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Process of Infection with Bacteriophage φX174 XXXVIII. Replication of φX174 Replicative Form In Vivo

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The replication of bacteriophage φX174 replicative-form DNA has been studied by structural analysis of pulse-labeled replicative-intermediate molecules. Such intermediates were identified by pulse-labeling with [^{3}H]thymidine and separated into four major fractions (A, B, C, and D) in a propidium diiodide-cesium chloride buoyant density gradient. Sedimentation analysis of each of these fractions suggests the following features of φX replicative-form DNA replication in vivo. (i) At the end of one cycle of replication, one daughter replicative form (RFII) contains a nascent plus (+) strand of the unit viral length, and the other daughter RFII contains small fragments of a nascent minus (−) strand. (ii) Asymmetry is also associated with production of the first supercoiled RFI after addition of pulse label in that only the minus strand becomes radioactive. (iii) A supercoiled DNA (RFI') seems to occur in vivo. This DNA is observed at a position of greater density in a propidium diiodide-cesium chloride buoyant density gradient than normal RFI. (iv) A novel DNA component is observed, at a density greater than RFI, which releases, in alkali, a plus strand longer (1.5 to 1.7 times) than the unit viral length. These results are discussed in terms of the possible sequence of events in φX174 replicative-form replication in vivo.

During infection of *Escherichia coli* with bacteriophage φX174, DNA replication has been shown to have three distinct steps (24): (i) single-stranded circular viral DNA is converted upon entry into the cell to double-stranded circular replicative form (RF) by preexisting host enzymes; (ii) RF molecules then replicate semi-conservatively to produce 20 to 30 progeny RF molecules per cell; (iii) progeny single-stranded circular viral DNA is synthesized at the final step.

In this paper we are concerned with the second step, namely, φX RF replication in vivo. To explain the sequence of events in φX RF replication, a rolling-circle molecule was proposed as a replicative intermediate (9). The rolling-circle model predicted covalent joining of the old and new strands (to produce a DNA strand longer than the unit viral length) and asymmetry of RF replication. Previous laboratory observations of φX RF replication have been mainly interpreted in accord with this model in spite of the disagreement about which strand of RF is elongated in such a rolling-circle intermediate (4, 18, 23).

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We have performed a series of experiments to study additional details of the φX RF replication mechanism. Infected cultures were pulse-labeled with [^{3}H]thymidine for intervals of 5 to 25 s to identify replicating RF intermediate molecules. After extraction and purification, the pulse-labeled DNA was separated into several different fractions in a propidium diiodide (PDI)-CsCl buoyant density gradient, and each fraction was analyzed further by sucrose velocity sedimentation and CsCl equilibrium centrifugation.

The results described in this paper support an asymmetric model of φX RF replication in that at the end of one cycle of replication the two RF partners differ in their structure and in the strand specificity of the pulse-label incorporation. It is suggested that one daughter RF contains a nascent plus (+) strand of the unit viral length and the other daughter RF contains small fragments of a nascent minus (−) strand. During RF replication in vivo a closed DNA (RFI') of low superhelicity seems to occur. This closed DNA and the normal intracellular supercoiled RF (RFI) are also produced in an asymmetric manner in that the pulse label is incorporated only into the minus strand.

We also describe a novel DNA intermediate

that is observed at a position of greater density in the PDI-CsCl buoyant density gradient than the normal RFI and releases, in alkali, a DNA piece of the plus strand longer than the unit viral length. This novel DNA is considered to be different from the rolling-circle molecule currently envisaged. These results are related to a partial sequence of events in φX RF replication in vivo.

MATERIALS AND METHODS

Phage and bacterial strains. A lysis-defective amber mutant of phage φX174, *am*3 (13), was used. *E. coli* H502 (*hcr*$^-$,*endo* I$^-$, *thy*$^-$,*su*$^-$) was used as the nonpermissive host strain for φX*am*3.

Medium and solutions. TPA medium is minimal TPG medium (26) plus 2.7 g of a mixture of 20 natural L-amino acids (Nutritional Biochemicals Corp.) per liter. Tris-EDTA is 0.05 M Tris-hydrochloride-0.005 M EDTA, pH 8.1. Tris-EDTA-borate is Tris-EDTA containing 0.05 M tetrasodium borate, pH 8.1.

Reagents. PDI was purchased from Calbiochem. Carrier-free [^{32}P]phosphate, [*methyl*-^{3}H]thymidine, [*methyl*-^{14}C]thymine, and [*methyl*-^{3}H]thymine were purchased from Schwarz/Mann. The specific activities were as noted throughout.

Infection, labeling, and DNA extraction. In a standard experiment, *E. coli* H502 was freshly grown to 5.0 × 10^8 cells/ml in 1,000 ml of TPA plus thymine (5 μg/ml) at 36 C, concentrated to 2.5 × 10^9 cells/ml in 200 ml of fresh TPA plus thymine (1 μg/ml), and treated with mitomycin C (Nutritional Biochemicals Corp.), 100 μg/ml, for 30 min (with occasional shaking) to specifically inhibit host DNA synthesis (20). The mitomycin C-treated cells were washed once with 200 ml of TPA plus thymine (1 μg/ml) and suspended in 1,000 ml of the same medium. After warming up for 15 min at 36 C, chloramphenicol dissolved in the same medium (2 mg/ml) was added to the culture at 5 min before infection to produce a final concentration of 35 μg/ml. Chloramphenicol at 35 μg/ml does not inhibit RF replication but blocks φX single-stranded DNA synthesis (26).

The culture, thus treated, was infected with φX*am*3 at a multiplicity of 5. At 2 min after infection at 36 C, 0.6 mCi of [^{14}C]thymine (100 μCi/ml, 38 mCi/mmol) was added to the culture at a final activity of 0.6 μCi/ml for long-term labeling of DNA synthesis (2 to 20 min). At 20 min after infection, the culture was pulsed by the addition of [^{3}H]thymidine (500 μCi/ml, 16 Ci/mmol) for 5 s (or longer, depending on the experiment) at a final activity of 10 μCi/ml. The pulsed culture (1,000 ml) was immediately poured into, and mixed well with, the crushed ice of Tris-EDTA (300 ml) containing 0.22 M NaN_3-0.013 M KCN in a bucket cooled in a dry ice-methanol bath. The pulsed culture could be cooled to 0 C within 10 s. A stainless-steel bucket was used to avoid possible breakage of the container during this process.

