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Nature of φX174 Linear DNA from a DNA Ligase-Defective Host

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Linear DNAs have been prepared from φX phage and from φX RF II (double-stranded circular form of φX DNA, formed during infection and nicked in one or both strands) molecules derived from infection at the restrictive temperature of *Escherichia coli ts7*, a host mutant with a temperature-sensitive DNA ligase activity. The linear DNA from these phages can be circularized by annealing with fragments of φX RF DNA produced by the *Haemophilus influenzae* restriction nuclease. The circularization experiment indicated that the site of breakage of the linear phage DNAs is not unique nor confined to a particular region of the genome. These linear DNAs were less than 0.1% as infective as circular phage DNA. The linear, positive strand of late RF II DNA, however, is uniquely nicked in the region of the φX genome corresponding to cistron A. Although a low level of infectivity is associated with the linear DNA derived from late RF II, this infectivity appears to be a result of the association of linear positive and linear negative strands during the infectivity assay.

φX174 is a small icosahedral bacteriophage containing a single-stranded circular DNA. During the infection of the host *Escherichia coli*, φX174 is able to produce multiple copies of its DNA. Three stages in this DNA replication process may be distinguished. (i) Upon infection, the DNA strand complementary to the parental circular single-stranded DNA is synthesized to form a double-stranded circular DNA (replicative form [RF]); (ii) the double-stranded RF undergoes semiconservative replication to form a pool of progeny RF DNAs; and (iii) single-stranded progeny viral DNAs are synthesized by an asymmetric replication process, using the complementary strand of RF as a template and packaged into phage in circular form. Baas and Jansz (2) determined from genetic studies of the replication of heteroduplex DNA that the origin of φX semiconservative DNA replication is probably in or near cistron A. With the use of the restriction nuclease from *Haemophilus influenzae* to analyze RF II DNA isolated late in the infection cycle, Johnson and Sinsheimer (12) demonstrated that a specific discontinuity exists in this RF II. The region containing the discontinuity has been shown to correspond genetically to the cistron A region (4). By inference, there appears to be a specific origin of late single-stranded DNA synthesis which is located in or near cistron A.

E. coli ts7 was isolated from *E. coli* TAU-bar, after mutagenesis, as a temperature-sensitive mutant defective in a late step in the dark repair of UV damage (19). The DNA ligase of *E. coli ts7* was shown to be temperature-sensitive in this mutant (17, 20). A study of the structure of φX DNAs isolated during φX replication in *E. coli ts7* has suggested that the host *E. coli* DNA ligase participates in φX DNA replication (21, 22). Schekman and Ray (22) observed that approximately 20% of the φX phage produced at the restrictive temperature in *E. coli ts7* contained linear rather than circular DNA. Schekman and Ray (22) also observed infectivity associated with the linear DNA of these phage with intracellular single-stranded DNA from φX-infected *E. coli ts7* and with linear DNA prepared from RF II from *ts7*-infected cells. Other workers (7, 23) have reported similar infectivities of linear DNAs from RF IIs isolated from other hosts.

We have attempted to determine the position, or positions, of the nick in these linear DNAs with respect to the φX genetic map. We have also reinvestigated the infectivities of these several nicked, single-stranded DNAs.

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MATERIALS AND METHODS

Phage, bacteria, and media. *E. coli ts7* was isolated and characterized by Pauling and Hamm (19) after mutagenesis of the parent strain *E. coli* TAU-bar (10). *E. coli* H502 (thy$^-$, uvr A$^-$, endo I$^-$, and su$^-$) was the host for the preparation of ^{14}C-labeled *ts79* phage. Spheroplasts were prepared from *E. coli* K12W6. *E. coli* HF4714 (su$^+$) and *E. coli* C (su$^-$) were used to titer *am3* and *ts79* phage, respectively. ϕX*am3* is an amber mutant in cistron E having a lysis-defective phenotype. ϕX*ts79* is a temperature-sensitive mutant in cistron G. *E. coli ts7* and TAU-bar were grown in TPA media (14) supplemented with 2 μg of thymine and 40 μg of uracil per ml. *E. coli* H502 was grown in TPA media supplemented with 2 μg of thymine per ml. *E. coli* K12W6 was grown in 3XD media (8). *E. coli* HF4714 and C were grown in KC broth (18).

Preparation of linear DNA from phage. *E. coli ts7* was grown at 30 C in 2 liters of TPA supplemented with 2 μg of thymine and 40 μg of uracil per ml, with vigorous aeration to 10^8 cells/ml. The temperature of the culture was raised to 38 C, and the culture was gently aerated for 45 min. ϕX*am3* was added at a multiplicity of infection of 20; 5 mCi of [^{3}]thymidine was added simultaneously. The infection and labeling were allowed to proceed at 38 C for 90 min with vigorous aeration. The infection was stopped, and the cells were cooled by adding one-tenth volume of 0.5 M potassium cyanide and 0.03 M sodium azide and quickly immersing the culture in a dry ice-methanol bath. The cells were pelleted by centrifugation at 7,000 rpm for 15 min in a Sorvall GS3 rotor, washed once with 200 ml of 0.05 M sodium borate, pH 8.5, and 0.005 M EDTA, and resuspended in 20 ml of the same buffer. The cells were lysed by the addition of 0.2 ml of a 10-mg/ml solution of lysozyme and incubation at 5 C for 1 h followed by freeze-thawing twice in a dry ice-methanol bath. The cell lysate was vortexed vigorously, and the cell debris was removed by centrifugation at 15,000 rpm for 20 min in a Sorvall SS34 rotor. To each milliliter of supernatant 0.605 g of CsCl was added, and the solution was centrifuged in the type 50 Spinco rotor for 40 h at 25,000 rpm. The phage band was collected, dialyzed, and centrifuged through a 5 to 20% neutral sucrose gradient in the SW25.2 Spinco rotor at 25,000 rpm for 3 h. The phage peak was pooled and dialyzed, and 0.605 g of CsCl per ml of solution was added. A CsCl equilibrium density gradient was formed by centrifugation again, as described above, and the phage band was pooled and dialyzed.

The DNA was extracted from phage using phenol, freshly distilled under nitrogen and equilibrated with 0.1 M Tris, pH 8.1, and 0.005 M EDTA. Extractions were performed at 37 C. An equal volume of phenol was added to the phage solution, and the phases were gently mixed for 20 min. The water phase was reextracted with an equal volume of phenol, whereas the original phenol phase was reextracted with an equal volume of 0.1 M Tris, pH 8.1, and 0.005 M EDTA, equilibrated with phenol. The procedure was repeated a third time. The aqueous phases were pooled, and the DNA was precipitated by the addition of one-tenth volume of 3 M sodium acetate, pH 5.5, and 2 volumes of isopropanol, followed by refrigeration overnight at −20 C. The precipitated DNA was resuspended in 5 mM Tris, pH 8.1, and 0.5 mM EDTA. Linear DNA was purified from circular DNA by one or more alkaline sucrose gradients.

