*MULTIPLE-LENGTH RINGS IN PREPARATIONS OF φX174 REPLICATIVE FORM**

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The bacterial virus φX174 has been shown, in studies by Sinsheimer and his co-workers,[1-3] to contain single-stranded DNA in the form of a covalently closed circle. During DNA synthesis, a double-stranded species is formed, RF,[4] that serves not only as a template for its own replication, but also for that of single-stranded viral DNA.[6, 7] Preparations examined thus far by electron microscopy have been found to consist of circles with a contour length of 1.64 μ.[8] We have prepared RF by a modification[9] of the method of Jansz, Pouwels, and Schiphorst,[10] and by a new procedure employing ethidium bromide in the manner suggested in a recent paper by Radloff, Bauer, and Vinograd.[11] These preparations, when examined in the electron microscope by a modified protein monolayer method,[12] show not only forms I, II, and III,[4] as previously described, but also a significant number of circular forms of double, triple, and several times the most frequent length. Some additional properties of RF and of the multiple-length forms are described in this communication.

Materials and Methods.—Preparations: Cultures of *E. coli* C and φX174 were obtained from Dr. Robert L. Sinsheimer, chloramphenicol from Parke, Davis and Co., and ethidium bromide from Boots Pure Drug Co. Ltd. RNase was purchased from Sigma Chemical Co., cesium salts from Gallard-Schlesinger Chemical Mfg. Corp., and cytochrome *c* from Nutritional Biochemicals Corp. Hercules nitrocellulose, "cubed, 1/4 sec" was obtained from Randolph Products Co.

Ultracentrifugation: Analytical sedimentation was carried out in the Spinco model E ultracentrifuge equipped with a photoelectric scanning system (Beckman Instruments Co.). Velocity runs were made by the banding method introduced by Vinograd *et al.*[13] and further developed by Studier.[14] A volume of 10 μl was layered onto 0.35 ml of 1 *M* NaCl containing 0.05 *M* phosphate buffer, pH 6.7 in a double-sector cell. Analytical buoyant density runs were made at 39,640 rpm using either CsCl or Cs_2SO_4. Five cells, each with an upper sapphire 1° window, were run simultaneously. Densities were calculated either from a marker or from the input density according to the method of Ludlum and Warner.[15] Equilibrium sedimentation in CsCl containing ethidium bromide (100 μg/ml) was performed in the Spinco model L ultracentrifuge using a type 65 angle head rotor at 50,000 rpm and 20°. The bands were directly visible when they contained about 20 μg of DNA; lower amounts became visible upon illumination at 365 mμ with a Mineralight lamp. Drops were collected by means of a microsiphon. A measured volume of a slurry of Dowex-50 (Na^+ form) was added to each fraction in order to remove the ethidium cation. The resin was allowed to settle and 0.1-ml aliquots of the supernatant were examined spectrophotometrically at 260 mμ. As long as the resin was in sufficient excess over the ethidium, a constant blank reading was obtained. Sucrose density gradients were run at 7° in the Spinco SW 65 rotor at 65,000 rpm. Following drop collection, samples were assayed by spectrophotometry or, in the case of P^{32}-labeled preparations, by scintillation counting.

Electron microscopy: The spreading procedure[12, 16] was used in the examination of all preparations. The spreading solution contained 1–2 μg/ml DNA, in1 *M* ammonium acetate, and 0.01% cytochrome *c*. The pH was adjusted to 5 with acetic acid unless otherwise specified. The subphase consisted of 70 ml 0.3 *M* ammonium acetate at the same pH as the spreading solution. It was contained in a Teflon-coated aluminum trough (coated by Tripoint, Inc., Commack, N.Y.). In order to prevent stretching of the DNA filaments, the salt concentration was not allowed to drop below 0.3 *M*.[17] A wetted glass ramp[12] reaching into the surface of the subphase was used

to apply the spreading solution. After spreading a sample of less than 0.1 ml, the surface film was slightly compressed by bars, so that talcum particles previously sprinkled on the surface became immobile, marking the film boundary. A diffusion procedure[18] has also been employed on several preparations and will be used in more detailed studies.