The pulsed culture was then pelleted in the cold and washed three times with cold Tris-EDTA-borate. The cells were lysed by a modified Hirt procedure (12) as follows. The cells were suspended in Tris-EDTA to a final volume of 55 ml (9.1 × 10^8 cells/ml). To this cell suspension were added 4 ml of 0.8 M EDTA (pH 8.1), 0.5 ml of tRNA (10 mg/ml in Tris-EDTA, heat-treated at 75 C for 20 min; Sigma Chemical Co.), and 5.0 ml of egg white lysozyme (10 mg/ml in Tris-EDTA; crystallized three times, grade I, Sigma). After 10 min at 36 C, 17.5 ml of Pronase (3 mg/ml in Tris-EDTA, heat-treated at 75 C for 20 min; grade B, Calbiochem) and 9.0 ml of 12% sodium dodecyl sulfate in Tris-EDTA were added to the lysates with thorough mixing by gentle rolling. The lysates were then incubated at 36 C for 4 h. At the end of the digestion, 4.0 ml of 5 M NaCl in Tris-EDTA was added to the clear cell lysates.

The clear cell lysates thus obtained were kept at 0 C overnight to precipitate sodium dodecyl sulfate and then centrifuged at 15,000 rpm for 40 min at 0 C. A major portion (ca. 90%) of host DNA pelleted in the sodium dodecyl sulfate precipitate, whereas the φX DNA (90 to 95%) remained in the clear supernatant.

The supernatant (85 ml) was then extracted once with 1 volume of redistilled phenol saturated with Tris-EDTA, at room temperature by gentle rotation for 10 min. The DNA was precipitated from the aqueous phase by adding 2 volumes of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.5) and by cooling to −20 C for 15 h.

The recovered DNA precipitate was dissolved in small volumes of Tris-EDTA, dialyzed against Tris-EDTA overnight, and concentrated to 3 ml, by the use of Ficoll (Pharmacia Fine Chemicals), in the cold. The concentrated DNA solution was treated with RNase (50 μg/ml; heat-treated at 75 C for 20 min; type 1-A, crystallized five times, Sigma) for 10 min at 36 C. To the DNA solution thus treated was added Sarkosyl NL-30 (Geigy Chemical Corp.) to a final concentration of 0.5%.

Centrifugation analyses and radioactivity measurements. Neutral sucrose gradients were linear with a gradient from 5 to 20% sucrose in 0.3 M NaCl-Tris-EDTA, pH 8.1. Alkaline sucrose gradients were linear with a gradient from 10 to 30% sucrose in 0.01 M EDTA, pH 12.7. The pH of the sample was adjusted to 12.7 with KOH before centrifugation, and 100 μg of heat-denatured calf thymus DNA was usually added as a carrier. To trap all fast-sedimenting DNA (if any), 0.5 ml of perfluorokerosene (high-boiling fraction, Pierce Chemical Co.) was placed at the bottom of the gradient as a cushion. Cellulose nitrate centrifuge tubes were coated with dimethyldichlorosilane (Bio-Rad Laboratories). Other centrifugation conditions are described in the figure legends.

PDI-CsCl buoyant density gradient centrifugations were performed as follows. The DNA sample in Tris-EDTA was mixed with 0.145 ml of PDI (5 mg/ml) in Tris-EDTA and diluted to 6.0 ml with Tris-EDTA. CsCl (5.010 g) was then added to produce the initial mean density of 1.515 gm/cm^3. The final concentration of PDI was 100 μg/ml and the final volume of the gradient was 7.260 ml. Equilibrium centrifugation

was performed with a fixed-angle type 65 Spinco rotor at 40,000 rpm for 42 h at 5 C. Cellulose nitrate tubes were used after dimethyldichlorosilane coating.

When necessary, PDI was removed from the DNA by passage through a Dowex 50W-X2 (Bio-Rad Laboratories) column. Dowex 50W-X2 was washed with 1 N KOH, adjusted with concentrated HCl to neutral pH, and washed with 10X SSC (1X SSC = 0.15 M NaCl plus 0.015 M sodium citrate). The DNA that eluted in the void volume of the Dowex column was dialyzed against Tris-EDTA and, if necessary, concentrated in the cold by using Ficoll.

Alkaline CsCl equilibrium centrifugation was performed as follows. After adjustment to pH 13.0 with KOH, the DNA sample was made up to 3.00 ml with 0.05 M potassium phosphate-0.005 M EDTA, pH 13.0. CsCl, 4.038 g, was added to the 3.00-ml alkaline DNA solution to produce the initial mean density of 1.750 gm/cm^3. The equilibrium sedimentation was carried out at 50,000 rpm for 24 h and then at 40,000 rpm for 36 h at 15 C in a type 65 Spinco rotor with polyallomer tubes. The gradient (4.022 ml) was collected dropwise (8 drops per fraction) directly on Whatman 3MM filter paper disks (2.4-cm diameter) and treated and assayed as described below. Calf thymus DNA, 400 μg, was added to the gradient as a carrier.

For radioactivity measurements, an aliquot of each fraction was placed directly on a filter paper disk (2.4-cm diameter, Whatman 3MM), washed twice with 5% trichloroacetic acid (10 ml per paper disk) and then with methanol (5 ml per paper disk), dried (65 C, 30 min), and counted in toluene scintillation fluid (Liquifluor, Nuclear-Chicago, Inc.). The window of each channel was so set that the efficiencies for ^{3}H, ^{14}C, and ^{32}P were, respectively, 60, 75, and 85% compared with those at the wide-open window. Spillover of ^{14}C into ^{3}H was 18% and among others it was below 1%.

Preparation of labeled φX DNA markers. ^{14}C- and ^{32}P-labeled single-stranded φX viral DNAs were extracted from ^{14}C- and ^{32}P-labeled φX*am*3 particles by the method of Guthrie and Sinsheimer (11). ^{32}P-labeled *am*3 particles were prepared as described previously (15). For ^{14}C-labeled *am*3 particles, *E. coli* H502 freshly grown to 4.0 × 10^8 cells/ml in 40 ml of TPA plus thymine (5 μg/ml) was transferred to a fresh 40-ml solution of TPA plus cold thymine (0.5 μg/ml) and [^{14}C]thymine (5.1 μg/ml or 1.25 μCi/ml; specific activity, 30 mCi/mmol) and infected with *am*3 at a multiplicity of 3; after incubation for 2.5 h at 36 C, ^{14}C-labeled particles were prepared as described above.

^{32}P-labeled complementary minus strand DNA was synthesized in vitro as described previously (5). For ^{32}P-labeled RFI and RFII, *E. coli* H502 freshly grown to 5.0 × 10^8 cells/ml in 20 ml of TPA with a 1/10 phosphate content plus thymine (10 μg/ml) was infected with *am*3 for 90 min at 36 C in the presence of chloramphenicol (35 μg/ml) and of carrier-free ^{32}P (1.5 mCi). The labeled φX DNA was extracted from the infected cells by the Brij procedure (19), treated with RNase (50 μg/ml), and purified by sedimentation through a neutral sucrose gradient. The RFI and RFII peak fractions were dialyzed against Tris-EDTA and stored at 2 C as ^{32}P-labeled RF markers.

