating ϕX at a normal rate following synchronized infection were allowed to incorporate [^{3}H]dT for 50 sec during the period of single-stranded circle synthesis.

After the pulse, the infected complexes were harvested and broken open with lysozyme and detergent. The unfractionated cell lysate was sedimented through a neutral velocity gradient, and the pulse-labeled DNA forms were recovered in a continuum of structures (Figure 2, fractions 18–42). The pulse-labeled DNA sedimented heterogeneously from 16 S, (the velocity of unit ϕX duplex rings) up to about 30 S (the velocity expected for relatively massive replicating intermediates).

That the pulse-label is contained in *viral* DNA forms is demonstrated by the DNA–DNA hybridization study of Fig. 2. The data show that the [^{3}H]dT

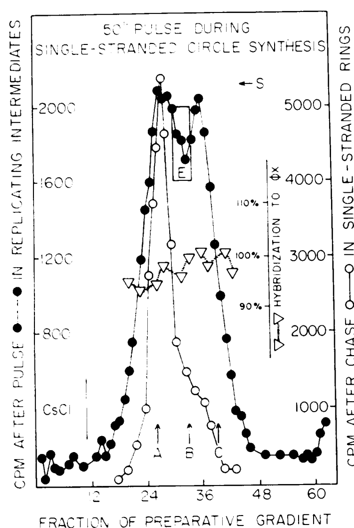

FIG. 2. Infection and pulse-labeling of intermediates. Cells were infected with ϕX and exposed to tritiated thymidine briefly during the period of single-stranded circle synthesis: *E. coli* strain HF 4704 (*hcr$^-$ thy$^-$*) was grown to a titer of 3 × 10^8/ml at 28°C in 100 ml of mT3XD medium.[5] During the final 20 min of growth, mitomycin C (Calbiochem) was present at 80 μg/ml. This antibiotic selectively inhibits host DNA synthesis while allowing a normal ϕX life cycle.[5,11] The cells were washed, resuspended in 25 ml of holding buffer[12] containing mitomycin, starved for 40 min, and then infected at a multipilcity of 2 with ϕX am3, a mutant that cannot lyse the host cell. After 15 min, when more than 99% of the phage had eclipsed, an equal volume of double-strength m3XD (containing 1 μg/ml of thymine) was added to initiate phage growth.

50 min later the infected cells were maturing 4 ϕX/cell per min, indicating a normal infection. At this time, [^{3}H]dT was added to the culture (Schwartz Bioresearch, Orangberg, N. Y., 5 mCi, 16 Ci/mmole). 50 sec after the addition of label, half of the culture was pipetted directly into an equal volume of acetone at −70°C to stop incorporation. The other half of the culture was allowed to continue incorporation for an additional 10 min in the presence of a thousand-fold excess of nonradioactive thymidine.

The cultures were harvested by centrifugation, washed, and resuspended at 4 × 10^9/ml in lysis buffer. (100 mM Tris(pH8)– 100 mM NaCl–10 mM KCN–10 mM Iodoacetate–1 mM EDTA). The infected complexes were then broken open with lysozyme (400 μg/ml, 37°C, 20 min) and detergent (2% sarkosyl, 65°C, 20 min) and, lastly, exposed to self-digested pronase (1 mg/ml, 4 hr, 37°C).

The unfractionated cell lysates were sedimented through preparative neutral velocity gradients (5–20% sucrose, 0.5 M NaCl, 1 mM EDTA, 0.1% sodium lauroyl sarcosinate in 0.05 M Tris, pH 8; underlaid with a saturated CsCl–sucrose cushion). Centrifugation was at 24,000 rpm for 17 hr at 8°C in the Beckman SW-25.1 rotor.

The solid dots show the distribution of [^{3}H]dT after the pulse, the open dots show the distribution after the chase. Although part of the pulse-label is sedimenting in the 27 S position characteristic of single-stranded circles, this label is actually present in rolling circle intermediates with long tails (see Fig. 3*A*).

To show that the pulse-label is present in ϕX positive strand base sequences, aliquots of each fraction were denatured, reneutralized, and hybridized[5] to membrane filters containing 1) immobilized positive and negative strands from ϕX duplex rings, or 2) only ϕX positive strands. The pulse-labeled strands hybridized with almost 100% efficiency to the filters which contained both ϕX positive and negative strands (*triangles*), but not at all (< 2%) to the filters which contained only positive strands (not shown).

has been incorporated entirely into ϕX DNA, not *Escherichia coli* DNA. And, as expected since labeling was carried out during the period of single-stranded circle synthesis, the replicating structures have incorporated radioactivity almost exclusively into positive strand base sequences.

