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Mechanism of Replication of Single-Stranded φX174 DNA

VII. Circularization of the Progeny Viral Strand

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Linear φX174 single-stranded DNA can be isolated from φX phage particles produced under various conditions. About half of the linear strands have a dGMP residue at the 5′ end, the remaining have roughly comparable amounts of dCMP, dTMP, and dAMP. The linear strands can be converted to covalently closed circular molecules by polynucleotide ligase, but only after they have been incubated with T4 DNA polymerase and deoxynucleoside triphosphates. Experiments with endonuclease R, the restriction enzyme from *Haemophilus influenzae*, indicated that the nucleotides incorporated into the DNA during this reaction were found predominantly in a limited region of the genome. The results suggest that the normal intermediate in single-stranded φX174 DNA synthesis may be a single-stranded linear molecule which is shorter than unit length and is intrinsically capable of circularization.

There are three stages in the replication of φX174 DNA: (i) the penetration of the single-stranded (SS) DNA into the cell and its conversion to the double-stranded replicative form (RF) DNA; (ii) the semiconservative replication of the RF molecules to generate a pool of some 10 to 50 RF molecules in the cell; (iii) the formation of progeny single-stranded DNA by an asymmetric replication of the RF (3, 16). During this third stage a covalently closed, circular SS DNA molecule is produced, and it is this event with which we are concerned here.

The synthesis of progeny SS DNA is known to require five viral-coded proteins, three of which are found in the mature virion (10) and to proceed via a linear intermediate which is infectious in spheroplasts (25, 26). The fact that linear SS DNA can be infectious suggests that there exists a mechanism, independent of the events involved in the actual synthesis of the molecule, for bringing the two ends together to form a circle, but the process used to bring the two ends together is not understood. In this work we present evidence that the structure of the linear strand enables the two ends of the molecule to hydrogen-bond to adjacent interior regions of the SS DNA molecule in a manner so that reaction with a DNA polymerase and polynucleotide ligase can join them. It is possible that this is the normal mechanism for circularizing a linear single-stranded DNA molecule.

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MATERIALS AND METHODS

Organisms. *Escherichia coli* C, φX174, and standard procedures for working with φX have been described (2). *E. coli tau-bar ts7* was isolated and characterized by Pauling and Hamm (18, 19). The lysis-defective amber mutant φX*am3* was isolated and characterized by Hutchison and Sinsheimer (9).

Enzyme reactions. Bacterial alkaline phosphatase (EC 3.1.3.1, Worthington, BAPC) was further purified on DEAE cellulose (31). Reactions were performed at 65 C for a total of 20 min; phosphatase was added to a concentration of 2 U/ml at 0 and 10 min. Deoxyribonuclease I (EC 3.1.4.5, Worthington, DPFF) was dissolved in 50 mM Tris-hydrochloride (pH 8) at 2 mg/ml and used at a concentration of 0.3 mg/ml for 2 h at 37 C with 5 mM $MgSO_4$. Venom phosphodiesterase (EC 3.1.4.1, Worthington, VPH) was similarly dissolved and added to the DNase I-digested sample at 180 μg/ml together with 0.5 mg each of dTMP, dCMP, dGMP, and dAMP per ml, and the reaction was continued at 37 C for 2 h more. Endonuclease R was purified from *Haemophilus influenzae* by the method of Smith and Wilcox (28), except that fractionation on an agarose column was replaced by a streptomycin precipitation and enzyme activity was monitored by the change in character of an alcohol precipitate of high-molecular-weight DNA (N. Axelrod, personal communication). Reactions with the nuclease and electrophoresis in acrylamide-

agarose gels were performed as described by Edgell et al. (4). T4 polynucleotide kinase (22), T4 polynucleotide ligase (32), T4 DNA polymerase (8), and *E. coli* DNA polymerase I (12) were purified and used as described in the references given. Details of these preparations will be published elsewhere. Gamma-^{32}P-ATP and alpha-^{32}P-dCTP were prepared and purified by the method of Glynn and Chappell (7) and a modified procedure of Symons (30; R. Schekman personal communication), respectively.

Thin-layer chromatography. Two-dimensional chromatography on PEI plates (Polygram Cell 300 PEI, Brinkman) was performed as described by Randerath and Randerath (21). The first dimension was developed with 1 M LiCl and the second dimension with a 9:1 mixture (vol/vol) of 1 M acetic acid and 3 M LiCl. Nucleotide spots were detected under UV illumination. The spots were excised and placed in scintillation vials; the nucleotides were eluted with 1 ml of 1 M NH_4HCO_3 and the radioactivity was determined with 10 ml of Aquasol (New England Nuclear) in a scintillation counter (Intertechnique SL36).

Centrifugation. Low-salt, alkaline sucrose gradients (linear) contained 0.1 M NaOH, 1 mM EDTA, and 5% to 20% sucrose. The sample to be centrifuged was denatured with 0.25 M NaOH and combined with 25 μg of carrier calf thymus DNA. Centrifugation in the Beckman SW50.1 rotor was for 5 h at 50,000 rpm and 5 C. Neutral sucrose gradients contained 1 M NaCl, 50 mM Tris-hydrochloride, pH 8, 1 mM EDTA, and 5% to 20% sucrose. Fractions of 0.1 ml were collected through a hole punched in the bottom of the tube and assayed for radioactivity on paper (11). Sedimentation in all gradients is toward the left.

Reagents. Borate is 50 mM sodium tetraborate. All Tris buffers were adjusted at room temperature to a pH of 8 with HCl.