Carbon-coated supports (platinum grids, Siemens type, with 7 or 9 holes, 70 μ diameter, or with slits 1 $\times$ 0.05 mm), or carbon-reinforced Formvar support films were used. Uranium was slowly deposited onto the specimens while the horizontally mounted, film-covered grids were rotated (60 rpm) in a metal, high-vacuum evaporator (Bendix-Balzers BA 350 S) at an 84° angle of incidence for 20–30 minutes.

Electron micrographs were taken with a Siemens Elmiskop IA microscope using a replica grating (2,160 lines/mm; Fullam, Inc.) as a reference, at magnifications of 7,205 $\times$ ($\pm$ 1%). Ratios of filaments and rings were counted, and contour lengths of the DNA molecules were measured, on tracings of enlarged images.

RF preparations: (*Method A*) RF was prepared according to a modification[9] of the method of Jansz *et al.*[10] In outline, this procedure involved infection of *E. coli* C (10^9 cells/ml) with ϕX174 at a multiplicity of 5, chloramphenicol (40 μg/ml) addition 6 minutes after infection, and vigorous aeration for 50 minutes. The washed cells were lysed with alkali at pH 12.5 and neutralized. The lysate was treated with RNase, heated to 80°, and after removal of a precipitate by centrifugation was chromatographed on Sephadex G-100. The excluded fraction was concentrated and passed through a nitrocellulose column which retained the single-stranded material. The eluate was concentrated by rotary flash evaporation at 30°. The average yield per liter of infected culture was 300 μg of RF.

P^{32}-labeled RF was prepared as described by Pouwels *et al.*[19] except that dephosphorylated casamino acids were not employed and 20 mc P^{32}-orthophosphate were added per liter of infected cells. A specific activity of 5,000 cpm/μg of DNA was obtained.

(*Method B*) RF was also prepared by a method employing ethidium bromide and not requiring exposure to pH 12.5. The method is based on the finding by Radloff *et al.*[11] that closed circles of double-stranded DNA bind less ethidium and, therefore, have a greater buoyant density than linear DNA. The washed cells were lysed with lysozyme. The lysate was treated with RNase and fractionated on Sephadex as described above. Form I of RF was separated from native host cell DNA and form II by centrifugation in CsCl containing 100 μg/ml of ethidium bromide. Two bands were obtained as illustrated in Figure 2*B*. The fractions corresponding to each of the two bands were pooled and the ethidium cation was removed with Dowex-50 (Na^+ form). The heavy band contained only form I as shown by band centrifugation. A yield of 45 μg of form I has been obtained from 500 ml of infected culture.

In both methods of preparation a considerable enrichment in RF, in relation to host cell DNA, is obtained by preferential precipitation of the latter in the centrifugation following RNase treatment.

Results.—Properties of RF: Band sedimentation analysis of material prepared by Method A indicated the presence of two components. A pattern is reproduced in Figure 1*A* in which form I, $s_{20,w} = 21S$, comprises about 90 per cent, and form II, $s_{20,w} = 17S$, 10 per cent of the sedimenting material. In six preparations, the amount of I varied from 60 to 90 per cent. Since any form II initially present in the lysate would be eliminated during alkali denaturation, its appearance in the final product is attributed to an introduction of single-strand breaks into form I during preparative manipulations. The buoyant densities of RF in Cs_2SO_4 and CsCl were found to be 1.427 and 1.707, respectively. A scan of a Cs_2SO_4 gradient is shown in Figure 1*B*. The absence of material banding near a density of 1.46 demonstrates a complete removal of denatured host cell DNA. Centrifugation of an RF preparation (Method A) in ethidium bromide-CsCl showed two bands corresponding to forms I and II (Figure 2*A*). This confirms the assignment of components identified by velocity sedimentation. The separation of the bands was 0.032 density units. This is similar to that observed for polyoma DNA and sup-