The acceptance of the pulse-labeled structures as replicating *intermediates* is based on the observation that when the 50-sec pulse was followed by a 10-min exposure to nonradioactive thymidine, label was chased out of the heterogenously-sedimenting structures and quantitatively reappeared in the 27 S position characteristic of single-stranded circles (Fig. 2, fractions 24–30).

Long Positive Strands. Fig. 3 shows that the replicating intermediates contain, as one component, positive strands that are longer than unit length. Intermediates of various sizes were recovered from areas *A*, *B*, and *C* of Fig. 2, and denatured into their component polynucleotide strands with alkali. The released strands were then sedimented through secondary neutral velocity gradients, together with a marker for the position of unit length genomes.

Fig. 3*C* shows that the slowest sedimenting intermediates, upon alkaline denaturation, yielded pulse-labeled positive strands that were near unit length or slightly longer. In contrast the faster-sedimenting intermediates, representing over 90% of the pulse-labeled structures of Fig. 2, released radioactive positive strands which sedimented in advance of the marker (fractions 28–30) and were thus judged to be longer than unit length.

The lengths of the longest pulse-labeled positive strands can be estimated from the empirical finding of Studier[6] that a flexible polynucleotide chain in a

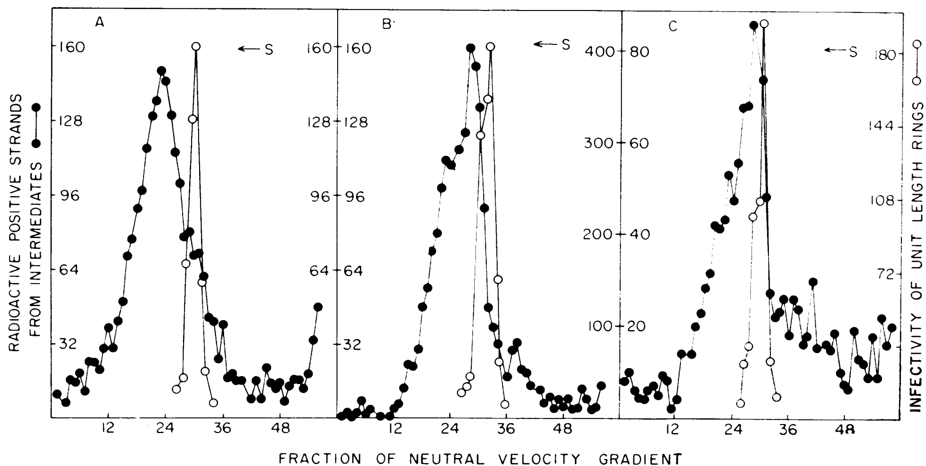

FIG. 3. Long positive strands. Pulse-labeled replicating intermediates were recovered from areas *A*, *B*, and *C* of the gradient shown in Fig. 2, and denatured into their component polynucleotide strands with 0.25 M NaOH. After 4 min at 37°C the solutions were reneutralized with HCl. The single strands from the intermediates were then sedimented through secondary neutral velocity gradients (10–30% sucrose, 1 M NaCl, 1 mM EDTA, 0.1% sodium lauroyl sarcosinate; for 2.5 hr. 64,000 rpm, 8°C, in the Beckman SW-65 rotor).

Alternate fractions of the gradients were assayed for radioactivity (representing the pulse-labeled positive strands from the intermediates) and for infectivity to spheroplasts[7] of marker ϕXh4 positive strand circles (which had been added prior to alkaline denaturation to define the sedimentation velocity of unit-length ϕX strands).

neutral pH, high ionic strength gradient will sediment 49% faster as its mass increases from m to 2 m. Thus, while a unit length single-stranded rod (or ring) sediments at 27 S, a double-length single strand is expected to sediment at 40 S. 40 S, in fact, is the velocity of the fastest sedimenting strands from the most massive replicating intermediates (Fig. 3*A*, fractions 12–15).

Circular negative strand templates: The long positive strands of the intermediates were readily seen (Fig. 3) because they acquired label during a pulse. However, the negative strand templates from which they were synthesized are unlabeled and thus somewhat more difficult to detect. To account for the synthesis of the long positive strands, we would expect that the negative templates must be redundant; but, this could be achieved either with DNA strands that are longer-than-unit-length or circular. In the event that the negative templates are circular, they should be visible by virtue of their infectivity to spheroplasts.