Centrifugation procedures. Neutral sucrose gradients contained 0.01 M Tris, pH 8.1, 4 mM EDTA, and a linear gradient of 5 to 20% sucrose. Alkaline sucrose gradients contained 0.2 N KOH, 4 mM EDTA, and a linear gradient of 10 to 30% sucrose. To separate circular and linear single-stranded DNA, the single-stranded DNA was centrifuged at 38,000 rpm in an SW40 or SW41 Spinco rotor for 17 h at 15 C. Generally, 0.15-ml fractions were collected, starting from the bottom of the tube, with the aid of a pump. If fractions were collected from the top, an auto-Densi-Flow apparatus (Buchler Instruments) was used. Positive and negative ϕX DNA strand separation was achieved by CsCl equilibrium density centrifugation of poly r(U,G)-ϕX DNA complexes described previously by Baas and Jansz (1). ϕX RF II DNA was denatured by heating to 85 C for 10 min in 0.01 M Tris, pH 8.1, and 4 mM EDTA, in the presence of 25 μg of poly r(U,G) per ml (base ratio G-U, 1.0:1.2; Miles Laboratories). The poly r(U,G)-ϕX DNA complexes were formed by quick cooling in ice water. CsCl was quickly added to a final density of 1.73, and a density equilibrium gradient was formed by centrifugation at 40,000 rpm in a Spinco type 50 rotor for 60 h at 5 C. Fractions (0.25 ml) were collected starting from the bottom of the tube.

Circularization of linear DNA with Hind fragments. Purified *Hin*d restriction nuclease fragments of ϕX*am3* RF I DNA were provided by Lloyd H. Smith. The fragments were isolated by continuous gel electrophoresis through 5% agarose gels and were shown to be pure by ethidium-bromide staining of fragments rebanded on polyacrylamide slab gels. Linear, positive-strand DNA was mixed with one of the *Hin*d nuclease fragments in a molecular ratio of 10 fragments to one linear DNA. If a combination of Hin II nuclease fragments was to be annealed with the linear DNA, then 10 molecules of each fragment were added per molecule of linear DNA. The fragments in the mixture were first denatured by dialysis of the solution against 90% formamide, 0.12 M Tris, pH 8.5, and 0.012 M EDTA for 3 h at room temperature. Annealing was then performed by dialysis against two changes of 50% formamide, 0.12 M Tris, pH 8.5, and 0.012 M EDTA for 2 h each. The annealed DNAs were stored at −10 C overnight in 50% formamide, 0.12 M Tris, pH 8.5, and 0.012 M EDTA and then combined with cytochrome *c* and spread onto 20% formamide, 12 mM Tris, pH 8.5, and 1.2 mM EDTA as described by Davis, Simon, and Davidson (5) for Kleinschmidt electron microscopy.

All linear DNAs used for these studies showed less than 2% circularization in the absence of *Hin*d fragments. Random linears were used as controls and were generated by either of two means: (i) X-irradiation of circular phage DNA to nick one-third of the molecules or (ii) immersing circular DNA in boiling water to nick approximately one-third of the molecules. The linear DNAs generated by these procedures were purified by sedimentation through alkaline sucrose

gradients, and both DNAs showed a similar random nature in circularization with the *Hind* fragments.

The efficiency of circularization by the method described was determined by annealing the randomly nicked linear DNAs to a combination of R_1, R_2, and R_3 fragments which are known to be 18.5, 14.0, and 12.4% of the φX genome, respectively (A. S. Lee and R. L. Sinsheimer, Proc. Nat. Acad. Sci. U.S.A., in press). In the presence of these fragments, 25% of the unit length φX DNA observed in the electron microscope was circular. The efficiency of circularization is therefore estimated to be 55%.

Infectivity assay of φX DNA. The infectivity of φX DNA was determined by the procedure of Guthrie and Sinsheimer (9). In experiments where both φX*am*3 and φX*ts*79 DNA were employed, the incubations with spheroplasts were carried out at 30 C. The two types of phage progeny were then distinguished by plating on *E. coli* C at 30 C and on *E. coli* HF4714 at 40 C.

Preparation of late FF II DNA. Late RF II from *E. coli* H502 was prepared as described by Johnson and Sinsheimer (12), except that the last two purification steps were (i) centrifugation through a neutral sucrose gradient in an SW25.2 Spinco rotor at 25,000 rpm for 16 h at 5 C and (ii) centrifugation through a high salt (1 M NaCl) neutral sucrose gradient in an SW27 Spinco rotor at 25,000 rpm for 16 h at 5 C, rather than two low salt isokinetic sucrose gradients. The late RF II from *E. coli ts*7 was prepared similarly except that *E. coli ts*7 was grown at 30 C in TPA supplemented with 40 μg of thymine and 40 μg of uracil per ml to 10^8 cells/ml. The temperature of the culture was raised to 38 C and maintained at that temperature for 45 min with gentle aeration, at which time φX*am*3 phage was added at a multiplicity of infection of 20.

Enzyme reactions. *H. influenzae* (Hin) restriction nuclease, *Hin*d, was purified as described by Smith and Wilcox (24) and was provided by Paul H. Johnson. The reaction mixture for *Hin*d digestion (100 μliters) was 7 mM Tris, pH 7.4, 50 mM NaCl, 7 mM mercaptoethanol, 7 mM $MgCl_2$, between 0.1 and 2 μg of DNA, and approximately 0.001 to 0.1 U of *Hin*d. Incubation was for 5 h at 37 C. Reactions were stopped by adding one-fifth volume of 5A buffer (see electrophoresis section) and sucrose to 5%. *E. coli* DNA polymerase I (EC 2.7.7.7) was purified by the method of Jovin et al. (13) to Fraction VII purity and was a gift of Arthur Kornberg. Reaction conditions are described in the figure legends. Calf thymus primer DNA was prepared by digestion of calf thymus DNA (Sigma) with pancreatic DNase I (Sigma) until 30% of the optical density at 260 nm was made acid soluble (6).