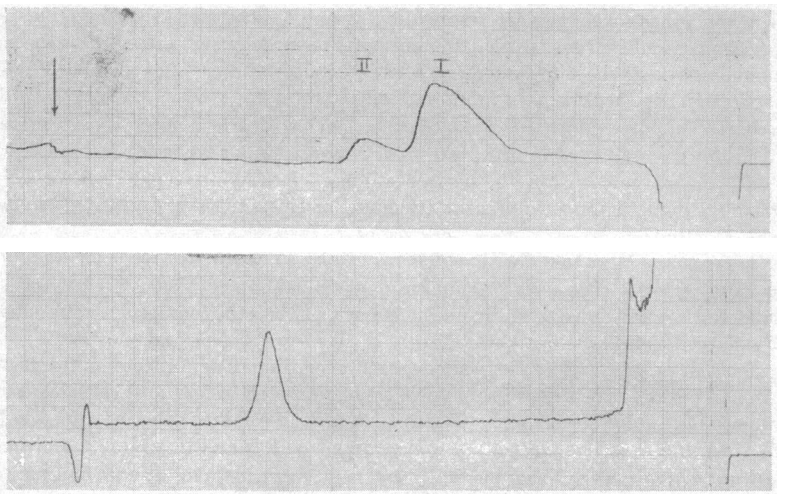

FIG. 1.—Ultracentrifuge scans of RF preparations prepared by Method A. *Upper scan:* Band velocity sedimentation after 39 min at 50,740 rpm and 20°. 10 μl of a solution containing 1 μg of DNA was layered onto 1 *M* NaCl, 0.05 *M* phosphate buffer, pH 6.7, in a 12-mm double-sector cell. The meniscus is indicated by an arrow. The 21*S* peak corresponds to form I and the 17 *S* peak to form II. *Lower scan:* Cs_2SO_4 equilibrium density gradient after 20 hr at 39,460 rpm and 25°. 0.4 μg DNA was centrifuged to equilibrium in Cs_2SO_4 with a density of 1.45 over a small volume of FC 43 fluid in a double-sector cell against Cs_2SO_4 of the same density. The buoyant density of the band was 1.427.

ports the prediction of Vinograd *et al.*[5] that form I of RF-ϕX contains supertwists. The number of supertwists has been estimated by Crawford and Waring[20] to be about 12 right-handed twists for a molecular weight of 3.4×10^6 daltons. A similar estimate has been made by Bauer and Vinograd.[21]

In developing the preparative procedures described as Method B, the ethidium separation was first applied to the Sephadex eluate obtained by Method A. Two bands were obtained having the same densities as those in Figure 2*A*. The light band was shown by electron microscopy to contain denatured DNA, presumably derived from *E. coli*, in addition to RF. The heavy band consisted entirely of form I, as shown by velocity sedimentation and electron microscopy. When introduced at an earlier stage of the preparative procedure the ethidium bromide technique eliminates the need for an alkali extraction step, and preparations made in this manner have followed the procedure outlined in Method B. The separation of RF (in this case entirely form I) from host-cell DNA, is shown in Figure 2*B*. The product obtained in this way had a sedimentation coefficient of 21*S* and a buoyant density in Cs_2SO_4 of 1.428.

Electron microscopy: In electron micrographs, RF appears almost entirely as rings. Double- and multiple-length rings are randomly scattered over the fields and were present in all preparations obtained by both Methods A and B with a frequency of 2–3%. A field chosen to include three double-length rings is shown in Figure 3*A*, and one with a triple-length ring in Figure 3*B*. The rings appear untwisted in preparations spread at pH 5, in spite of the demonstration by centrifugation that the RF is 60–90% form I. In samples spread at pH 7, twisted rings