Preparation of linear strands. *E. coli ts7* was grown in 10 liters of TKCaB (1) at 30 C in a 14-liter New Brunswick magnaferm fermentor. When the cells reached about 3×10^8 to 4×10^8 cells/ml the temperature was raised to 37 C. After 30 min at 37 C the cells were infected with φX*am*3 at a multiplicity of infection of 10; at the same time 1 mCi of ^{3}H-thymidine was added. The infection was allowed to proceed at 37 C with vigorous aeration for 150 min. The cells were cooled and collected in a Sharples continuous flow centrifuge. The pellet was resuspended in 100 ml of 50 mM Tris-hydrochloride, pH 8, and homogenized with a Teflon homogenizer. To the suspension was added 600 ml of 50 mM Tris-hydrochloride-20 mM EDTA (pH 8) and 20 ml of an 8 mg/ml lysozyme solution. The cells were stirred gently at 4 C for several hours and then vigorously for a few minutes to reduce the viscosity. The debris was removed by centrifugation for 30 min at 10,000 rpm in the Sorvall GSA rotor. The supernatant fluid was centrifuged for 3 h at 35,000 rpm in the type 42 Beckman rotor at 4 C, and the resulting pellet was suspended in 70 ml of borate, homogenized, and stirred gently for 24 h. Debris was again removed by centrifugation in the Sorvall SS34 rotor for 20 min at 10,000 rpm, and then the supernatant fluid was centrifuged in the type 42 rotor as above. The pellet was then resuspended in 15 ml of borate, homogenized, and stirred gently for 24 h at 4 C. The debris was removed in the SS34 rotor as above and then the supernatant fluid was banded in CsCl (0.625 g per g of solution) in the type 60 rotor for 27 h at 33,000 rpm. The phage band was collected and dialyzed against 50 mM borate-5mM EDTA. The equilibrium CsCl density gradient centrifugation and dialysis were repeated once.

The DNA was extracted from the particles with an equal volume of phenol in the presence of 1% sodium dodecyl sulfate; the phenol had been redistilled, stored under argon, and equilibrated with borate prior to use (24). The first extraction was performed at 37 C for 30 min with gentle agitation. The phases were separated, the aqueous phase was similarly re-extracted with an equal volume of phenol, and then both phenol phases were extracted with an equal volume of borate at 55 C. The aqueous phases were pooled and the DNA was precipitated with 2 vol of isopropanol and 0.1 vol of 3 M sodium acetate (pH 5.5). The precipitated SS DNA was dissolved in 50 mM Tris-hydrochloride-20 mM EDTA (pH 8) and centrifuged on 34-ml neutral sucrose gradients in the SB110 rotor of the IEC B60 ultracentrifuge (22,000 rpm, 10 C, 14 h). Fractions of 1 ml were collected and assayed, and the SS DNA-containing fractions were pooled and precipitated with isopropanol-sodium acetate.

Circularization of the linear strands. Linear strands with a 5′ terminal phosphate were incubated with 50 mM Tris-hydrochloride (pH 8), 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μM each of dCTP, dTTP, dGTP, ATP, and dATP, 2 μliters of ligase, and 2 μliters of T4 DNA polymerase in a 20- to 40-μliter volume containing about 1 μg DNA. When labeling the DNA with ^{32}P-dCTP, the reaction (150 μliters) contained in addition 14 μM dCTP at 10 mCi/μmol, 4 μliters of T4 DNA polymerase, 10 μliters of T4 ligase, and 10 mM $(NH_4)_2SO_4$. Reactions were performed at 37 C for 4 h.

RESULTS

Presence of linear strands in phage particles. *E. coli ts7* is a temperature-sensitive mutant of *E. coli* tau-bar (18) that contains a temperature-sensitive polynucleotide ligase (E. B. Konrad, P. Modrich, and I. R. Lehman, in press; 17). The properties of this mutant are consistent with the idea that it has a reduced amount of ligase activity in vivo, at the permissive as well as at the restrictive temperature (18). Nevertheless, φX is able to replicate in this mutant at the high temperature (24, 25) although the yield of infective progeny phage is reduced in proportion to the length of time the cells have been exposed to the high temperature (D. T. Denhardt, unpublished data).

In a population of phage particles synthesized in the mutant at the restrictive temperature a greater proportion of the phage DNA is found in