RESULTS

Use of PDI-CsCl buoyant density gradient. The φX174 DNA forms were sedimented to equilibrium in CsCl buoyant density gradients containing various PDI concentrations. Figure 1 presents the buoyant separations of purified RFI, RFII, and single-stranded viral DNA in the presence of PDI at 100 μg/ml. The relative buoyant density shift was the largest for RFII, smaller for single-stranded DNA, and the least for RFI. The buoyant separations among φX DNA forms suggest that, in addition to the superhelicity of the closed DNA (2, 10), single-strandedness in the DNA structure would also influence the buoyant density in the presence of PDI, and thus replicative intermediates with a significant single-stranded region might be separated from other DNA forms in PDI-CsCl buoyant density gradients. The rolling-circle replicative intermediates for φX single-stranded viral DNA synthesis (3, 9, 15, 25) that contain single-stranded tails extending from duplex rings could, in fact, be separated in the PDI-CsCl buoyant density gradient as a broad shoulder extending from the RFII peak to the position where single-stranded DNA would band (unpublished data).

Profiles of the buoyant separations of φX DNA pulse-labeled during RF replication. All of the labeled φX DNA fractions in a neutral sucrose gradient were pooled and subjected to buoyant separation in PDI-CsCl density gradients. Before the separation, an aliquot of the pooled φX DNA was sedimented to equilibrium in an alkaline CsCl gradient to separate the plus and the minus strands. It was confirmed that both the short (^{3}H counts) and long (^{14}C counts) pulse labels in the whole φX DNA were almost equally distributed between the plus and the minus strands.

The φX DNA labeled in a 5-s pulse (^{3}H) during RF replication was separated into three distinct fractions in a PDI-CsCl density gradient (Fig. 2a): a peak (fraction A) at the nicked RF (RFII) position (ρ = 1.516 gm/cm^3); a broad shoulder (fraction B) that extends from the RFII peak to the position where single-stranded DNA would band (ρ = 1.555 gm/cm^3); and a peak (fraction D) at a buoyant density (ρ = 1.597 gm/cm^3) heavier than that of the normal, intracellular supercoiled RFI (ρ = 1.578 gm/cm^3). The DNA pulsed for 20 s contained an additional component (fraction C) peaking at the RFI position (ρ = 1.578 gm/cm^3, Fig.

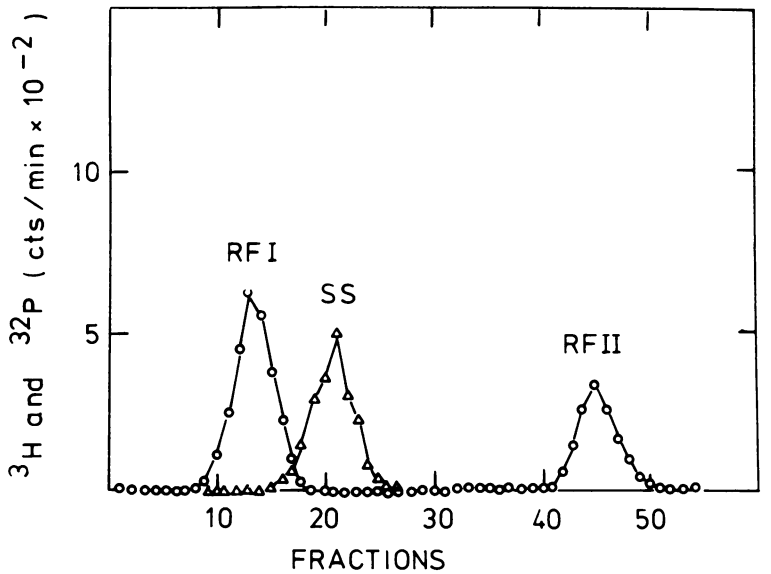

FIG. 1. *Relative buoyant separations of purified RFI, RFII, and single-stranded φX DNA in a PDI-CsCl buoyant density gradient. Purified ^3H-labeled RFI, ^3H-labeled RFII and single-stranded ^{32}P-labeled φX viral DNA were sedimented to equilibrium in a CsCl density gradient containing PDI, 100 μg/ml. The gradient was collected dropwise from the bottom of the tube directly onto Whatman 3MM filter paper disks. Symbols: O, ^3H; Δ, ^{32}P.*

2b). As the pulse time increases, pulse counts are incorporated increasingly into RFI and RFII peaks. The progeny RF molecules (^{14}C counts) are mostly closed during RF replication in the presence of chloramphenicol (Fig. 2a and b).

Analysis of RF intermediates. The four fractions (A, B, C, D) described above (Fig. 2a and b) were analyzed further by sucrose velocity sedimentation and alkaline CsCl equilibrium centrifugation.

Fractions A, B, and C. Fraction A was separated at the RFII position in the PDI-CsCl density gradient (Fig. 2). The pulse-labeled DNA from this fraction also sedimented sharply as a single peak at the RFII position (16*S*) in a high-salt neutral sucrose gradient. In an alkaline sucrose gradient, most pulse label from RFII cosedimented sharply with the linear single-stranded φX DNA of viral length (data not shown). As shown in Fig. 3, when this linear single-stranded DNA of viral length was sedimented to equilibrium in an alkaline CsCl gradient, the pulse label was found principally with the plus strand. (The asymmetry of thymine content would lead to 57% of incorporated thymine into the plus strand.) It seems that asymmetry is associated with the production of the plus and the minus strands of new RFII molecules.

The pulse-labeled DNA from fraction B sedimented as a broad peak spreading from RFII up to single-stranded DNA markers in a high-salt neutral sucrose gradient (Fig. 4a). In an alkaline sucrose gradient, almost all of the pulse label sedimented as small fragments of 7 to 12*S* (Fig. 4b). Furthermore, these DNA fragments cobanded with the ^{32}P-labeled minus strand marker in an alkaline CsCl buoyant density gradient (Fig. 4c). These results, taken together with the buoyant separation in the PDI-CsCl density gradient, suggest that the pulse-labeled DNA from fraction B is composed of RF molecules that contain small fragments of a nascent minus strand and appreciable single-stranded regions.