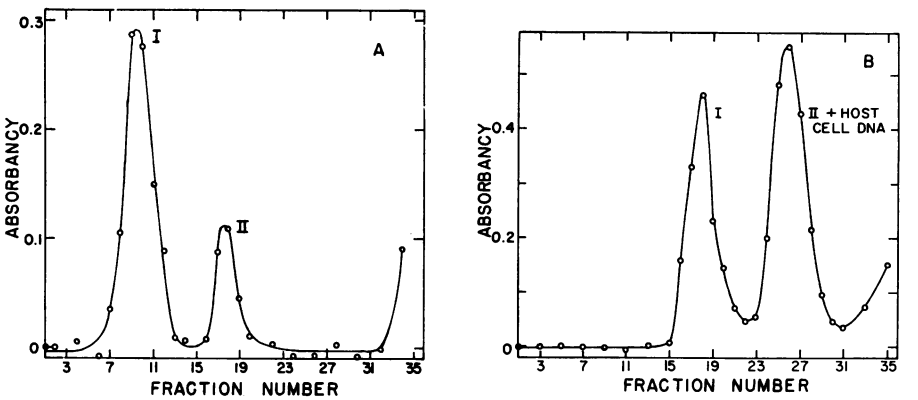

FIG. 2.—Equilibrium density gradients in CsCl containing ethidium bromide. The solutions contained 100 μg/ml of ethidium bromide and were centrifuged for 40 hours at 50,000 rpm. The top of the gradient is on the right. (*A*) Purified RF (30 μg, Method A) in 5.0 ml CsCl, density 1.58. The denser band contains 76% of the DNA and consists of form I. The same preparation was found to contain 70% of form I by velocity band sedimentation. Optical densities were obtained as described in the text. An absorbancy blank of 0.12 has been subtracted. Band I has a buoyant density of 1.612 and band II of 1.580. (*B*) Preparation of form I, RF, by Method B. Concentrated eluate containing 65 μg of DNA from the Sephadex column step of Method B was centrifuged in 5.0 ml of CsCl, density 1.60. Band I has a buoyant density of 1.598 and the light band one of 1.568.

predominate, as has been reported by others for RF-ϕX and other circular DNA's.[5, 22-24] This is illustrated in Figure 3*C*. Similar preparations, in which most of the rings were untwisted, were also obtained when the diffusion procedure was carried out at pH 5. Multiple-length rings were present in *both* bands of an ethidium bromide gradient of purified RF, such as that shown in Figure 2*A*. There is no indication of a greater fraction of single-strand scissions in the multiple-length rings as compared to those of normal length. Statistical studies were not attempted.

The length distributions in an RF preparation consisting of about 70% form I, as shown both by band sedimentation and ethidium bromide separation, is summarized in Figure 4. The mean values found were 1.63, 3.15, and 4.56 μ for the single, double, and triple lengths. The latter two are slightly less than exact multiples of the single size. Higher multiples occur, but not with sufficient frequency for statistical treatment. Linear forms were present to the extent of 1.4%. Most of these were derived from broken rings as indicated by the length distribution given in Figure 4*C*. Occasionally longer, linear forms (up to 17 μ) were present, presumably derived from host cell DNA.

Since neither Method A nor B employs the commonly used phenol extraction, a purified RF sample (Method A) was treated with phenol, extracted with ether and reexamined as a control. Multiple-length forms were still evident in electron micrographs.

A control (noninfected) culture of *E. coli* grown to 10^9 cells/ml and exposed to chloramphenicol, was processed by Method A and the fractions corresponding to those ordinarily containing RF were concentrated and examined for circular DNA. None was found at a level of concentration equivalent to 20-fold that of the usual method.