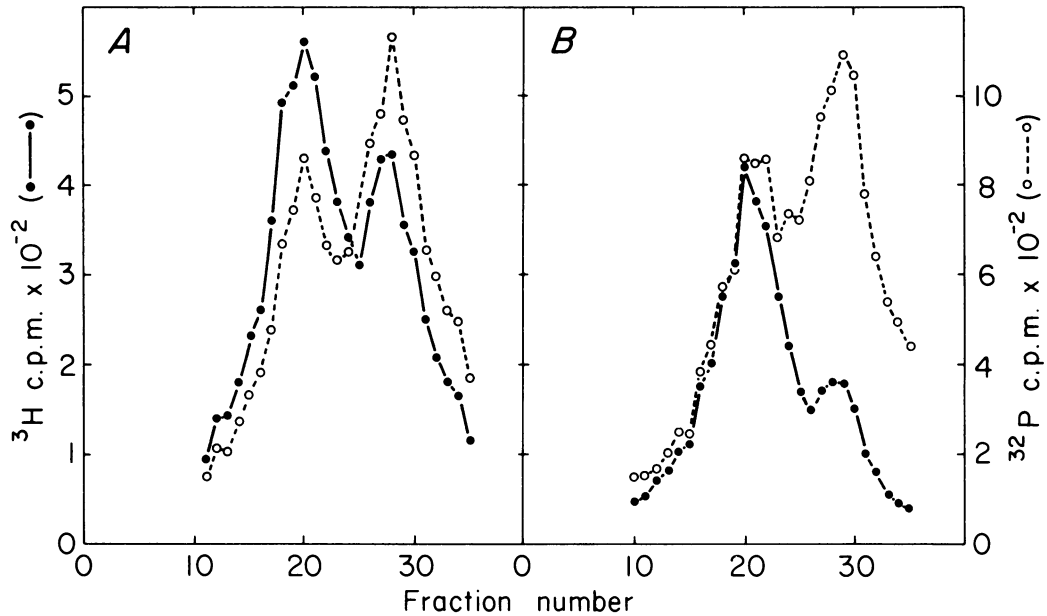

FIG. 1. *Presence of linear strands in phage particles. Cultures of infected cells were labeled for 10 min during SS DNA synthesis with ^{3}H-thymidine. Phage particles were purified from the cells, and the SS DNA was extracted from them with phenol-sodium dodecyl sulfate. The ^{3}H-labeled viral DNA (●——●) was then centrifuged on a low-salt alkaline sucrose gradient with ^{32}P-labeled (○----○) circular and linear strands as markers. The faster-sedimenting circular molecules are around fraction 20. A, DNA from phage particles synthesized in E. coli ts7 at 37 C. B, DNA from particles synthesized in E. coli C.*

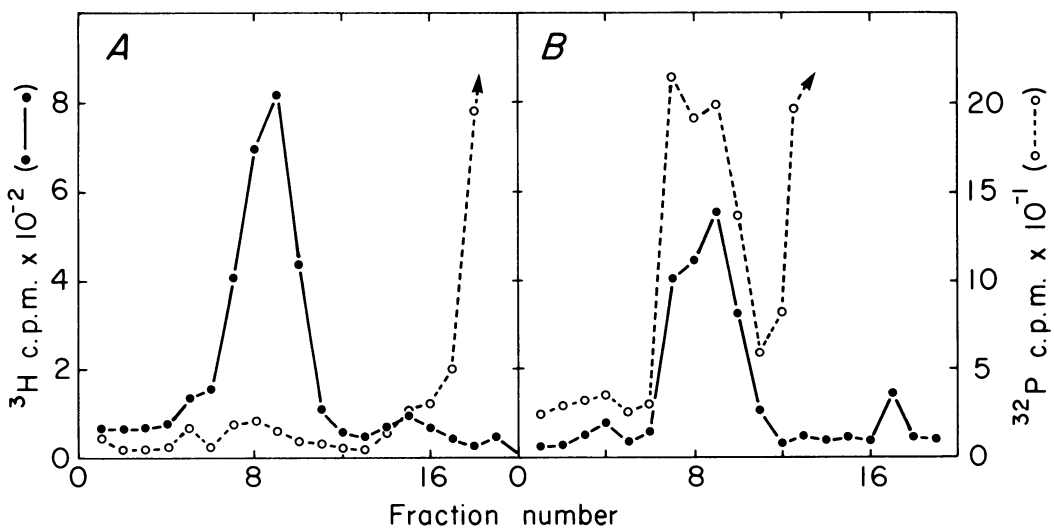

FIG. 2. *Presence of a 5'-terminal phosphate on the linear strands. ^{3}H-labeled linear strands from ϕX particles synthesized in the ligase-deficient host at 37 C were prepared as described in Materials and Methods. One portion was treated with phosphatase, and the DNA was separated from the phosphatase by banding in CsCl. Both phosphatase-treated and untreated samples were then incubated with polynucleotide kinase and gamma-^{32}P-ATP and centrifuged on a neutral sucrose gradient for 3 h at 50,000 rpm. A, – Phosphatase; B, plus phosphatase. Symbols: ●——●, ^{3}H; ○----○, ^{32}P.*

a linear form compared to phage synthesized in wild-type cells. An experiment showing this is presented in Fig. 1. For the experiment in panel A the cells were grown at 30 C and then shifted to 37 C at the time of infection with φX*am*3 (lysis-defective); 150 min later ^{3}H-thymidine was added for 10 min. The cells were disrupted, the phage particles were purified, and the viral DNA was extracted. The SS DNA was then centrifuged on a low-salt, alkaline sucrose gradient with marker DNA. The distribution of the radioactivity in this gradient (Fig. 1A) shows that about 40% of the ^{3}H is in linear strands and 60% in the faster-sedimenting circular strands. Similar observations have been made by Schekman and Ray (25).

Figure 1B shows that linear strands are also found in phage particles synthesized in *E. coli* C. However, in the wild-type cell a smaller proportion of the total viral DNA is found in linear strands, and it is not clear whether these linear strands are equivalent to the intermediate found in the ligase-defective host or whether they are the result of adventitious breakage of the DNA during extraction and isolation, or both.

Circularization of the linear strands. To facilitate the exploration of a variety of conditions for obtaining circularization of the linear strands, the following procedure was adopted. Initially it was determined that the 5′ end was phosphorylated by comparing the amount of ^{32}P incorporated into the linear strands by polynucleotide kinase and gamma-^{32}P-ATP before and after treatment of the linear strands with alkaline phosphatase. Fig. 2 shows that non-phosphatase-treated linear strands (Fig. 2A) are a very poor substrate for the polynucleotide kinase in comparison to phosphatase-treated (Fig. 2B) strands. Therefore, linear strands pretreated with phosphatase were incubated with polynucleotide kinase and gamma-^{32}P-ATP and purified by sedimentation velocity centrifugation on neutral sucrose gradients. The linear strands carrying ^{32}P on their 5′ ends were subsequently incubated under various conditions with polynucleotide ligase, and, after the ligase reaction, the DNA was again exposed to phosphatase to determine whether any ^{32}P had become resistant to the action of the phosphatase. Resistance to phosphatase would be expected if circularization had occurred, but would not in itself prove circularization. The data presented in Table 1 indicate that only in reactions which contained T4 DNA polymerase as well as ligase did some of the terminal ^{32}P phosphate become phosphatase resistant.