When progeny RF was labeled with [^{14}C]-thymine from 2 to 20 min after infection in the presence of chloramphenicol (35 μg/ml), most of the ^{14}C label appeared in fraction C (Fig. 2a and b). This fraction was separated at the RFI position in the PDI-CsCl density gradient. That the labeled DNA in fraction C is closed RFI was strengthened by results of the velocity sedimentation analysis of DNA in neutral (21*S*) and alkaline (54*S*) sucrose gradients (data not shown). However, nascent RFI molecules were not immediately produced after very short pulses (Fig. 2a); about a 20-s pulse time was needed until pulse label began to appear in RFI (Fig. 2b). Furthermore, most of the pulse label in the first RFI cobanded with the minus strand marker, whereas the long-term (^{14}C) label was distributed equally in the plus and minus strands (Fig. 5). Thus, as

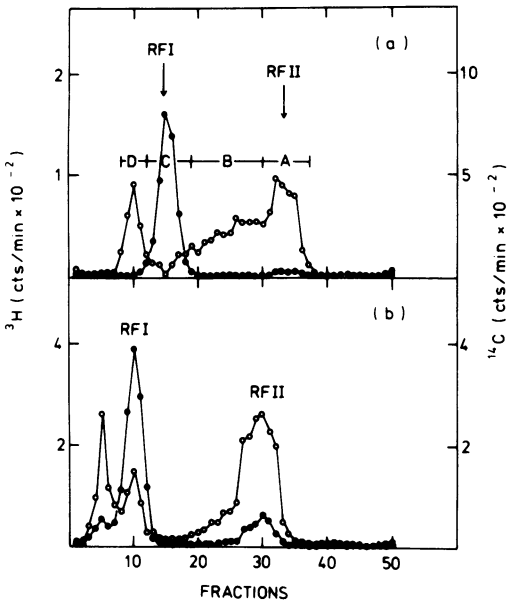

FIG. 2. *Buoyant separations in PDI-CsCl density gradients of φX DNA components pulse-labeled during RF replication. A mitomycin-treated E. coli H502 culture (1 liter of 5.0 × 10⁸ cells/ml in TPA plus thymine, 1 μg/ml) was infected with φXam3 in the presence of chloramphenicol, 35 μg/ml. At 2 min after infection, [¹⁴C]thymine was added to the infected culture (0.6 μCi/ml). At 20 min after infection, the culture was pulsed with [³H]thymidine (10 μCi/ml) for the indicated time. The labeled DNA was extracted as described in the text and purified further by sedimentation through 56 ml of 5 to 20% neutral sucrose gradients in 0.3 M NaCl Tris-EDTA (with 6 ml of CsCl cushion; density, 1.45 gm/cm³) at 25,000 rpm and 5 C for 20 h in an SW25-2 Spinco rotor. The labeled φX DNA fractions from the sucrose gradients were pooled, dialyzed against Tris-EDTA, and concentrated by Aquacide II (Calbiochem) in the cold. The labeled φX DNA was then sedimented to equilibrium in the PDI-CsCl density gradients (40,000 rpm, 42 h, 5 C, a type 65 Spinco rotor). The gradients were collected and assayed for radioactivities as described in the legend of Fig. 1. (a) A 5-s pulse, 20-μl fraction assayed; (b) a 20-s pulse, 10-μl fraction assayed. Symbols: ○, ³H; ●, ¹⁴C.*

reported previously (4), asymmetry is associated with the production of the first supercoiled RFI after addition of pulse label in that only the minus strand is radioactive.

Fraction D. The DNA in fraction D seems to exist transiently during φX RF replication. When a short pulse label (5 s) of [³H]thymidine was given to the φX-infected culture, a large proportion (30%) was incorporated in the DNA of this fraction; this proportion was progressively reduced as the pulse time increased (Fig. 2a and b). Repeated sedimentation of the DNA in fraction D to equilibrium in the PDI-CsCl density gradient confirmed its unique buoyant density in comparison with other φX DNA forms (Fig. 6).

When the DNA in fraction D after a 5-s pulse was sedimented in an alkaline sucrose gradient, a large proportion of the pulse label (63.6%) was released as a fast-sedimenting, single-stranded DNA of greater (1.5 to 1.7 times) length than the unit viral length, while the rest of the pulse label (36.3%) sedimented onto the perfluorokerosene cushion at the bottom of the gradient (Fig. 7a). That the fast-sedimenting pulse label represented an alkali-denatured RFI was shown by sedimenting the DNA (from the fraction D of a 10-s pulse) in an alkaline sucrose gradient for a shorter time. This DNA from a 10-s pulse released, in alkali, 44% of the pulse label as a long single-stranded DNA; the rest (56%) remained in the denatured RFI (Fig. 7b). The proportion of pulse label released as a long single-stranded DNA progressively decreased and that in denatured RFI increased as the pulse time increased (Fig. 7a, b, and c). No small fragments that would sediment slower than the linear single-stranded DNA of viral length were released from the pulse-labeled DNA in this fraction.

The change of relative distribution of the pulse label between released single-stranded DNA and denatured RFI, dependent on the pulse time, indicated that the DNA in fraction D is not a homogeneous component. In a separate experiment, an infected culture was pulse-labeled for 25 s, and fraction D was isolated from an initial PDI-CsCl buoyant density gradient and again sedimented to equilibrium in the PDI-CsCl buoyant density gradient. Not only the short pulse label (³H) but also the long-term label (¹⁴C) of progeny RF was evident in the peak of the fraction D (Fig. 6; also see Fig. 2b). Incorporation of the long-term label, as well as the short-pulse label, into this fraction ruled out the possibility that the appearance of fraction D was somehow an artifact associated with the short pulse.

The strand specificities of the label in the released single-stranded DNA and in the denatured RFI from fraction D were examined in alkaline CsCl buoyant density gradients. Before centrifugation, several nicks were introduced by boiling for 15 min. In fraction D from a 25-s pulse, a major portion of the pulse label was incorporated into the minus strand and only a small portion (approximately 30%) was incorporated into the plus strand (Fig. 8a). However, incorporation of the long-term label (¹⁴C) into both strands was equal. (Some