Enrichment of multiple-length forms: DNA (RF) preparations enriched in

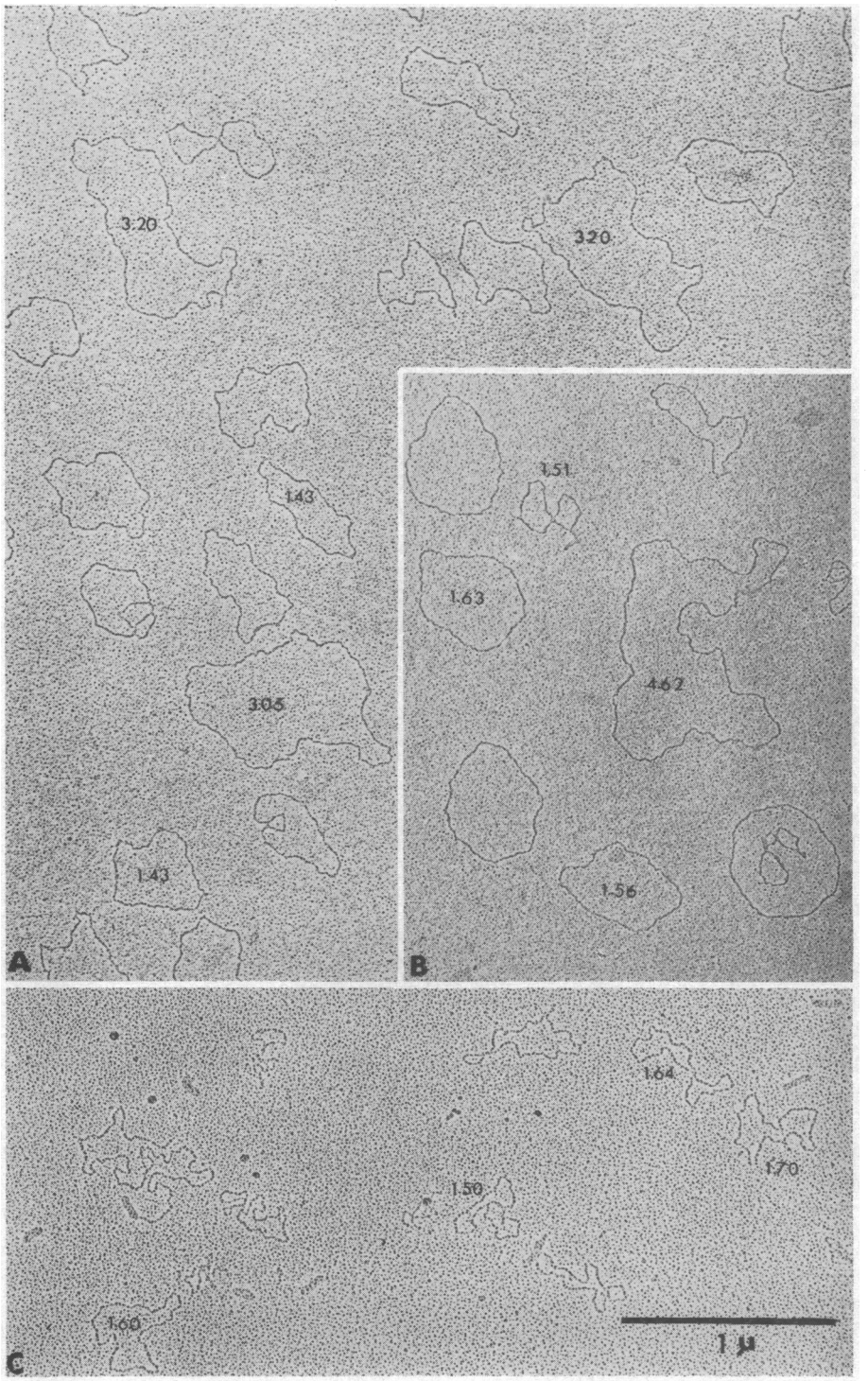

FIG. 3.—Electron micrographs showing multiple-length rings in an RF preparation. Magnification, 30,000 ×. Some contour lengths in microns are given on the figure. *A* and *B* show fields containing multiple-length rings from a preparation spread at pH 5. The same preparation spread at pH 7 showing predominantly twisted rather than open circles is shown in *c*.

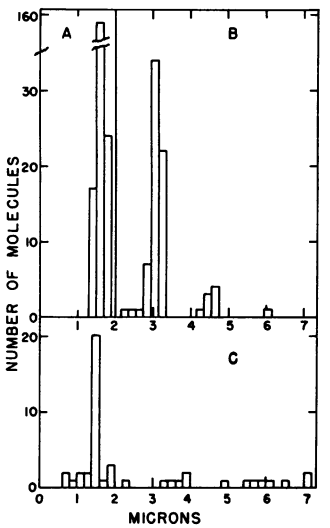

FIG. 4.—Contour length distribution in one RF preparation. Out of 3,263 molecules counted, 3,118 were single-length rings (95.5% of the total molecules), 100 were multiple-length rings (3.1%), and 45 were linear forms (1.4%). (*A*) *Single-length rings:* Of the 3,118 single-length rings, 200 fully opened forms were measured for the length distribution. Mean length: 1.63 ± 0.08 μ. (*B*) *Multiple-length rings:* All of the 100 multiple-length rings were measured. Mean lengths: 2×, 3×, and 4× rings, respectively, were 3.15, 4.56, and 6.0 μ. (*C*) *Linear forms:* All of the 45 linear forms were measured. A peak corresponding to single-length rings is evident.

multiple-length circles were prepared from sucrose gradients. P^{32}-labeled preparations were employed in order to facilitate the monitoring of minor fractions. In a sucrose gradient (Figure 5*A*) the main peak sediments at 21*S* and has a slower moving shoulder of form II at 17*S*. Although radioactivity above background is evident below the main peak, it is not well defined. The fractions containing the faster-moving material were combined and dialyzed. After concentration, the preparation was rerun on a sucrose gradient (Figure 5*B*). A well-defined peak was obtained at about 30*S*. Fractions 1–10 were pooled, dialyzed and concentrated. Four per cent of the radioactivity applied to the first sucrose gradient (Figure 5*A*) was recovered in this preparation. Electron microscopic examination of this material showed that more than half of the rings were of multiple-length. There was, however, a considerable contamination by long filaments, presumably native *E. coli* DNA.