To prove circularization, the ^{32}P-labeled DNA was centrifuged on an alkaline sucrose gradient before and after treatment with phosphatase. These gradients are presented in Fig. 3. Figure 3A shows that prior to phosphatase-treatment most of the ^{32}P is in approximately unit-length, linear strands; Fig. 3B shows that after phosphatase treatment the phosphatase-resistant ^{32}P sediments predominantly in the same position as circular strands. This experiment shows that linear strands isolated from particles produced in the ligase-deficient host are intrinsically capable of becoming covalently closed circular SS DNA, but only after reaction with a DNA polymerase as well as with polynucleotide ligase.

Analysis of the 5′ terminus of the linear DNA. The linear strands were labeled with a 5′ terminal ^{32}P as previously described and digested with pancreatic DNase I and venom phosphodiesterase, and the resulting mononucleotides were chromatographed in two dimensions on a thin-layer PEI plate as described in Materials and Methods. The mononucleotides

TABLE 1. *Conversion of the terminal 5′-^{32}P-phosphate to a phosphatase-resistant form*

Incubation Mixture	Expt 1[a]		Expt 2[a]	
	^{3}H	^{32}P	^{3}H	^{32}P
Complete[b]	2,451	325	1,970	147
−Ligase-T4 DNA polymerase	2,698	42	1,930	11
−Ligase			1,794	59
−T4 DNA polymerase	2,400	21	1,960	19
−DNA			50	14
−Phosphatase	2,648	1,830	2,350	1,125

[a] The ligase-polymerase reaction was incubated for 5 h in experiment 1 and 24 h in experiment 2. After the phosphatase reaction 25 μliters of DNA (1 mg/ml) in 0.1 M EDTA (pH 7), 0.5 ml of water, and 0.5 ml of 10% trichloroacetic acid were added consecutively at 0 C. The trichloroacetic acid precipitates were collected on glass fiber filters and washed with four 3-ml portions of 5% trichloroacetic acid and three 3-ml portions of cold ethanol. The filters were dried, and the amount of ^{3}H and ^{32}P was determined in a scintillation spectrometer and measured in counts per minute.

[b] The complete reaction mixture contained salts and enzymes as described in Materials and Methods. After incubation with ligase and polymerase at 25 C, 30 μliters of Tris-hydrochloride (50 mM, pH 8) and 5 μliters of bacterial alkaline phosphatase were added and the mixture was incubated at 65 C for 15 min. In experiment 2 a second 5-μliter portion of phosphatase was added and a second 15-min incubation was performed.

[c] The preparation of T4 DNA polymerase used contains a small amount of polynucleotide ligase.

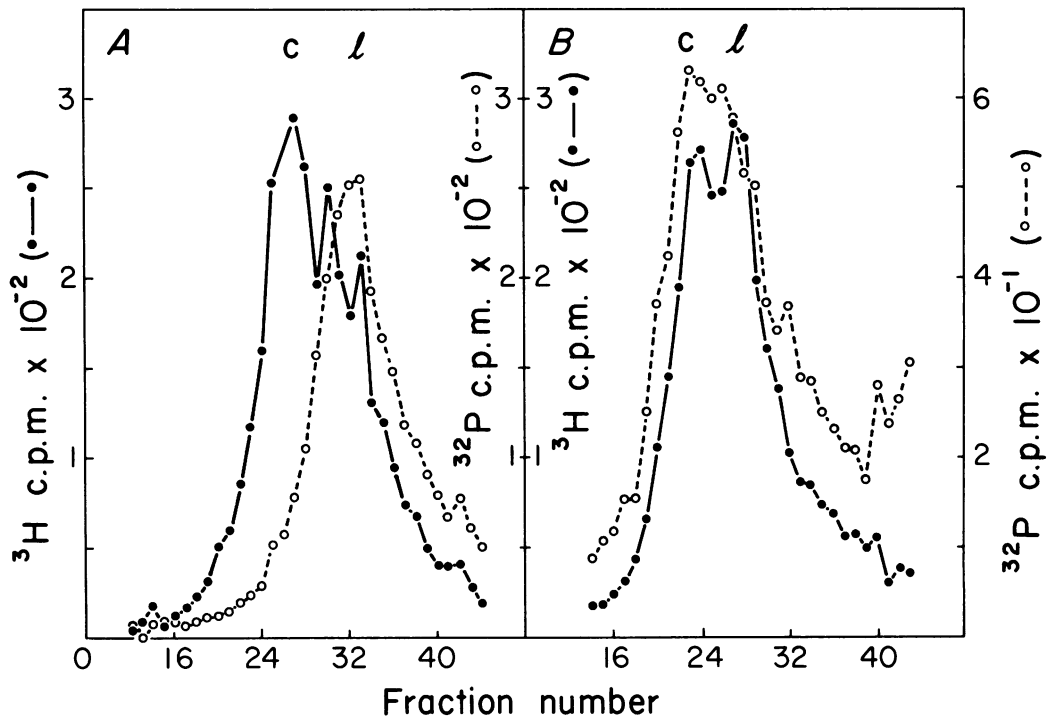

FIG. 3. *Circularization of the linear strands. Linear strands were prepared, and the 5' terminus was labeled with ^{32}P after treatment of the DNA with phosphatase as described in the legend to Fig. 2. The terminally labeled strands were then incubated with T4 DNA polymerase and polynucleotide ligase as described in Materials and Methods. Frame A shows the sedimentation pattern of this DNA. Frame B shows the sedimentation pattern of the same DNA after it had been treated with phosphatase. Both gradients are low-salt alkaline sucrose gradients. Symbols: O----O, ^{32}P-labeled DNA; ●——●, ^{3}H-labeled linear (l) and circular (c) strands added as markers.*

were located by examination of the plates under UV illumination, and the radioactivity was eluted and counted. Table 2 summarizes the data of a number of experiments, all of which lead to the conclusion that nearly half of the linear strands have a G residue at the 5' terminus; the complete ϕX genome contains 23% G (27).