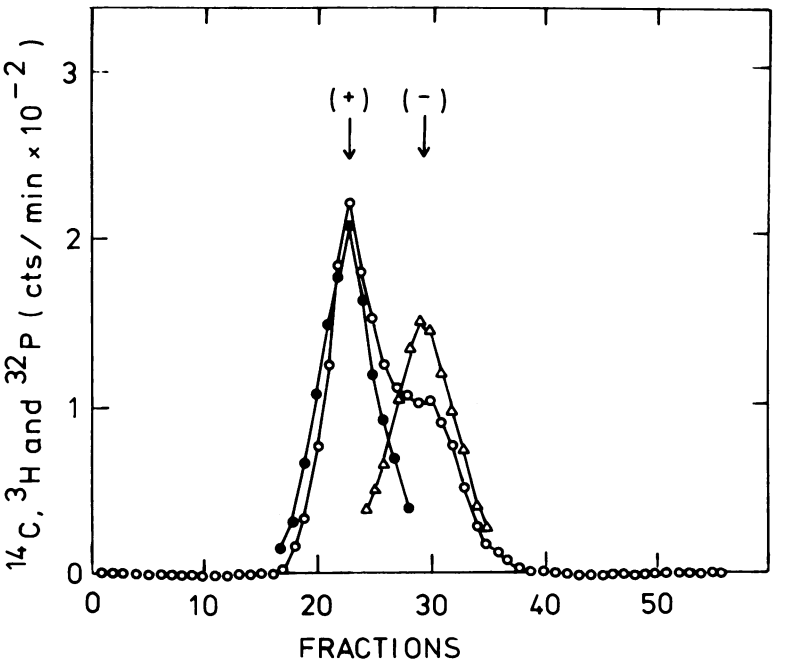

FIG. 3. *Strand specificity of the pulse label incorporation into the nascent RFII. In an experiment similar to that described in the legend of Fig. 2, φX DNA was pulse-labeled with [^{3}H]thymidine for 10 s at 20 min after infection and separated in a PDI-CsCl buoyant density gradient. The labeled φX DNA fraction corresponding to fraction A (RFII) in Fig. 2 was sedimented in an alkaline sucrose gradient (11 ml, 38,000 rpm, 18 h, 15 C). The labeled DNA of the unit viral length (14S) was then sedimented to equilibrium in an alkaline CsCl buoyant density gradient as described in the text. The gradient was collected and assayed for radioactivity as described in the legend of Fig. 1. ^{14}C and ^{32}P labels represent the viral and complementary strand DNA markers, respectively. Symbols:* O, ^{3}H; ●, ^{14}C; Δ, ^{32}P.

difference of ^{14}C counts in the two strands is due to the difference of thymine base ratio [24]). In Fig. 8b and c it is also shown that most of the short pulse label (^{3}H) released as single-stranded DNA was in the plus strand, whereas most of the short pulse label in denatured RFI was in the minus strand. Most of the long-term label (^{14}C) released as single-stranded DNA was also in the plus strand. Long-term label in the denatured RFI, however, was incorporated equally into the plus and minus strands (Fig. 8c). That almost 74% of the short pulse label in fraction D from a 25-s pulse remained in denatured RFI in alkali (Fig. 7c) explains why the entire fraction D showed asymmetric incorporation of the major pulse label into the minus strand (Fig. 8a).

Fraction D is not an artifact because of the presence of chloramphenicol (35 μg/ml) during φX RF replication. The profile of the buoyant separations of φX DNA pulse-labeled in the absence of chloramphenicol was similar to that shown in Fig. 2, and the pulse-labeled DNA of fraction D also released in alkali a long DNA piece of plus strand nature (data not shown).

From the buoyant separation in the PDI-CsCl density gradient, the RFI DNA in fraction D should contain supercoiled DNA of low superhelicity; such RF should have a lower sedimentation coefficient than does the normal RFI (fraction C). As shown in Fig. 9, in a high-salt neutral sucrose gradient fraction D from a 25-s pulse almost cosedimented with the RFII marker despite the fact that 74% of the pulse label appeared in denatured RFI in alkali.

From what has been described above it is clear that the DNA in fraction D consists of at least two types of DNA, which happen to have the same buoyant density in the PDI-CsCl density gradient: (i) DNA (closed or open) that releases, in alkali, long single-stranded DNA of the plus-strand nature. (ii) supercoiled RF (RFI') of lower superhelicity than the normal RFI (fraction C). This DNA might be the precursor of the normal RFI. It is pulse-labeled first in the minus strand as the normal RFI.

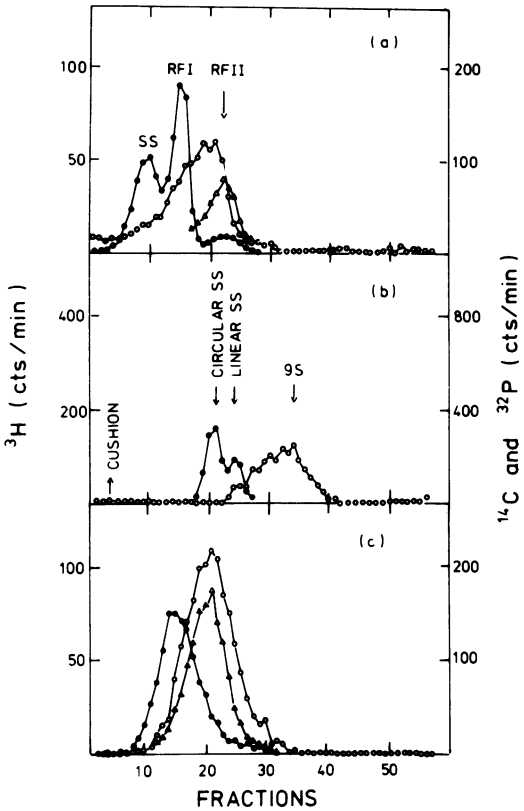

FIG. 4. *Sedimentation studies of the labeled φX DNA in fraction B. The labeled φX DNA fraction corresponding to fraction B from a 10-s pulse with [^{3}H]thymidine at 20 min after infection (in the presence of chloramphenicol) was subjected to the sedimentation analyses. (a) High-salt neutral sucrose gradient (12 ml, 29,000 rpm, 18 h, 5 C, SW41 Spinco rotor). ^{14}C-labeled RFI, ^{32}P-labeled RFII, and ^{14}C-labeled single-stranded φX DNA markers were added to the gradient before run. (b) Alkaline sucrose gradient (11 ml, 38,000 rpm, 18 h, 15 C, SW41 Spinco rotor). ^{14}C-labeled single-stranded φX viral DNA (circular and linear, of the unit viral length) and 100 μg of calf thymus DNA were added to the gradient. The gradient contained 0.5 ml of a perfluorokerosene cushion. (c) Alkaline CsCl buoyant density gradient. The small fragments of labeled φX DNA (fractions 28 to 40) from (b) were dialyzed against Tris-EDTA and mixed with ^{14}C-labeled viral and ^{32}P-labeled complementary strand ΦX DNA markers and 400 μg of calf thymus DNA as a carrier before centrifugation. The gradients were collected and assayed for radioactivities as described in the legend of Fig. 1 (for further details about the centrifugation procedures, see the text). Symbols: O, ^{3}H; ●, ^{14}C; Δ, ^{32}P.*

DISCUSSION

The differential relative buoyant density shifts in the CsCl density gradient containing PDI enable discrete separations of φX RF and single-stranded DNA molecules. In addition, RFII molecules with appreciably long single-stranded tails and incomplete RFII molecules with single-stranded regions can be separated from the other DNA forms as a broad shoulder spreading toward higher density from the RFII peak (Fig. 2 and unpublished data). We have also observed that short pulse-labeled DNA of *E. coli* 1000 (*polA1*), which gives rise to small fragments (22), is separated in a PDI-CsCl buoyant density gradient as a broad shoulder between the buoyant density positions of φX RFII and single-stranded φX DNA (unpublished data). It was reported previously that such short pulse-labeled duplex DNA contains single-stranded gaps between Okazaki fragments (28).