Gel electrophoresis. The electrophoresis buffer was prepared by a fivefold dilution of 5A buffer (96.9 g of Tris base, 54.4 g of sodium acetate·$3H_2O$, 14.9 g of EDTA·$2H_2O$, 40 g of sodium dodecyl sulfate and water to 4 liters and adjusted to pH 7.8 with acetic acid). Gels of 5% polyacrylamide were prepared by mixing 3 ml of 5A buffer, 6.5 ml of H_2O, 5 ml of acrylamide-bis solution (15 g of acrylamide, 0.75 g of bis-acrylamide dissolved in water to 100 ml), 50 μliters of *N,N,N′,N″*-tetramethylethylenediamine, and 0.5 ml of 10% ammonium persulfate. The liquid solution was poured into Plexiglas tubes (1 by 15 cm) sealed at the lower end with Parafilm. After the gels were formed, a piece of dialysis tubing was attached to the open end of the tube with a rubber band, the Parafilm was removed from the bottom, and the gels were tapped gently down to the end of the tube covered by dialysis tubing. The gels were prerun at 10 V for 3 h using 1A electrophoresis buffer. Samples were gently layered onto the gels and were electrophoresed at 50 V for approximately 12 h. The gels were frozen on dry-ice slabs and sliced with a Mickle gel slicer. The gel slices were shaken at room temperature in 5 ml of NCS counting solution (see radioactive methods) overnight and then counted.

Radioactive material and scintillation counting. [^{3}H]thymidine (specific activity, 54 Ci/mmol) and [^{14}C]thymine (specific activity, 51 mCi/mmol) were purchased from Schwarz/Mann. Deoxyribonucleoside triphosphates, labeled in the alpha position with ^{32}P, were purchased from ICN. The original specific activities were greater than 50 Ci/mmol. Radioactive DNAs from sucrose gradients were assayed by spotting a 10- to 100-μliter sample on filter paper disks, which were washed as previously described (3) and counted in a toluene-based scintillation fluid: 4 g of 2,5-diphenyloxazole, and 50 mg of 1,5-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. Polyacrylamide gel slices were counted in NCS scintillation fluid: 90 ml of NCS solubilizer (Amersham/Searle), 10 ml of H_2O, 42 ml of Liquifluor (New England Nuclear), and 858 ml of toluene per liter of solution.

UV irradiation of DNA. Samples (0.3 ml) of DNA dissolved in 0.05 M Tris, pH 8.1, were placed on watch glasses, 3.8 cm in diameter, which were centered in glass petri dishes (10 cm in diameter). The petri dishes were placed under a UV lamp, and the samples were exposed to 19 ergs of UV light per mm^2 per s for varying periods of time up to 5 min. The DNAs were then assayed for biological activity.

RESULTS

Linear DNA in phage particles from E. coli ts7. The presence of linear DNA in phage particles produced in the ligase-defective host mutant, *E. coli ts*7, as reported by Schekman and Ray (22), is demonstrated in Fig. 1. Cells of *E. coli ts*7 infected with φX*am*3 phage at a nonpermissive temperature (38 C) and labeled with [^{3}H]thymidine (see Materials and Methods) were combined with ^{14}C-labeled, φX*am*3-infected cells of *E. coli* TAU-bar, the parent strain of *E. coli ts*7. The cells were lysed and the phage were co-purified and phenol-extracted, and the radioactive DNA was sedimented through an alkaline sucrose gradient to separate circular and linear forms (see Fig. 1). Approximately 30% of the ^{3}H-labeled phage produced at 38 C in *E. coli ts*7 contained linear DNA. In contrast, less than 3% of the ^{14}C-labeled phage DNA from *E. coli* TAU-bar was in the linear form. Whether 3% of the phage

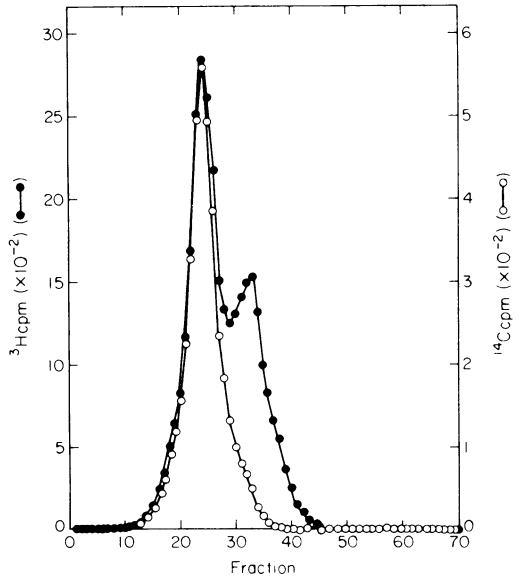

FIG. 1. *Linear DNA in phage from E. coli ts7. Infected cells of E. coli ts7 and E. coli TAU-bar were combined and the phage were co-purified. Phage proteins were extracted with phenol, and the phage DNA was sedimented through an alkaline sucrose gradient to resolve circular and linear forms. Symbols: [^{3}H]DNA of phage from E. coli ts7, (●); [^{14}C]DNA of phage from E. coli TAU-bar, (○).*

from TAU-bar actually contains linear DNA or whether the low percent of linear DNA is produced as an artifact during phage purification, phenol extraction, or sedimentation is not known. It is clear, however, that a significant proportion of phage from *E. coli ts7* contains linear DNA.

An important step in the procedure for obtaining linear phage DNA from *E. coli ts7* (see Materials and Methods) is to maintain the culture at the restrictive temperature (38 C) for approximately 45 min prior to infection. In addition, we have observed a larger proportion of linear phage DNA when the culture has been grown at 30 C (rather than the more permissive temperature of 25 C) prior to the temperature shift to 38 C. These culture conditions (30 C) are not ideal for *E. coli ts7*, since microscopic examination of the culture shows a large number of cells which have lost their ability to divide, forming long "chains" of cells. The phage yield at 38 C from an *E. coli ts7* culture was approximately 2% that from an *E. coli* TAU-bar culture when the cultures were grown at 30 C to a similar cell density (determined using a Coulter counter) and infected similarly at 38 C. The exact percent of linear DNA observed in the phage from *E. coli ts7* varies from experiment to experiment. Thirty percent of linears has been the maximum observed, and sometimes as few as 10% of the phage DNA molecules are in linear form. In the experiments described in this paper, only phage preparations containing 20% or more linear DNA have been used.

Location of the ends of linear DNAs. Several experiments were conducted to determine if this linear DNA from ϕX grown in *E. coli ts7* was non-specifically nicked or was a unique species of linear DNA possessing a discontinuity in a specific region of the ϕX genome. This determination was possible through the use of the restriction nuclease from *H. influenzae*, *Hin*d, which cleaves circular double-stranded ϕX DNA into 13 specific fragments ranging in size from 1,000 to 80 nucleotides (Lee and Sinsheimer, Proc. Nat. Acad. Sci. U.S.A., in press).