Nature of the noncircularized DNA. Since only a portion (10–20%) of the linear DNA could be converted to a circular form by T4 DNA polymerase and ligase (Table 1 and Fig. 3), an investigation was undertaken into the nature of the noncircularized DNA. In this experiment the linear strands were reacted with T4 DNA polymerase and ligase except that alpha-^{32}P-dCTP was used in the incubation mixture. The labeled DNA resulting from the reaction was then reacted with *E. coli* DNA polymerase, unlabeled deoxynucleoside triphosphates, and ligase to convert as much as possible to a double-stranded form. Figure 4 shows analyses of the DNA at the various stages of these reactions. In Fig. 4A the alkaline sucrose sedimentation pattern of the ^{32}P-labeled DNA after reaction with T4 DNA polymerase is shown and compared with ^{3}H-labeled unit-length circular and linear strands. Figure 4B shows a similar

TABLE 2. *The nucleotide at the 5' terminus of the linear single-stranded DNA*

Nucleotide	1[a]	2	3	4	5	6	7	Avg[b]	8[c]
dGMP	41	40	48	47	39	46	43	43.4	34
dCMP	18	16	28	21	19	27	29	22.6	21
dAMP	25	30	13	14	18	12	15	18.1	18
dTMP	16	15	12	18	24	14	13	16.0	27

[a] Numbers 1 to 7 represent 7 different determinations of the percentage of each ^{32}P-labeled nucleotide. Preparations 1 to 5 were all phosphatase treated, labeled with gamma-^{32}P-ATP and polynucleotide kinase, and then digested. Preparations 6 and 7 were derived from preparations 4 and 5 after circularization of the linear strands with T4 DNA polymerase and ligase and then removal of all unprotected phosphate with phosphatase.

[b] The average of preparations 1 through 7.

[c] The SS DNA substrate for this reaction was not treated with phosphatase prior to the labeling with gamma-^{32}P-ATP and polynucleotide kinase. It was from the same preparation as no. 5.