These observations indicate that single-strandedness in duplex DNA influences these relative buoyant density shifts. In addition, the relative buoyant density shift in the presence of intercalative dye is influenced by the extent of dye binding as determined by the degree of superhelicity (or other constraints to unwinding) and to a lesser extent by guanine plus cytosine content and molecular weight (2, 10). The isolation, from an ethidium bromide-cesium chloride buoyant density gradient, of closed DNA molecules of different superhelicity has been reported among closed DNA forms of simian virus 40 and PM2 (6, 8). Here we have also described the isolation, by PDI-CsCl buoyant density gradient centrifugation, of a closed φX DNA of superhelicity lower than that of normal RFI that is accumulated in the infected cells.

Based on the results described in this paper we are able to point out several features of φX RF replication in vivo. (i) The first RFII detected after addition of a pulse label becomes radioactive preferentially in the viral plus strand. The size of the pulse-labeled plus strand is the unit viral length (14*S*). (ii) Small fragments of the complementary minus strand occur during RF replication and are mostly associated with RF II containing single-stranded regions. (iii) Asymmetry is also associated with the appearance of the first supercoiled RFI, after addition of pulse-label, in that only the minus strand is radioactive. (iv) A supercoiled form of RF (RFI') seems to occur in vivo, that has lower superhelicity than the normal RFI principally accumulated in the φX-infected cells. (v) An unusual DNA form of transient existence is associated with RF replication in vivo. This DNA is found in the PDI-CsCl buoyant density gradient at a greater density than the normal RFI (actually, at the same position as the

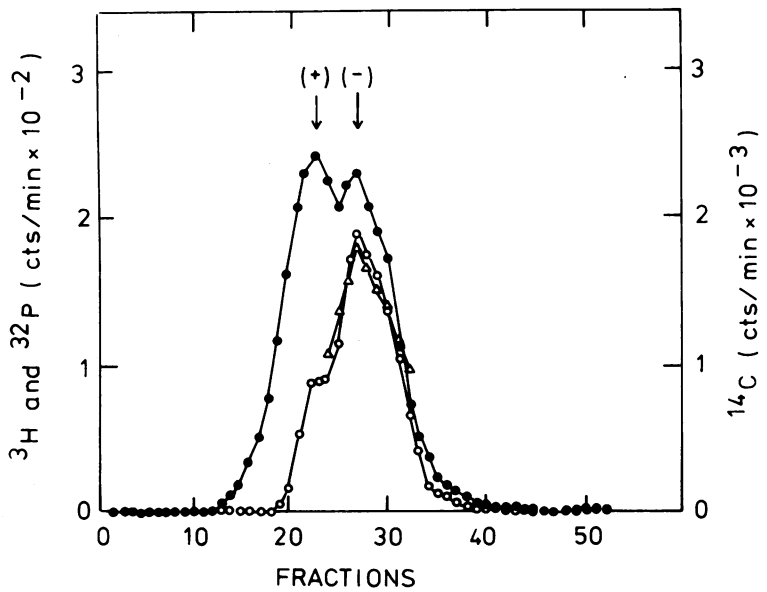

FIG. 5. *Strand specificity of the pulse label incorporation into the nascent RFI. In an experiment similar to that described in the legend of Fig. 3, the labeled φX DNA fraction corresponding to fraction C (RFI) from a 25-s pulse with [^3H]thymidine was sedimented to equilibrium in an alkaline CsCl buoyant density gradient. Before centrifugation, several nicks were given to the labeled RFI by boiling for 15 min. ^{14}C label represents the long-term label of RFI with [^{14}C]thymine from 2 to 20 min after infection, and ^{32}P label represents the complementary minus-strand marker. Symbols:* O, ^3H; ●, ^{14}C; Δ, ^{32}P.

RFI' of low superhelicity) and releases, in alkali, a plus strand longer (1.5 to 1.7 times) than the unit viral length.

These findings may tentatively be interpreted as showing that φX RF replication is an asymmetric event in that at the end of one cycle of replication one daughter RF contains a nascent plus strand of the unit viral length and the other daughter RF contains small fragments of a nascent minus strand. The latter RF is completed first to a closed RF.

This interpretation suggests that the plus strand replicates continuously and the minus strand replicates discontinuously. However, the possibility is not rigorously excluded that small fragments of the plus strand exist but are more rapidly joined to larger units than those of the minus strand and, thus, were not detected in the present experiments.

Eisenberg and Denhardt (7) have recently reported that there exist gaps at many locations in RFII, mainly in the minus strand, and that these gaps can be filled by joint action of DNA polymerase and DNA ligase, resulting in elongation of the small fragments into unit viral length. This observation strengthens the suggestion that one of the two nascent RF partners (incomplete RF) after a round of replication is a gapped molecule in which the minus strand is discontinuous.

As observed previously (4), the first supercoiled RFI produced after the addition of pulse label is radioactive only in the minus strand (Fig. 5). This asymmetry of the pulse label incorporation into the minus strand is also seen in the closed RFI' of low superhelicity (Fig. 8c). The closed RFI' is labeled in a short pulse faster than the normal RFI, whereas the normal RFI is accumulated predominantly in the φX-infected cells (Fig. 2a and b). It is plausible that the closed RF' exists transiently and is the precursor of the normal RFI. However, the possibility is not rigorously excluded that the closed RFI' of low superhelicity is formed by DNA ligase action (specifically to seal nicks on only the labeled minus strand of the nascent RFII) during φX DNA extraction from the infected cells.

The possibility that fraction D is not, in fact, φX DNA but an episomal element in the host cells is excluded by the observation that uninfected host cells, pulse-labeled under identical conditions, did not give rise to a peak of pulse label at the fraction D position.