Purified ϕX DNA fragments generated by *Hin*d cleavage of ϕX RF DNA were individually hybridized to purified single-stranded linear DNA. The resultant hybrids were observed under the electron microscope, and the percent of single-stranded DNA molecules which could be circularized by each pure fragment was determined. Three possible results could be obtained in such an experiment, depending on whether the DNA is specifically or non-specifically nicked. (i) If the linear DNA possessed a nick in a unique region of the ϕX genome, only one fragment would be expected to circularize the linear DNA upon hybridization. (ii) If the linear DNA possessed a unique nick but the nick was very close to a restriction nuclease cleavage site, none of the fragments would be expected to circularize the linear DNA. (iii) If the linear DNA were non-specifically nicked with respect to the ϕX genome, all fragments should circularize a proportion of the linear DNA and this proportion should be directly related to the size of the fragment.

Table 1 presents the results of hybridization experiments using the three largest fragments generated by *Hin*d cleavage of ϕX RF DNA. The fragments are designated as R_1, R_2, and R_3 in the order of decreasing size. The larger fragments were the most easily purified and were expected to give greater accuracy in determining the percent of circularization than smaller fragments.

Randomly nicked, linear ϕX DNA, produced by X-irradiation or boiling of circular phage DNA, was used to determine the efficiency of hybridization.

From the first column in Table 1, it is apparent that the linear phage DNA from *E.*

TABLE 1. *The circularization of linear single-stranded φX DNAs by hybridization to Hin II nuclease fragments*[a]

Hind fragment	% Circles observed with		% Circles for random linear DNA
	Linear DNA[b] of phage	Linear DNA[c] of RF II	
R_1	10	2	10 (expected)
R_2	8		8 (expected)
R_3	7	37	7 (expected)
$R_1 + R_2 + R_3$	26		25 (observed)

[a] The three largest double-stranded DNA fragments produced by *H. influenzae* restriction nuclease digestion of φX RF II, designated R_1, R_2, and R_3 in order of decreasing size, were hybridized to different linear single-stranded φX DNAs (see Materials and Methods). The percent of linear DNA circularized after hybridization with each fragment or combination of fragments was determined by electron microscopy. The linear phage DNA was extracted from φX *am*3 phage produced at the restrictive temperature in *E. coli ts*7 and purified from circular DNA by sedimentation through two alkaline sucrose gradients. Linear DNA of RF II, isolated from *E. coli* H502, was purified from circular DNA of RF II by one alkaline sucrose gradient. The linear DNA thus obtained was then banded in a CsCl density equilibrium gradient in the presence of poly r(U,G), and the fractions containing the positive strands were pooled. The positive-strand linear DNA was resedimented in alkaline sucrose for a second purification of linear DNA from circular DNA. Randomly nicked control DNA was obtained by limited X-irradiation or boiling of purified circular φX *am*3 phage DNA (see Materials and Methods), and the linear DNA was purified from residual circular DNA by two alkaline sucrose gradients. The amount of circularization of randomly generated linear DNA with a combination of R_1, R_2, and R_3 is empirically 25%. Since R_1, R_2, and R_3 constitute 18.5, 14.0, and 12.4% of the φX genome, respectively, the efficiency of circularization is estimated to be 55%; the expected percent of circularization of each fragment then for randomly nicked DNA is listed in the last column.

[b] A residual 0.5% contamination of the linear DNA with circular DNA was subtracted to obtain the values in this column.

[c] A residual 0.7% contamination of the linear DNA with circular DNA was subtracted to obtain the values in this column.

*coli ts*7 is circularized by each fragment in decreasing proportion corresponding to the decreasing size of the fragment. The effects of each fragment are additive, since hybridization with a combination of the large fragments resulted in a percent of circularization which is closely the sum of the percents obtained with individual fragments. These values may be compared with those in the last column which lists the percent of circularization to be expected for a randomly nicked linear DNA. The two values correspond well, and it may be concluded that the linear phage DNA from *E. coli ts*7 is non-specifically nicked.

Conversely, the linear positive strand of RF II isolated from *E. coli* H502 late in infection has a relatively specific discontinuity in the R_3 region, since the R_3 fragment preferentially circularizes the linear DNA. The R_1 fragment correspondingly has a decreased ability to circularize the linear DNA. This result confirms and extends the previous observation (12) that RF II isolated late in infection contains a specific discontinuity in the R_3 region.

Additional evidence demonstrating the non-specific nature of the linear DNA of φX phage from *E. coli ts*7 was provided by converting the linear single-stranded DNA to a linear double-stranded form using *E. coli* DNA polymerase I. The linear DNA was incubated at 15 C in the presence of four deoxyribonucleoside triphosphates, including [^{32}P]dCTP, calf thymus DNA primer approximately 50 nucleotides in length, and *E. coli* DNA polymerase I. DNA synthesis proceeded to approximately onefold synthesis and stopped. The product of this reaction was analyzed by sedimentation velocity in a neutral sucrose gradient shown in the lower panel of Fig. 2. The product sediments slightly slower than circular double-stranded RF II DNA, included in the gradient as a marker, and sediments in the position expected for a linear double-stranded RF III DNA. The radioactive material sedimenting near the top of the gradient is calf thymus primer DNA labeled during the polymerization reaction. As a control, circular phage DNA was converted to a double-stranded form with *E. coli* DNA polymerase I in the same manner. The product of this reaction is shown in the top panel of Fig. 2 and, as expected, sediments as an RF II DNA.

The double-stranded DNA polymerase products were then digested with the *Hind* restriction nuclease in the presence of an in vivo synthesized ^{3}H-labeled RF I marker. The fractionation of the Hin II digestion products on 5% polyacrylamide gels is shown in Fig. 3. The top panel is the fragment profile for the ^{32}P-labeled, in vitro synthesized, circular double-stranded RF II DNA and ^{3}H-labeled RF I marker. The fragment profiles are very similar. There is, however, a tendency for the in vitro synthesized DNA to be more resistant to Hin II digestion as noted by the shoulders in front of the two larger fragment peaks. These two shoulders are frequently observed as partial digestion products