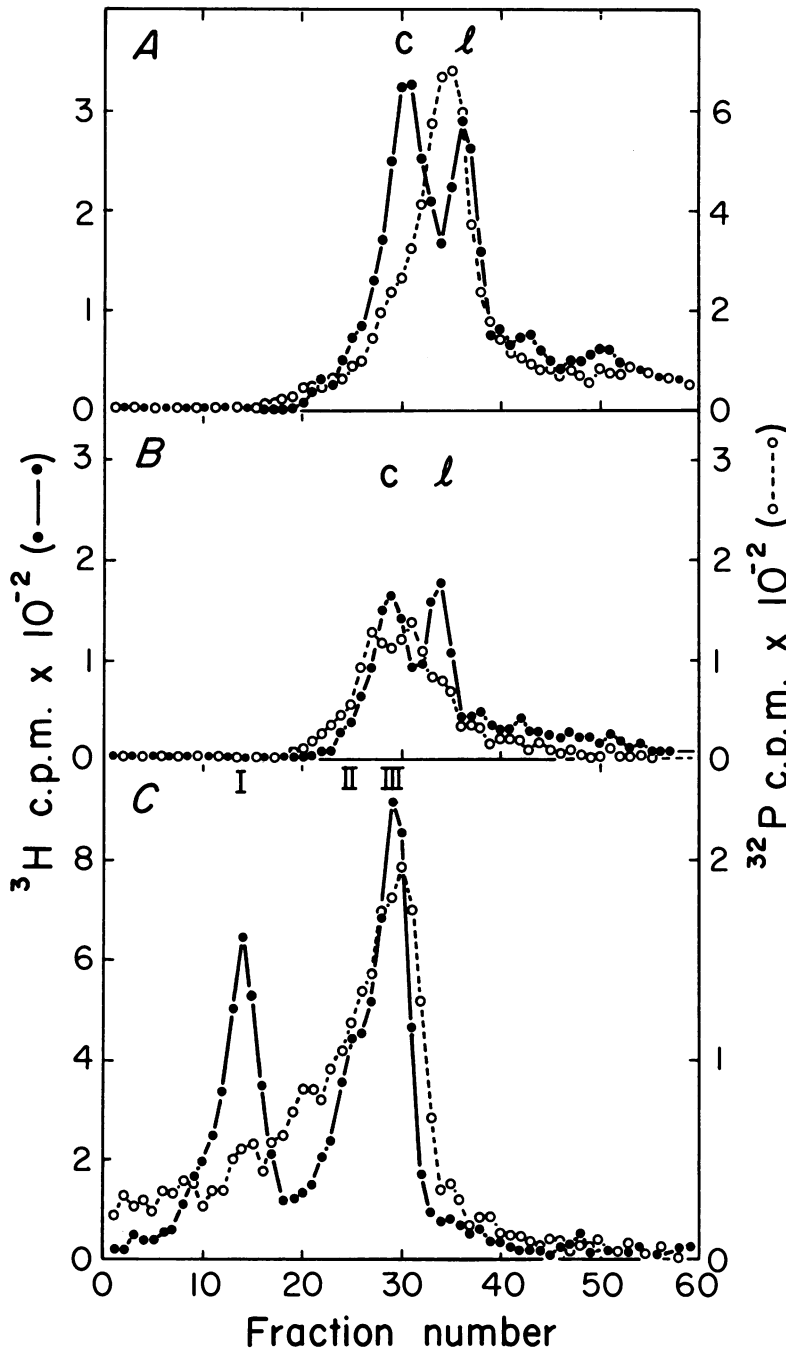

FIG. 4. *Sedimentation analysis of linear strands after incubation with T4 DNA polymerase. Linear strands were prepared and incubated with T4 DNA polymerase, ligase, ^{32}P-dCTP, and other reagents as described. The labeled DNA was purified by sedimentation on neutral sucrose gradients. A, Low-salt, alkaline sucrose gradients of this ^{32}P-labeled DNA with marker ^{3}H-linear (l) and circular (c) strands. The labeled DNA was then incubated further with E. coli DNA polymerase 1, ligase, oligonucleotides, and unlabeled triphosphates (24) to form RF. The DNA from this reaction was purified again by centrifugation on neutral sucrose. Samples of this DNA were then run on alkaline and neutral sucrose gradients with appropriate markers. B, Alkaline sucrose gradients of the ^{32}P-labeled DNA with ^{3}H-marker circular and linear strands. C, Neutral sucrose gradient of the ^{32}P-labeled DNA with φX ^{3}H-RF I, II, and III markers (13). The gradients in all three parts were centrifuged in the SW40 rotor for 13 h at 38,000 rpm. ●——●, ^{3}H; ○----○, ^{32}P.*

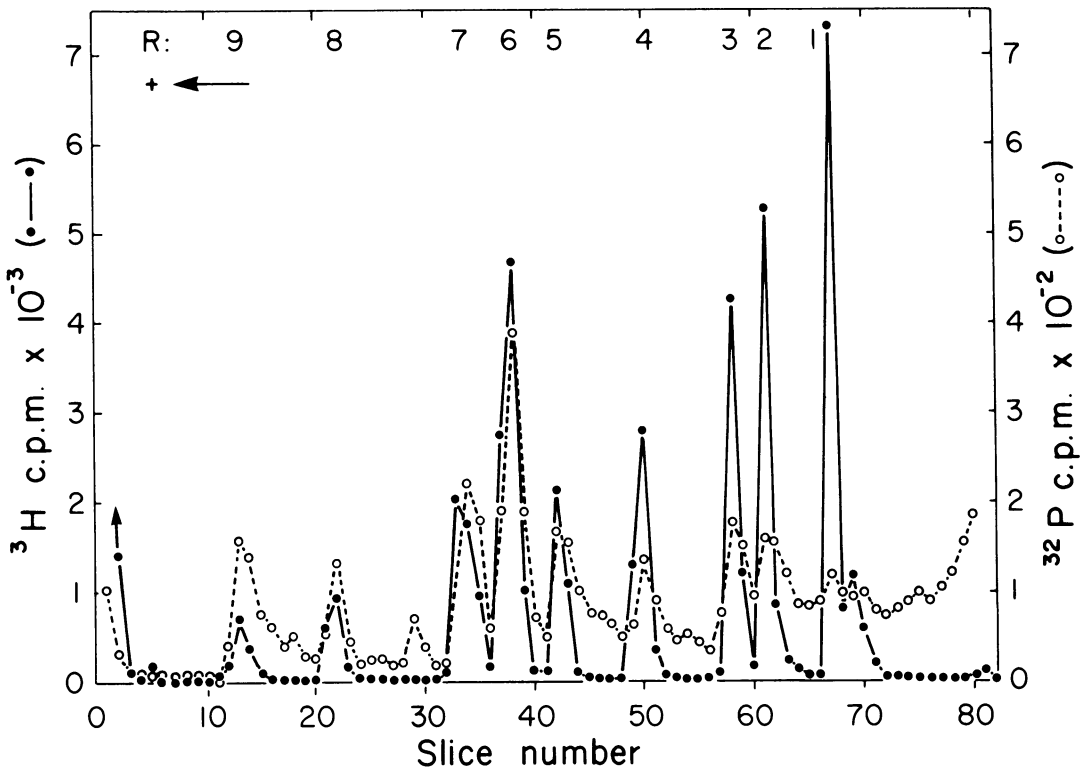

FIG. 5. *Analysis of ^{32}P-labeled RF DNA with endonuclease R. The ^{32}P-labeled RF DNA was prepared as described in the text and the legend to Fig. 4. It was digested with endonuclease R and subjected to electrophoresis on acrylamide-agarose gels (4) along with a digest of uniformly labeled ^{3}H-RF DNA. Symbols: ●——●, ^{3}H; ○----○, ^{32}P. Peak 7 contains two fragments (7.1 and 7.2) and peak 6 contains three fragments (6.1, 6.2, 6.3) (reference 4).*

gradient of the material after the reaction with *E. coli* DNA polymerase I. Figure 4C shows a neutral sucrose gradient of the same material as was run in Fig. 4B but this time with ^{3}H-labeled RF I, II, and III (RF III is a linear duplex the length of the ϕX genome). These gradients, collectively, suggest that the majority of the linear strands were not converted to circular RF II molecules but rather to "hair-pin"-like RF III molecules composed of longer than unit-length linear strands that were self-complementary and thus able to fold back upon themselves to form double-stranded RF III molecules of roughly unit length. In the alkaline gradient of Fig. 4B, linear strands twice the molecular weight of ϕX DNA would sediment somewhat faster than the circular unit-length molecules, roughly around fraction no. 26.