Neither the structure of the novel DNA in fraction D nor its involvement in the RF replication events is clear. Its position in the PDI-CsCl density gradient is most simply interpreted as indicative of a limited binding of PDI, more comparable to that of RFI than RFII,

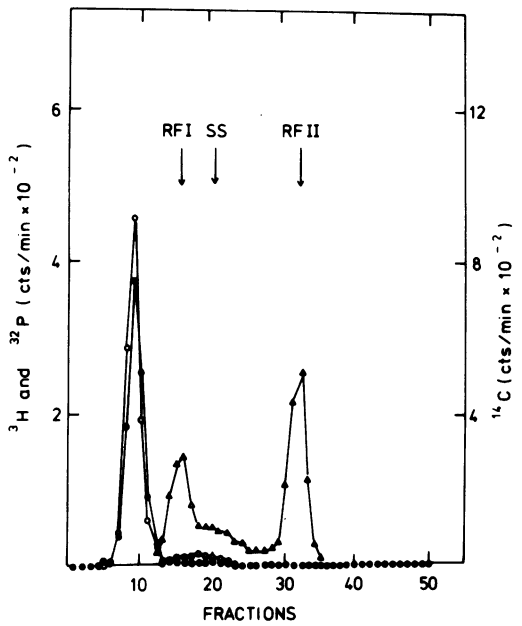

FIG. 6. *Buoyant separation of the pulse-labeled DNA in fraction D in the PDI-CsCl density gradient. In an experiment similar to that described in the legend of Fig. 2, φX DNA was pulse-labeled for 25 s with [^{3}H]thymidine at 20 min after infection (in the presence of chloramphenicol) and fractionated in a PDI-CsCl buoyant density gradient. The labeled φX DNA fraction corresponding to fraction D (Fig. 2) was again sedimented to equilibrium in a PDI-CsCl density gradient to confirm the unique density of this DNA fraction. ^{14}C label represents the long-term label of φX DNA with [^{14}]thymine from 2 to 20 min after infection. ^{32}P-labeled RFI, ^{32}P-labeled RFII, and ^{32}P-labeled single-stranded φX viral DNA markers were added to the gradient. Other experimental conditions were the same as those described in the legend of Fig. 1. Symbols: O, ^{3}H; ●, ^{14}C; Δ, ^{32}P. Arrows show the buoyant positions of the RFI, RFII, and single-stranded φX viral DNA.*

together with some single-stranded character. It may have a structure similar to that observed in bacteriophage M13 single-stranded DNA synthesis (14) or to that observed (R. Espejo and R. L. Sinsheimer, J. Mol. Biol., submitted for publication) during φX single-stranded DNA synthesis—an RF ring in which both ends of a nicked, elongated viral strand are free in the solvent, unbonded to the complementary strand but hydrogen-bonded to each other. The resistance to free rotation of one strand about

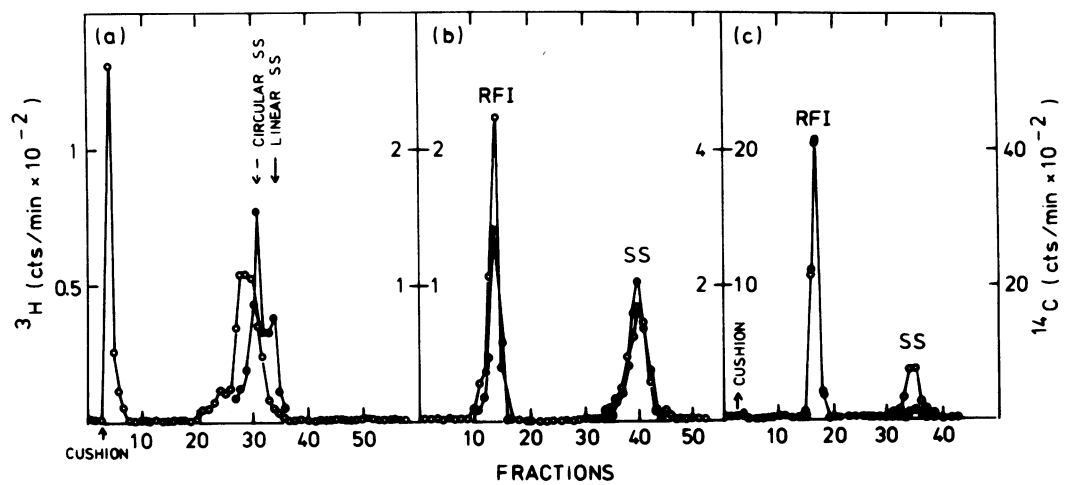

FIG. 7. *Velocity sedimentation of the pulse-labeled φX DNA of fraction D in alkaline sucrose gradients. In Fig. 2, 4, and 6, φX DNA was pulse-labeled for 5, 10, and 25 s, respectively, with [^{3}H]thymidine at 20 min after infection in the presence of chloramphenicol (35 μg/ml) and fractionated in PDI-CsCl buoyant density gradients. After PDI removal, dialysis against Tris-EDTA, and concentration by Ficoll, the labeled DNA fractions corresponding to fraction D were subjected to sedimentation through 11 ml of 10 to 30% alkaline sucrose gradients in an SW41 Spinco rotor under the conditions indicated below. The gradients contained calf thymus DNA carrier (100 μg) and 0.5 ml of a perfluorokerosene cushion when so indicated. The gradients were collected and assayed as described in the legend of Fig. 1. (a) DNA pulse-labeled for 5 s. Sedimentation was at 41,000 rpm and 15 C for 15.5 h. ^{14}C-labeled single-stranded viral DNA (circular and linear) marker was added. ^{3}H-pulse-labeled super coiled RF denatured in alkali was trapped on the perfluorokerosene cushion. (b) DNA pulse-labeled for 10 s. Sedimentation was at 41,000 rpm and 15 C for 5 h. ^{14}C-labeled single-stranded viral DNA and ^{14}C-labeled RFI markers were added. (c) DNA pulse-labeled for 25 s. DNA separated twice in PDI-CsCl buoyant density gradients (Fig. 6) was used. ^{14}C label represents the long-term label of φX DNA with [^{14}C]thymine from 2 to 20 min after infection. Sedimentation was at 27,000 rpm and 15 C for 11.5 h; 20 μl of each fraction was assayed. Symbols: O, ^{3}H; ●, ^{14}C*