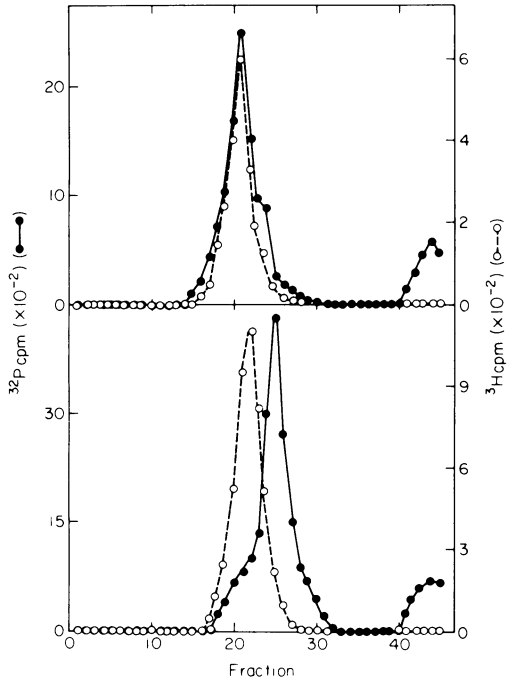

FIG. 2. *Polymerase I reaction products using circular and linear phage DNA templates. Purified circular and linear phage DNA (2 μM nucleotide equivalents each) from E. coli ts7 were incubated for 5 h at 15 C in a 100-μliter reaction mixture containing 67 mM potassium phosphate, pH 7.4, 15 mM $MgCl_2$, 1 mM mercaptoethanol, 1 μM calf thymus DNA primer, 25 μM each of dATP, dTTP, dGTP, and 17 μM [^{32}P]-dCTP (specific activity, 14.1 Ci/mmol) and 2.5 U of E. coli DNA polymerase I. The reaction was stopped by the addition of 20 mM EDTA, and the reaction products were purified from unreacted nucleoside triphosphates by passage through a 1- by 70-cm glass-bead column (pore size, 24 nm). A portion of the products were sedimented in a neutral sucrose gradient in the presence of a ^{3}H RF II marker (○). Upper panel: ^{32}P product of circular DNA (●). Lower panel: ^{32}P product of linear DNA (●).*

in limited *Hin*d digestion reactions. The increased resistance of the in vitro synthesized DNA to *Hin*d digestion may be related to the presence of primer DNA resulting in single-stranded tailed structures. Similarly, the higher background between fragment peaks observed with the in vitro synthesized RF II DNA may be a result of interference by primer DNA, leading to partial digestions and altered mobility of fragments.

The lower panel of Fig. 3 is the fragment profile for the linear double-stranded RF III DNA synthesized in vitro with DNA polymerase I, using linear phage DNA from *E. coli ts7* as a template. ^{3}H-labeled RF I marker DNA was included in the reaction, and the ^{32}P-labeled linear RF III fragments correspond closely to the marker profile. Comparison of the upper and lower panels of Fig. 3 shows that the double-stranded linear DNA and double-stranded circular DNA synthesized in vitro have virtually indistinguishable fragment patterns. These results confirm the circularization experiments, demonstrating that the linear DNA of phage from *E. coli ts7* are non-specifically nicked.

The lack of infectivity of linear DNA. Since Schekman and Ray (22) reported that the linear DNA of phage from *E. coli ts7* showed a low level of infectivity, it was of interest to study the infectious properties of the linear phage DNA from *E. coli ts7* in detail. Approximately equal amounts of ^{3}H-labeled *am3* phage from *E. coli ts7* and ^{14}C-labeled *ts79* phage from *E. coli* H502 were combined and phenol-extracted. The ^{14}C-labeled *ts79* phage DNA served as a circular infectivity marker as well as a radioactive aid in determining the amount of purification of the ^{3}H-labeled linear DNA. Purification of the linear DNA was achieved by three successive

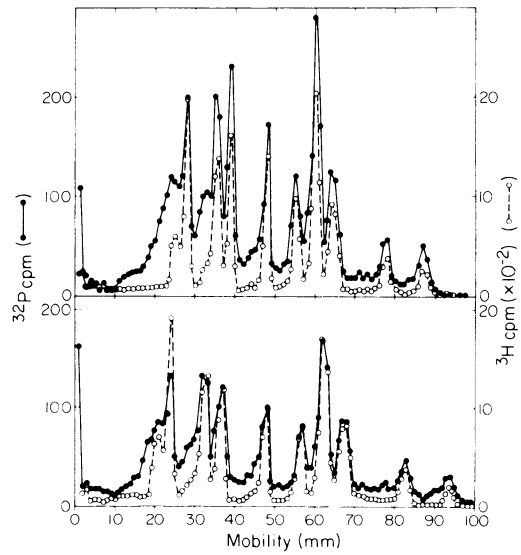

FIG. 3. *Hin II restriction nuclease fragments of in vitro synthesized RF II and RF III DNA. The double-stranded ^{32}P-labeled DNA polymerase products (●) described in the legend to Fig. 2 were digested with Hind restriction nuclease in the presence of ^{3}H-labeled RF II marker DNA (○). The digestion products were electrophoresed through 5% polyacrylamide gels for 14 h at 50 V. Upper panel: ^{32}P RF II DNA synthesized in vitro using purified circular DNA of phage from E. coli ts7. Lower panel: ^{32}P RF III DNA synthesized in vitro using purified linear DNA of phage from E. coli ts7.*

velocity sedimentations through alkaline sucrose gradients.

The radioactive profile of the first alkaline sucrose gradient appears in the top left panel of Fig. 4. The ^{3}H-labeled *am3* phage from *E. coli ts7* initially contained approximately 25% of linear DNA, whereas the ^{14}C-labeled *ts79* phage DNA showed only approximately 2% linear strands. There should, therefore, have been at least a 10-fold change in the ratio of *am3* infectivity to *ts79* infectivity between the circular DNA peak and the linear DNA peak if the two linear phage DNAs were infectious. However, since the overlap of infectivity from the circular peak to the linear peak was so great (data not shown), two more purification steps were required.

Peak fractions of linear DNA were pooled and resedimented on a second gradient shown in the upper right panel of Fig. 4. From the degree of purification of the ^{14}C-labeled *ts79* linear DNA, it can be estimated that the molar ratio of ^{3}H-labeled *am3* circular DNA to linear DNA is now approximately 1 to 7.

As a final purification step, the peak linear DNA fractions were pooled again and resedimented in a third alkaline sucrose gradient. The gradient was collected from the top to avoid tailing of circular DNA into the region of linear DNA. The positions of radioactivity-labeled