Pulse-labeled ϕX replicating intermediates were obtained from region *B* of Fig. 2 and, after repeated purification by sedimentation, denatured into their component polynucleotide strands with alkali. Fig. 4*A* displays the sedimentation profiles of both the radioactive *and* the infective strands from the replicating

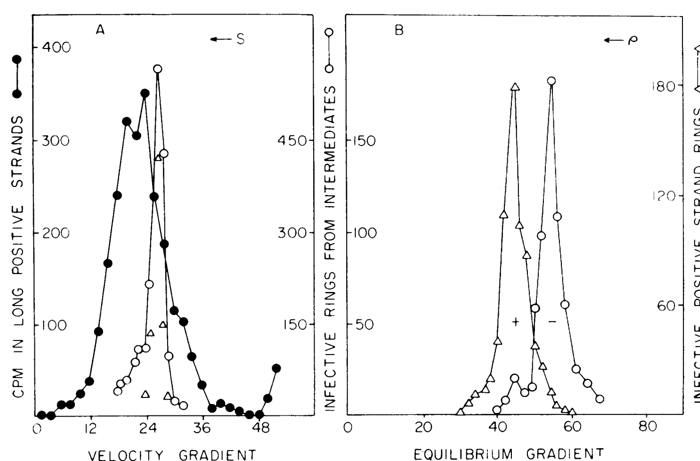

FIG. 4. Circular negative strands. (*A*) Replicating intermediates were recovered from area *B* of Fig. 2 and denatured with alkali. The released single strands were then sedimented through a neutral velocity gradient. The pulse-labeled positive strands (solid dots) are seen sedimenting ahead of unit-length ϕX strands. The unit length position is marked by two kinds of infective single-stranded circles: marker ϕXh4 positive strand rings, and ϕX am-3 rings, derived from the intermediates. (*B*) The infective single-stranded circles (both those from the intermediates and also the marker positive strand rings) were recovered from fractions 25–28 (*A*) and centrifuged to equilibrium in alkaline CsCl. The material was taken up to 2500 μl with Na_3PO_4 containing 1 mM EDTA and 5 μg of denatured lambda phage DNA. 3.325 g of CsCl was added and the solution was centrifuged at 40,000 rpm for 60 hr at 17°C in the angle 60 rotor of the IEC B60 centrifuge.

Gradient fractions were assayed for the ability to infect spheroplasts and produce either am-3 phage (representing the rings from the replicating intermediates) or ϕXh4 phage (representing the marker positive strand circles). The rings from the intermediates separated from the positive strand circles and came to equilibrium in the negative strand position, (*B*, fractions 50–60).

intermediates. In addition to the long pulse-labeled positive strands (Fig. 4*A*, fractions 12–24) the denatured intermediates have indeed released an infective component; this is responsible for the ability of fractions 25–28 to generate ϕX am3 phage particles upon incubation with *E. coli* spheroplasts. The infective component from the intermediate is taken to be a single-stranded ring because it sediments with precisely the same velocity as the marker positive strand circles (which are identified in the spheroplast assay by their production of ϕX h4 phage).

The infective single-stranded circles were recovered from fractions 25–28 of Fig. 4*A*. They were then centrifuged to equilibrium in alkaline CsCl to determine whether those corresponding to the genotype of the replicating intermediates would form a single infectivity peak at the negative strand position.

In alkaline CsCl, as determined by Vinograd, Morris, Davidson, and Dove, ϕX positive and negative strands separate from each other and band at two different densities,[8] since the ϕX positive strand contains more thymine and guanine residues than the negative; above pH 12.5 the ring protons of these residues are titrated off and replaced by density-enhancing Cs^+ ions.

When the fractions of the alkaline CsCl gradient were assayed with spheroplasts for the ability to produce phage of the marker and experimental genotypes, it was found that the single-stranded circles from the intermediates had in fact separated from the marker positive strand circles. The infectivity corresponding to the genotype of the replicating intermediates had peaked in the negative-strand position (Fig. 4*B*, fractions 50–60).

In several experiments, the number of long positive strands (as judged by the radioactivity, content of fractions 12–24 of Fig. 4*A*) was compared to the number of negative strand circles (as judged by the amount of infectivity, in fractions 25–28 of Fig. 4*A*). In each case, the replicating structures contained equal numbers of long positive strands and negative template rings within a factor of two.