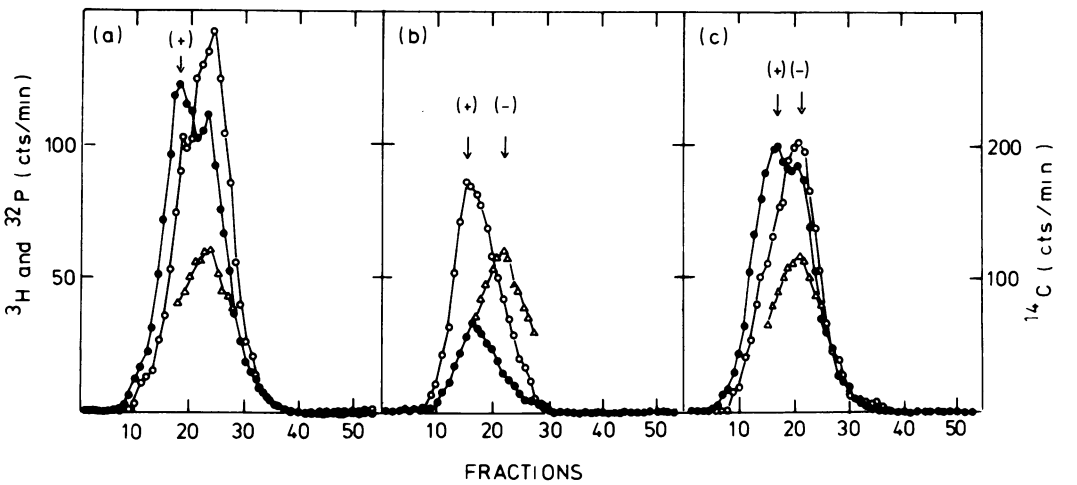

FIG. 8. *Strand specificity of pulse label in the DNA of fraction D. The fraction D component from a 25-s pulse with [^{3}H]thymidine (Fig. 6) and the single-stranded DNA and the denatured supercoiled RF released in alkali from fraction D (fractions 31 to 38 and 15 to 18 in Fig. 7c) were nicked by boiling in Tris-EDTA for 15 min and sedimented to equilibrium in alkaline CsCl density gradients, using the same procedures as described in the legend of Fig. 3. ^{14}C label represents the long-term label of φX DNA with [^{14}C]thymine from 2 to 20 min after infection. ^{32}P-labeled complementary minus-strand DNA marker was added. (a) Whole fraction D component from a 25-s pulse. (b) Single-stranded DNA piece released in alkali from fraction D. (c) Alkali-denatured supercoiled RF from fraction D. Symbols: ○, ^{3}H; ●, ^{14}C; △, ^{32}P. Arrows show the buoyant positions of the viral (+) and complementary (−) strand DNA.*

the other in such a structure inhibits PDI binding comparably to RFI.

We cannot be certain that this component is a true intermediate in the course of RF replication (although, in at least the shortest pulses used, the incorporation of label into this form must be included to account for equimolar synthesis of viral and complementary strands). Conceivably, even in the presence of chloramphenicol as employed here, a low level of abortive asymmetric viral strand synthesis (and subsequent degradation) may occur.

In the course of the experiments described in this paper, attempts have been made to isolate putative rolling-circle molecules. The rolling-circle molecules that are currently envisaged for φX replication (4, 9, 18, 23) would naively be expected at the buoyant position of RFII or as a dense shoulder spreading from RFII peak (fractions A or B in Fig. 2) in a PDI-CsCl buoyant density gradient, depending on the extent of single-strandedness in the DNA molecules. When φX DNA pulse-labeled for 5 s was separated in a PDI-CsCl buoyant density gradient (Fig. 2), no DNA was found (in either fraction A or B) that sedimented more rapidly than the RFII marker in a high-salt neutral sucrose gradient and also released, in alkali, a DNA piece longer than the unit viral length (Fig. 4b and unpublished data). DNA sedimenting faster than RFII in a high-salt neutral sucrose gradient was found in the fraction B (Fig. 4a). The fast-sedimenting DNA, however, did not release, in alkali, a DNA piece longer than the unit viral length. Instead, it released, in alkali, only small fragments of the minus strand (Fig. 4b and c). It is thought to be incomplete RFII (see above).

Schröder and Kaerner (23) have reported isolation of a pulse-labeled DNA from φX-infected cells, that sedimented ahead of RFII in a neutral sucrose gradient and released in alkali a plus-strand DNA longer than the unit viral length. This DNA molecule was interpreted as the rolling-circle molecule as proposed by Gilbert and Dressler (9) and Dressler and Wolfson (4). When the DNA of fraction D was sedimented in a high-salt netural sucrose gradient, an appreciable portion of the pulse-labeled DNA (^{3}H counts) was found to sediment faster than RFII (Fig. 9). It seems likely that this fast-sedimenting DNA is the novel DNA that releases, in alkali, long DNA of the plus-strand nature (see above) and that the long DNA piece isolated by Schröder and Kaerner (23) thus derives from the novel DNA of our fraction D. The rolling-circle molecules that are envisaged for φX RF replication would not band at the fraction D position in the PDI-CsCl buoyant density gradient. In spite of un-

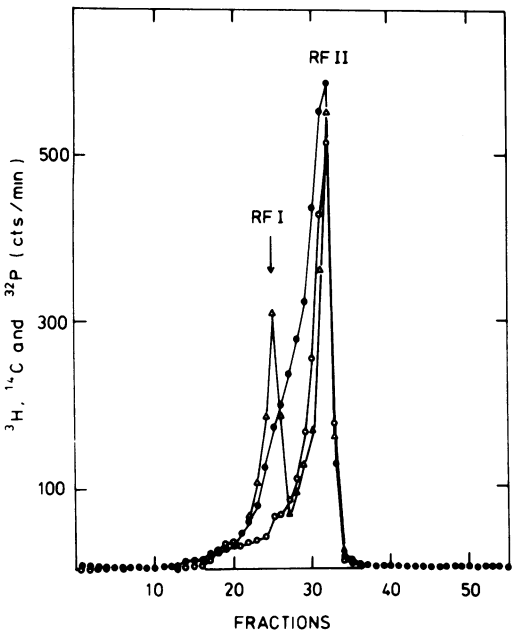

FIG. 9. *Sedimentation in a neutral sucrose gradient of the pulse-labeled DNA in fraction D. The whole fraction D component from a 25-s pulse (Fig. 6) was sedimented through 12 ml of a 5 to 20% sucrose gradient in 0.3 M NaCl Tris-EDTA at 31,000 rpm for 12 h at 5 C in an SW41 Spinco rotor. PDI was removed from the DNA before run. ^{32}P-labeled RFI and ^{32}P-labeled RFII markers were added. ^{14}C label represents the long-term label of φX DNA from 2 to 20 min after infection. The gradient was collected and assayed as described in the Legend of Fig. 1. Symbols: ○, ^{3}H; ●, ^{14}C; △, ^{32}P. Arrows show the sedimentation positions of RFI and RFII.*

certainty of its true involvement in the replication events, the structure of the novel DNA should be elucidated for further understanding of φX RF replication.

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