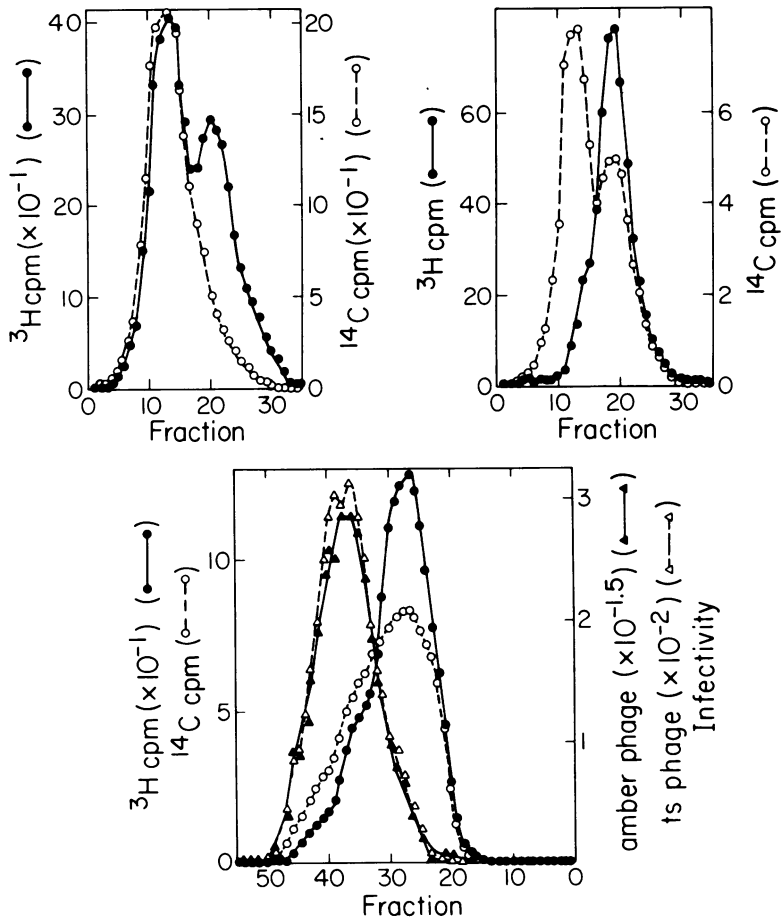

FIG. 4. *Infectivity study of phage DNA from E. coli ts7. ^{3}H-labeled am3 phage from E. coli ts7 were combined with [^{14}C]ts79 phage from E. coli H502, and phenol was extracted. The DNA was sedimented in an alkaline sucrose gradient to resolve circular and linear forms (top left panel). The peak linear DNA fractions were pooled and resedimented in a second alkaline sucrose gradient (top right panel). The peak fractions of linear DNA were pooled and resedimented on a third alkaline sucrose gradient (lower panel). All three panels show ^{3}H phage DNA (●) from E. coli ts7 and ^{14}C phage DNA (○) from E. coli H502. The lower panel also shows infectivity of am3 DNA (▲) from E. coli ts7 and infectivity of temperature-sensitive phage DNA (△) from E. coli H502.*

DNA and infectious DNA are shown in the lower panel of Fig. 4. From the distribution of radioactive label, it is calculated that the ratio of circular ^{3}H-labeled *am*3 DNA to linear ^{3}H-labeled *am*3 DNA is approximately 1 to 100.

Infectious *am*3 and *ts*79 DNA is present only in the region of circular DNA. Furthermore, the ratio of the infectivity of *am*3 and *ts*79 DNA is constant across the gradient. Since there is at least 10 times more *am*3 linear DNA than *ts*79 linear DNA, a change in the ratio of *am* to *ts* infectivity of at least one order of magnitude would be expected if the linear *am* DNA is infectious. The experiment demonstrates that the linear *am*3 phage DNA from *E. coli ts*7 is less than 0.1% as infectious as circular *am*3 phage DNA.

Several workers have reported that linear DNAs isolated from RF II prepared at various times in the infectious cycle and in various hosts are infectious (7, 22, 23). It was of interest to repeat and extend these observations. RF II, pulse-labeled with [^{3}H]thymidine 45 min after infection, was isolated from *E. coli ts*7 and purified. The preparation was determined to be approximately 90% pure RF II by electron microscopy. Velocity sedimentation in alkaline sucrose of the ^{3}H-labeled RF II in the presence of ^{14}C circular and linear marker DNA showed that the ^{3}H label sedimented as linear DNA (data not shown). To determine the infectious properties of linear DNA from late RF II, the ^{3}H-labeled RF II DNA was sedimented through an alkaline sucrose gradient. An infectivity assay across the gradient showed that greater than 90% of the infectious DNA was present under the circular peak (data not shown). Since the overlap of the circular DNA into the linear DNA would obscure a small amount of infectivity under the linear peak, the peak linear DNA fractions were pooled. Approximately half of the linear DNA was sedimented through a second alkaline sucrose gradient (Fig. 5, left). The other half was first hybridized to poly r(U,G), and the positive and negative strands were separated by equilibrium density centrifugation in CsCl. The positive strands were pooled and sedimented through a second alkaline sucrose gradient (Fig. 5, right).

The infectivity of the DNA of both sucrose gradients shown in Fig. 5 was determined using the same preparation of spheroplasts so the amounts of infectivity are comparable. In the left panel, the infectivity assay across the gradient of RF II linear DNA, purified by two sucrose gradients only, shows a distinct peak of infectivity under the linear DNA region as well as in the circular DNA region. It is difficult from the

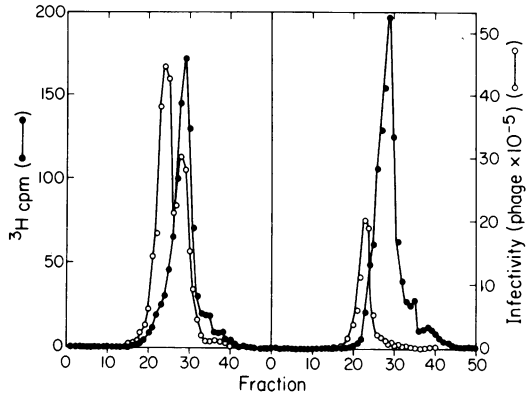

FIG. 5. *Infectivity of linear DNA from late RF II isolated from E. coli ts7. RF II, pulse-labeled with [^{3}H]thymidine 45 min after infection at 38 C in E. coli ts7, was purified, and the linear DNA was separated from circular DNA by sedimentation through an alkaline sucrose gradient. A portion of the linear DNA was sedimented through a second alkaline sucrose gradient without further treatment (left). Another portion of the linear DNA was hybridized to poly r(U,G), and the positive and negative strands were separated by equilibrium density centrifugation. The positive strands were pooled and sedimented through a second alkaline sucrose gradient (right). Symbols: ^{3}H linear DNA (●), infectivity* (O).

experiment to calculate the relative infectivity of the linear DNA versus circular DNA, but a maximum limit of approximately 5% can be set since 85% of the original circular DNA infectivity was removed by the first sucrose gradient. These results are in agreement with those of Schekman and Ray (22).

In the right panel of Fig. 5, the infectivity assay across the gradient of the linear positive-strand DNA from RF II shows only one peak of infectivity which corresponds to circular DNA. Comparison of the left and right panels shows that, by removing negative strand DNA from the preparation in the left panel, approximately one-half of the circular infectivity is removed and over 95% of the linear infectivity is removed. Unless we assume that linear negative strands are infective and positive strands are not, the experiment demonstrates that the infectivity of the linear DNA is dependent on the presence of both positive- and negative-strand DNA.