The 3′-OH end of the long strand is the growing end: The data of the previous two sections has shown that the replicating intermediate contains two components: a long positive strand and a circular negative strand. If the long positive strand is growing by chain elongation around the negative template ring, then replication must proceed with the displacement of one end of the long strand from the template. Which end of the long stand is displaced as a result of synthesis, and which retains its association with the template ring, can be determined by exposing the pulse-labeled replicating intermediates to exonuclease III.

Exonuclease III, as characterized by Richardson, Lehman, and Kornberg, is a highly specific nuclease that will depolymerize a polynucleotide strand from its 3′ terminus if, and only if, that terminus is double-stranded.[9] Thus, only if the long, pulse-labeled, positive strand of the intermediate has its 3′ terminus positioned upon the negative template ring will the enzyme be able to shorten the strand and release acid-soluble mononucleotides.

Fig. 5 shows the results of exposing pulse-labeled replicating intermediates to exonuclease III. The native intermediates were incubated with exonuclease

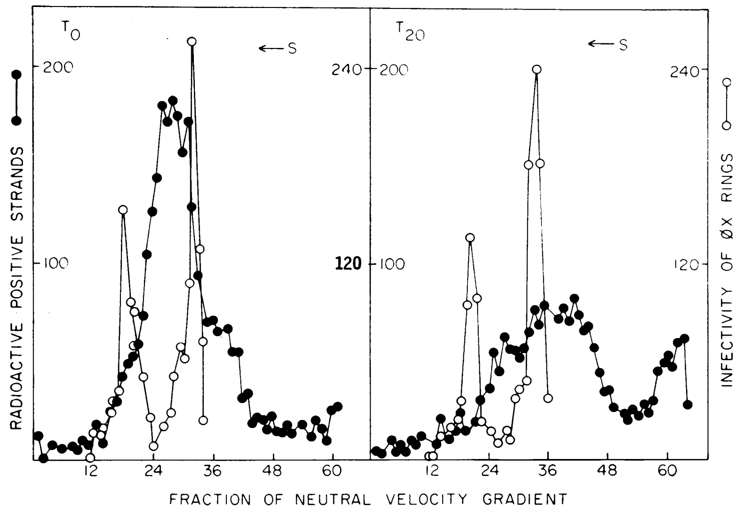

FIG. 5. The 3′ end of the long strand lies upon the template ring. Pulse-labeled intermediates were obtained from a preparative velocity gradient, identical to that shown in Fig. 2, and were diluted into a buffer for treatment with exonuclease III (70 mM Tris, (pH 8)–0.7 mM $MgCl_2$–10 mM mercaptoethanol).

ϕX positive strand rings and supercoils were added to the solution, then exonuclease III (the phosphocellulose fraction, from Drs. William Reznikoff and Charles A. Thomas, Jr.). The mixture was incubated at 37°C; aliquots were withdrawn at 0 and 20 min.

Enzymic digestion was stopped by the addition of sodium dodecyl sulfate to 0.5%, followed by alkaline denaturation of the sample (0.25 M NaOH). After reneutralization, the remaining DNA strands were sedimented through neutral velocity gradients.

Solid dots: radioactive positive strands from the pulse-labeled intermediates before digestion (*A*) and after digestion (*B*).

Open dots: infective supercoils (fractions 18–21) and single-stranded circles (fractions 32–35) present before and after exonuclease digestion.

for either 0 or 20 min, and then denatured. Their remaining polynucleotide strands were sedimented through neutral velocity gradients. Fig. 5*A* shows the sedimentation profile of the denatured reaction mixture after *zero minutes* of enzyme treatment. Pulse-labeled positive strands sediment in a distribution between unit length and twice-unit-length rods, the positions of which are defined by the infectivity to spheroplasts of marker single-stranded circles (27 S) and supercoils (40 S) present in the reaction mixture. Fig. 5*B* shows the effect of a limited (20 min) exposure of the intermediates to the enzyme: the long positive strands have been shortened, partially or completely. This result indicates that the long positive strands of the intermediates do in fact have their 3′ termini positioned upon the negative ring templates.

An important control is represented by the equal number of infective single-stranded circles and supercoils present at the beginning of enzyme digestion (Fig. 5*A*, *open dots*) and at the end (Fig. 5*B*, *open dots*). The fact that these infective forms were not destroyed indicates that during the exonuclease III digestion there was no significant nicking activity directed against either single-stranded or double-stranded DNA. Such nicks would lead to shortened positive strands, or to the creation of 3′-OH targets for exonuclease III attack in substrates that would otherwise be inert.