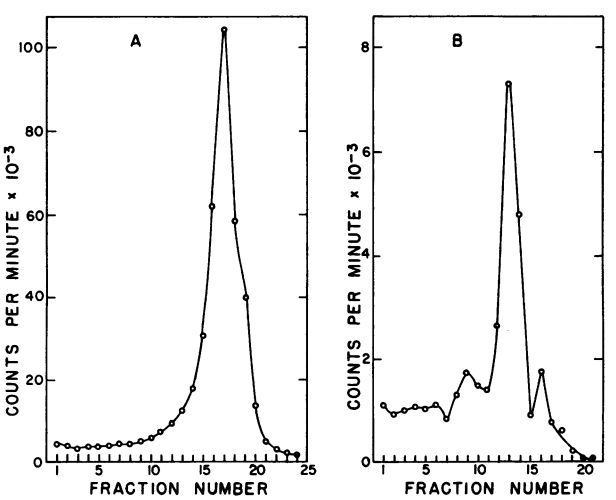

FIG. 5.—Sucrose gradient sedimentation of P^{32}-labeled RF. Five-ml linear gradients prepared from 5–23% (w/v) sucrose in SSC were centrifuged at 65,000 rpm and 25 fractions were collected. (*A*) RF (100 μg in 200 μl) was centrifuged for 97 min. Fractions 1–14 were pooled and dialyzed. After concentrating to a volume of 100 μl, the preparation was applied to another gradient. This was run for 140 min and is shown in (*B*).

Discussion.—The existence of a multiple-length distribution in circular double-stranded DNA preparations has been described in several reports. Radloff *et al.*[11] have found two series of circular DNA in HeLa cells. One series comprised small rings with a heterogeneous length distribution, while the other, from mitochondria, contained only double- and triple-length forms. Similar observations have been made on circular DNA from yeast mitochondria,[25] and circular DNA from a bacterial plasmid has been reported[26] to contain a small percentage of forms double the prevailing length. Multiple-length rings of viral DNA have not previously been isolated from infected cells.

The conclusion that the multiple-length forms described in this paper are related to RF-ϕX is based primarily upon the length distribution measurements, and upon the absence of such forms from noninfected cultures. This point could most clearly be resolved if the multiple-length forms prove to be infectious. They do not appear to be an artifact of a preparation procedure, since they have been found by two independent methods. The reason they have not been observed in previous preparations[19, 27, 28] is probably attributable to the use of MAK column chromatography. In these procedures RF is eluted only slightly ahead of native host cell DNA. Multiple-length forms would tend to be retarded more than single-length rings and would thus be discarded, particularly upon rechromatography and elimination of tail fractions.

Large molecules of DNA have been shown by Frankel[29] to accumulate in chloramphenicol-treated cultures of *E. coli* infected with T2. Similar structures have been noted by others in the presence or absence of chloramphenicol.[30, 31] In a recent report, Frankel[32] suggests that these are extended, repeating forms of the circularly permuted T4 genome. Multiple-length rings of RF might be structurally analogous to such forms and might represent intermediates in replication or recombination. Such a relationship would imply the existence of mechanisms both for formation of multiple-length circles and the reformation of those of normal length.

Summary.—Purified preparations of bacteriophage ϕX174 replicative form (RF) have been shown by electron microscopy to contain a small percentage of multiple-length circular molecules. Preparations enriched in multiple-length rings have been obtained.

Note added in proof: Drs. E. F. J. van Bruggen, H. S. Jansz, and P. H. Pouwels have informed us that they have also observed multiple-length rings of RF-ϕX and that these rings are not present in noninfected cultures.

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[4] Abbreviations used: RF, replicative form; MAK, methylated bovine plasma albumin-kieselguhr. Other abbreviations are those defined by the *J. Biol. Chem.* Following the terminology introduced by Vinograd *et al.*[5] for polyoma virus DNA, the double-stranded covalently closed

circles of RF will be referred to as form I, the circular structures with one (or more) single-strand scissions as form II, and the linear form derived from I or II as form III.

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