We calculate (29) that, during the process of RF III formation, about 600 to 1,000 nucleotides were added by the T4 DNA polymerase reaction and a further 1,000 to 4,000 nucleotides by the *E. coli* DNA polymerase I. Since it appears (Fig. 3B) that few or no double-length circles were produced, we conclude that most of the 3′ termini that were available for reaction with T4 DNA polymerase were produced by the formation of intramolecular complexes rather than intermolecular complexes.

The RF III molecules resulting from the successive reactions with T4 DNA polymerase and ^{32}P-dCTP, and then *E. coli* DNA polymerase I and unlabeled triphosphates, were treated with endonuclease R in order to localize the radioactivity incorporated during the reaction with T4 DNA polymerase. Figure 5 presents a gel pattern of the endonuclease R digest of the ^{32}P-labeled DNA together with ^{3}H-RF (uniformly labeled). It is evident that fragments R9, R8, and R7.1 have more label than fragments R3, R2, and R1, with the other fragments (R4-R6) having intermediate amounts of label. We conclude from this experiment that the label incorporated into the linear strands by T4 DNA polymerase I is in a limited region of the genome, and that the ends of the linear strands are not randomly located.

The various transformations undergone by

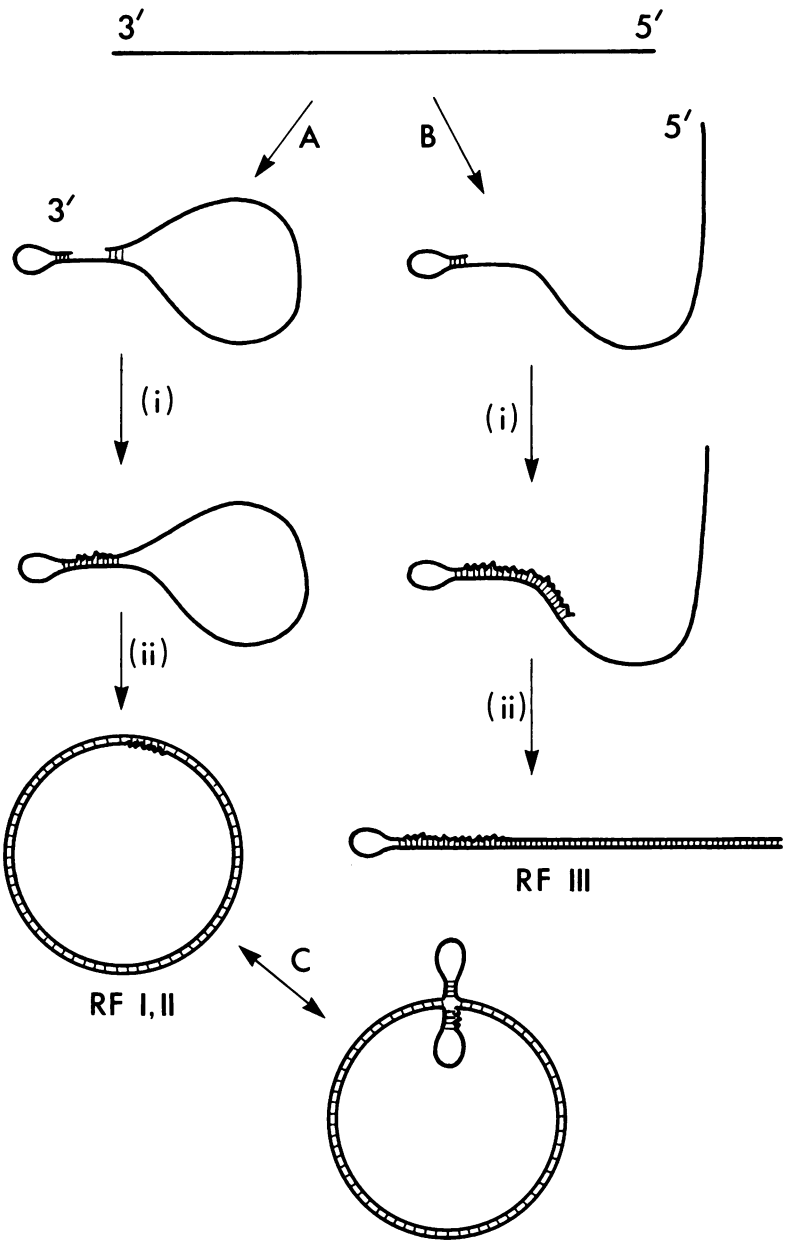

FIG. 6. *Conversion of linear strands to RF molecules. Pathway A represents the reaction sequence for 10 to 20% of the molecules in the population. Pathway B represents the reaction sequence for the majority of the molecules. (i) Reaction with T4 DNA polymerase, polynucleotide ligase, alpha* ^{32}*P-dCTP, dATP, dGTP, dTTP, and ATP. The squiggle (∿∿) represents incorporated radioactivity. (ii) Reaction with E. coli DNA polymerase I, oligonucleotide primers, ligase, and unlabeled triphosphates. Pathway A leads to the production of RF I and II molecules labeled with* ^{32}*P in a very limited portion of the genome. Pathway B, the predominant pathway, leads to the production of RF III molecules containing* ^{32}*P radioactivity in a larger region of the genome. It should be noted that the region of the genome which becomes labeled during the formation of RF III in pathway B corresponds to the region from about 9 o'clock to 11 o'clock in the circular RF I, II molecule pictured in pathway A. The entire genome does not become labeled because T4 DNA polymerase is not able, by itself, to replicate long stretches of single-stranded DNA. C illustrates the interconversion between two RF structures, one of which is a complete circular duplex, the other of which has a small "loop-out." Nucleotide sequences capable of forming loop-outs like these as a result of their rotational symmetry about a central region (equivalent to the presence of an inverted repetition) can be called palindromic sequences. The "loop-outs" are not drawn to scale, and the topology of the interlocked strands is not indicated.*