Electron microscopy: A collaboration was undertaken with Dr. Lorne MacHattie to process for the electron microscope several of the same preparations of replicating intermediates which had been analyzed by physcial chemical methods. Since the replicating intermediates were expected to contain single-stranded regions, the pulse-labeled structures were processed by the Westmoreland adaptation[10] of the Kleinschmidt technique. This procedure renders single-stranded DNA visible, though thinner and less rigid than duplex DNA.

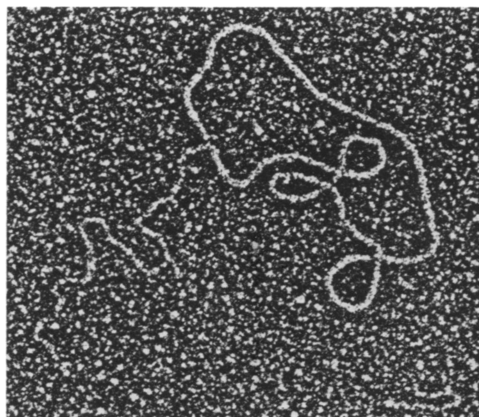

FIG. 6. A ϕX rolling circle.

In preparations of pulse-labeled intermediates, double-stranded circles with single-stranded tails were often seen (Fig. 6). Characteristically, about 20–40% of the viral structures isolated from the replicating intermediate region of a preparative velocity gradient (such as Fig. 2, area *E*) had this configuration. The remaining viral structures were nicked or supercoiled ϕX duplex rings, present in essentially equal numbers.

Discussion. This paper shows that nascent positive strand material for progeny ϕX chromosomes first appears in greater-than-genome-length polynucleotide strands. These strands are associated with, and presumably generated by, continuous copying around circular negative strand templates. In this synthesis, the 5′ terminus of the positive strand is displaced from the template ring as the 3′ end is simultaneously elongated. These results correspond to the rolling circle description of ϕX DNA synthesis. The properties of this actively replicating DNA molecule have also been studied by Knippers, Komano, Razin, Davis, and Sinsheimer for ϕX[13,14] and by Ray[15] and Wirtz and Hofschneider[16] for M13. Their findings are in close agreement with ours.

The DNA synthesis which occurs during bacterial mating provides another instance of replication that can be explained in terms of the rolling circle intermediate. When bacteria mate, a single preexisting strand of the male chromosome is detached and transferred, 5′-end first, to the female recipient. Simultaneously, a new copy of the transferred strand is synthesized and retained in the male. Because males can, during prolonged mating, transfer a second, linked set of markers to the female, it appears likely that the synthesis involves continuous copying around a circular template. These experiments and concepts have been established by Rupp, Ihler, Ohki, Tomizawa, and Fulton.[17–19]

Several systems which make double stranded DNA also appear to involve a rolling circle intermediate. Schnöss and Inman[20] have obtained electron microscopic evidence that the replication of phage P2 DNA proceeds via an intermediate which consists of a duplex ring with an attached double-stranded tail. Furthermore, studies of the late life cycle of phage lambda by Smith and Skalka[21] and Kiger and Sinsheimer[22] have yielded evidence for catomeric replicating

structures which may be duplex rings with double-stranded tails. Moreover, rolling circle structures have occasionally been observed among the replicating DNA of polyoma,[23] colicin factors,[24] and plasmids.[25]

For several other organisms studied thus far, however, the more frequently observed conformation for the replicating chromosome is the double-forked circle of the type originally found for *E. coli* by Cairns.[26] Hopefully, these two types of configurations for replicating DNA, and the different modes of replication which they imply, may be reconciled within a larger framework, but this remains for future work.

It is a privilege to acknowledge the collaboration of Walter Gilbert in this work, also, I am grateful to Dr. Lorne MacHattie for the application of his skill in electron microscopy. John Cairns and James Watson offered thoughtful readings of the manuscript, after John Wolfson had provided persistent encouragement for its preparation.

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* Paper I in this series, Gilbert and Dressler,[2] discusses the general properties and applicability of the rolling circle model. Paper III is Dressler and Wolfson (*Proc. Nat. Acad. Sci. USA*, **67**, 456 (1970)) and presents experiments in support of the rolling circle mechanism for the synthesis of ϕX *duplex* rings.

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