To substantiate this result, the linear, infective DNA from the gradient shown in the left panel was pooled and dialyzed. Samples were UV irradiated for varying periods of time (see Materials and Methods). The rate of UV inactivation of infectivity associated with the linear DNA was compared with that of control single-

stranded circular phage DNA and control RF II DNA. Under conditions where control single-stranded DNA was inactivated three orders of magnitude, control double-stranded RF II DNA was inactivated one-half order of magnitude and the linear infective DNA of RF II from *E. coli ts7* was inactivated approximately one order of magnitude. Thus, the linear DNA behaves more as a double-stranded DNA than as a single-stranded DNA. The probable interpretation of these experiments is that linear positive and negative strands of DNA anneal with one another, before or during the spheroplast incubation period, to form circular RF II structures which are infective. That these RF II structures do not behave completely as double-stranded DNA may reflect the presence of gapped regions of DNA or an incomplete annealing process resulting in partially single-stranded DNAs with exposed ends susceptible to exonuclease digestion. Such a partially single-stranded DNA might be expected to exhibit an efficiency of infectivity and a UV sensitivity intermediate between single- and double-stranded DNA. These results are totally consistent with the observations of Schekman and Ray (22) and Schröder and Kaerner (23), including the sensitivity of linear infectivity to exonuclease I treatment, if the annealing reaction occurs rapidly before or during the incubation of DNA with spheroplasts.

The nature of RF II isolated late in infection in E. coli ts 7. RF II DNA molecules isolated late in infection from *E. coli* H502 (a host with normal ligase activity) have a specific discontinuity in the positive strand (see Table 1; 12). These RF II molecules are the precursors of the single-stranded phage DNA. When they are pulse-labeled, the label appears almost exclusively in the positive strand (16), the asymmetric label representing phage single-stranded DNA synthesis. Since our results show that the linear DNA molecules found in phage from *E. coli ts7* are not nicked in a specific region of the genome but rather are randomly nicked, it was of interest to determine if the late RF II from *E. coli ts7* possessed a random or specific nick in its positive strand.

E. coli ts7, infected with *am3* phage at 38 C, was pulse-labeled with [^{3}H]thymidine for 10 s after 45 min of infection. The ^{3}H-labeled RF II DNA was purified (see Materials and Methods) and shown to be greater than 90% pure RF II by electron microscopy. Approximately 10% of the RF II DNA possessed visible single-stranded tails. A portion of the ^{3}H-labeled RF II DNA was combined with a mixture of ^{14}C-labeled RF I and RF II DNA marker, and the positive and negative strands were separated with the aid of poly r(U,G) by equilibrium density centrifugation in CsCl. Figure 6 is the radioactivity profile of the density gradient containing ^{3}H-pulse-labeled late RF II from *E. coli ts7* and uniformly labeled [^{14}C]RF I and RF II marker DNA. The ^{3}H pulse label of the late RF II from *E. coli ts7* appears preferentially in the positive strand. An identical pattern was obtained with late, pulse-labeled RF II from *E. coli* H502, a host with normal ligase activity (data not shown). *E. coli ts7* thus appears to be normal in terms of asymmetric single-stranded DNA synthesis.

The ^{3}H-pulse-labeled RF II DNA from *E. coli ts7* was sedimented through an alkaline sucrose gradient to separate the circular and linear strands of DNA. The ^{3}H-labeled DNA sedimented solely as linear DNA. Similar results were obtained with RF II from *E. coli* H502 (data not shown). To determine if the discontinuity of the linear DNA was in a specific region of the genome, the positive, linear-strand DNA was purified by two alkaline sucrose gradients and one CsCl equilibrium density centrifuga-

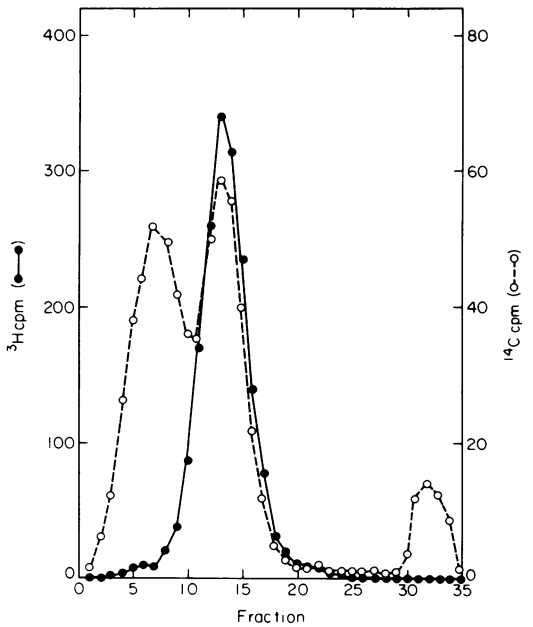

FIG. 6. *Strand distribution of pulse label in RF II DNA from E. coli ts7. Pure RF II DNA, pulse-labeled with [^{3}H]thymidine for 10 s after 45 min of infection at 38 C in E. coli ts7, was combined with a mixture of ^{14}C marker RF I and RF II DNA. The DNAs were denatured and hybridized to poly r(U,G). The resultant hybrids were separated by equilibrium density centrifugation in neutral CsCl. Symbols: ^{3}H-pulse-labeled, late RF II DNA from E. coli ts7, (●); ^{14}C marker RF I and RF II, (O).*

tion in the presence of poly r(U,G) to separate positive and negative strands. The resulting positive strand linear DNAs were hybridized to Hin II restriction nuclease fragments (Table 1). Hybridization with the largest Hin II fragment (designated in Table 1 as R_1) resulted in approximately 3% circularization, and hybridization with the third largest Hin II fragment (R_3) resulted in 33% circularization. These values may be compared with those of Table 1 (2 and 37%, respectively) for the linear, positive strand of late RF II from *E. coli* H502, a host with normal ligase activity. Although there is a small difference in the percentages of circularization obtained with the positive, linear strands of RF II from normal host the from mutant host, it is not large enough to be considered significant. The results clearly show that most of the positive, linear strands of RF II from *E. coli ts*7 possess a unique discontinuity like those of RF II from *E. coli* H502 and are distinctly different from the randomly nicked linear DNA found in phage from *E. coli ts*7.

A second method to demonstrate that the late RF II from *E. coli ts*7 possesses a discontinuity in a unique region of the genome involves the direct *Hin*d nuclease digestion of the late RF II, labeled specifically in the region of the discontinuity. Such specific labeling of late RF II was achieved by incubation with *E. coli* DNA polymerase I and deoxynucleoside triphosphates. The polymerase reaction was carried out at 15 C for 1 h. The DNA product was purified from unreacted deoxyribonucleoside triphosphates on a glass-bead column and digested with *Hin*d restriction nuclease in the presence of a ^{3}H-labeled RF II marker.