the linear strands in the reactions described in the text above and in Fig. 4 are illustrated in Fig. 6. The data presented in Fig. 4, together with the knowledge that only about 10 to 20% of the linear strands were circularized (Table 1), suggest that most of the linear single-stranded DNA molecules were converted to RF III-type structures as illustrated in pathway B. Consequently, digestion of these molecules with endonuclease R will yield labeled fragments derived predominantly from the region around 9 to 12 o'clock on the circular RF structure (Fig. 6, bottom left).

DISCUSSION

In 1959 Sinsheimer (27) presented evidence that the DNA of bacteriophage ϕX174 was single stranded. Two components were observed in the ultracentrifuge, a major component at 16*S* and a minor component at 14*S*, but the nature of the difference between the two components was not then determined. Subsequent studies (5) provided evidence that the more rapidly sedimenting 16*S* molecule was circular, whereas the second, slower, component was a linear molecule of the same molecular weight. All phage preparations examined contained at least 10 to 20% of the linear component, which was then thought to have been produced by random cleavage of the circular molecule. Our present results suggest an alternative explanation—that the linear strands were intermediates in SS circle formation. However, further studies will be required to distinguish these possibilities.

Nearly half of the linear strands have a phosphorylated deoxyguanosine residue at the 5′ end, although significant amounts of the other three deoxynucleotides are found as well. These linear strands are produced by the displacement of a viral strand from an RF molecule simultaneously with synthesis of a new viral strand in the RF (3), and the RF molecules produced have a gap in the newly synthesized plus strand (24). The gap is in the fragment R3 produced by endonuclease R (P. H. Johnson and R. L. Sinsheimer, Fed. Proc. **32**:491, 1973) and this fragment is known to carry markers from cistron A, the product of which is required for RF replication and SS DNA synthesis. Whether or not this gap is in the same position of the genome as the gap which must be closed to circularize the linear strands remains to be shown, but our result (Fig. 5) showing a deficiency of label in (among others) the R3 fragment derived from RF III molecules (see Fig. 6) is consistent with this possibility.

A puzzling aspect of our experiments is why nucleotides must be added to the linear strand before ligase can successfully join the two ends, since the linear strands were isolated under conditions of ligase deficiency. One explanation could be that, as a result of the ligase-deficiency, there were an increased number of nicks and gaps in the cellular DNA and that this reduced the number of DNA polymerase molecules that were effectively available to close the gap in the ϕX DNA.

The nucleotides at the 5′ ends of the RF II molecules and of the replicating intermediates during single-stranded DNA synthesis have been separately determined by Knippers et al. (14). Consonant with their finding of a 5′ terminal deoxyguanosine in the replicating intermediates is our observation of a 5′ terminal G in the linear SS DNA. However, their finding of a 5′ terminal deoxycytidine in the RF II molecules and their observation that in both types of molecules the 5′ ends were not phosphorylated are at present not in harmony with our data.

The results we have obtained support the idea that circularization of the linear ϕX SS DNA is accomplished by bringing the two ends of the linear DNA in proximity by complementary base pairing in the manner illustrated in Fig. 6. Consistent with this model are the observations of an exonuclease I-resistant region in ϕX SS DNA (5, but see 20 also) and of a double-stranded core in preparations of SS ϕX DNA digested with *Neurospora crassa* SS-specific endonuclease and *E. coli* exonuclease I (23). Also, this mechanism of circularization would explain why linear ϕX SS molecules with appropriately located ends were infectious in spheroplasts (25, 26). The linear SS nucleic acid genomes of other organisms also may be capable of circularization by a similar mechanism. For instance, the circular SS molecules of adeno-associated virus observed in the electron microscope (15) may have the structure pictured in Fig. 6. This could easily be tested by experiments similar to those we have described in this paper.

The palindromic sequence implied by our experiments (see Fig. 6) may explain certain aspects of the behavior of superhelical ϕX RF. For instance, the number of superhelical turns in superhelical ϕX RF would be a function of the number of base pairs present in the "looped-out" structure pictured at the bottom of Fig. 6, and it may be that this "looped-out" region is the region sensitive to the SS-specific endonuclease (13). Although the looped forms are thermodynamically less stable than a complete duplex because of the reduced stacking forces and the reduced number of hydrogen bonds,

they could be readily stabilized intracellularly by the interaction of specific proteins with the loops or by stress induced in a covalently closed circular duplex by duplex unwinding resulting from transcription or replication. The potential significance of such structures in DNA is great. Although some proteins (e.g., repressors) almost certainly recognize specific base sequences, other proteins (perhaps for example those involved in the initiation of synthesis of Okazaki fragments) may recognize a specific type of looped structure that need not have an unique nucleotide sequence.

The existence of linear strands of less than unit length is not easily reconciled with a rolling-circle (6) mechanism of SS DNA synthesis proceeding via a viral strand that is cut into portions of appropriate lengths. Possibly the longer than unit-length strands observed result from faulty termination and are not normal intermediates in replication, or, alternatively, there is more than one mechanism of SS synthesis.

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