Figure 7 shows the fractionation of the *Hin*d nuclease digestion products by gel electrophoresis through a 5% polyacrylamide gel. The ^{32}P counts of the polymerase reaction appear in a high proportion in fragments 3, 5, and 8, whereas fragments 6 and 7 contain a lower amount of ^{32}P label and fragments 1, 2, 4, 9, and 10 contain essentially no ^{32}P label. A similar pattern is observed for RF II from *E. coli* H502 and corresponds closely to the extended repair reaction observed by Johnson and Sinsheimer (12). This result confirms that most of the late RF II from *E. coli ts*7 possesses a discontinuity in a specific region (the R_3 region) of the ϕX genome.

DISCUSSION

ϕX phage produced at the restrictive temperature in *E. coli ts*7 contain a small but significant proportion of linear DNA, the exact proportion being dependent on the temperature and incubation conditions. Hybridization of the linear phage DNA and Hin II nuclease fragments shows a circularization pattern similar to that produced with randomly nicked viral DNA (Table 1). The failure to observe a deficiency of any particular DNA fragment, after conversion of the linear phage DNA to a double-stranded form using *E. coli* DNA polymerase I and digestion of this RF III structure with *Hin*d nuclease, substantiates the conclusion that the linear phage DNA from *E. coli ts*7 is not nicked at a unique site or in a particular small region of the genome.

Although Iwaya et al. (11) observed that a low percent of terminal 5′-^{32}P label on linear DNA from *E. coli ts*7 phage was converted to an alkaline phosphatase-resistant form after T4 DNA polymerase and ligase treatment, a control with randomly nicked linear viral DNA was not studied; it is therefore difficult from their experiments to conclude that the linear phage DNA has some specific terminal structure other than that of randomly nicked DNA. By analysis of the 5′ terminal nucleosides of linear phage DNA from *E. coli ts*7 (by alkaline phosphatase treatment, followed by polynucleotide kinase and labeling with gamma-[^{32}P]rATP [data not shown]), we determined, in accord with Iwaya

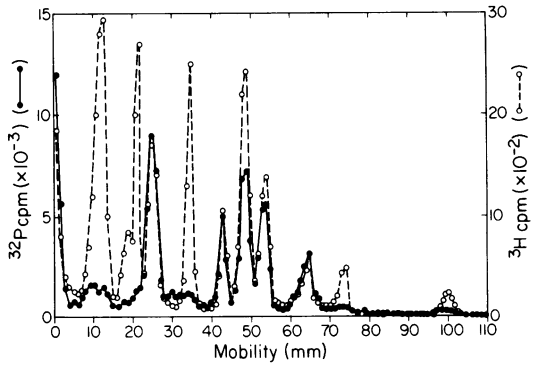

FIG. 7. *Hin II restriction nuclease fragments of polymerase-repaired, late RF II from E. coli ts7. Pure RF II (4.0 μM nucleotide equivalents), isolated 45 min after infection from E. coli ts7, was incubated for 1 h at 15 C in a 100-μliter reaction mixture of 67 mM potassium phosphate buffer, pH 7.4, 15 mM $MgCl_2$, 1 mM mercaptoethanol, 25 μM each of dCTP, dGTP, dTTP, and 1 μM ^{32}P-dATP (specific activity, 12 Ci/mmol), and 0.6 U of E. coli DNA polymerase I. The reaction products were separated from unreacted nucleoside triphosphates by a glass-bead column, combined with ^{3}H marker RF II DNA, and digested with Hin II restriction nuclease (see Materials and Methods). The digestion products were electrophoresed through 5% polyacrylamide gels for 12 h at 50 V. Symbols: ^{32}P-labeled polymerase product of late RF II, (●); ^{3}H-marker RF II DNA, (○).*

et al. (11), that there is a predominance of pG ends (approximately 40% of the 5′ ends are pG), but we do not interpret this to represent a specific end group.

The linear DNA of phage from *E. coli ts*7 is less than 0.1% (our limit of detection) as infective as circular phage DNA. The experiments of Schekman and Ray (22) concerning the infectivity levels of linear phage DNA from *E. coli ts*7 are not sufficiently detailed to permit a complete comparison with our results. However, our use of a circular, infectivity marker DNA with a differential radioactive label has permitted a very accurate determination of the level of infectivity of the linear phage DNA (unless it can be argued that the repeated centrifugations needed to remove the circular DNA differentially destroyed the intrinsic infectivity of the linear DNA). Any level of infectivity of less than 0.1% would be of questionable significance, since a very low level of negative-strand DNA may be present in phage particles.

The linear DNA derived from RF II, isolated late in infection from *E. coli ts*7, does provide a low level of infectivity (5% or less of the infectivity of circular DNA). These results are in agreement with the observation of Schekman and Ray (22). Similar infectivities have been observed for linear DNAs derived from RF II isolated from two hosts other than *E. coli ts*7 (7, 23) so that this effect is not host specific. However, we believe the infectivity observed for the linear DNA derived from RF II from *E. coli ts*7 is dependent on the presence of both negative- and positive-strand φX DNA in the linear preparation, since separation of negative-strand DNA resulted in a selective loss of infectivity associated with linear DNA (see Fig. 5). This conclusion was substantiated by a study of the rate of UV inactivation of the infectivity associated with the linear DNA. This rate of inactivation more closely resembled that for double-strand DNA than for single-strand DNA.

These results may seem to conflict with the observation that infectivity associated with linear DNA derived from RF II DNA is sensitive to *E. coli* exonuclease I digestion (22, 23). However, the results are consistent with these observations if the positive and negative linear DNAs anneal during the incubation of the DNA with spheroplasts and the annealed forms are the infective agents. Whether the annealing reaction is spheroplast facilitated is undetermined. The speed with which annealing occurs in the presence of spheroplasts would determine whether a log plot of DNA dilution versus phage output can distinguish between single- and double-strand infectivity (22).

Our results demonstrating that the linear DNA of phage from *E. coli ts*7 is not uniquely nicked were surprising, since the positive strand derived from RF II isolated from *E. coli* H502 during the period of single-stranded DNA synthesis possesses a unique nick. We considered the possibility that the RF II isolated from the mutant host, *E. coli ts*7, during single-stranded DNA synthesis did not possess a unique nick, thus explaining the presence of randomly nicked phage DNA as a result of aberrant DNA replication. The bulk of pulse-labeled late RF II from *E. coli ts*7, however, does possess a specific nick in its positive strand in the *Hin*d fragment 3 region and is asymmetrically replicated. The mechanism of φX single-stranded DNA termination and circularization still remains a question.

ACKNOWLEDGMENTS

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