*MULTIPLE-LENGTH RINGS OF φX174 REPLICATIVE FORM, II. INFECTIVITY**

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The replicative form, RF,[1] of the bacterial virus φX174 consists of double-stranded, covalently closed, circular DNA with a mean contour length of 1.64 μ.[2-4] In a recent communication we have shown that preparations of RF also contain 2–3 per cent of circular molecules having double, triple, and several times the most frequent length.[5] The conclusion that these rings are related to RF-φX is supported both by their stepwise contour length distribution and by their absence from non-infected cultures. In the work reported here this relationship has been more directly confirmed by the isolation of double-length circles and the demonstration that they are infectious in the spheroplast assay.

Materials and Methods.—These were the same as previously described,[5] except where specifically noted. Sucrose-density gradients were run in the Spinco SW41 rotor at 40,000 rpm. In place of shadowing, for electron microscopy, the DNA was stained with uranyl acetate in acetone according to a new procedure developed by Gordon and Kleinschmidt.[6] Open cyclic forms are more frequently seen in some preparations spread at pH 5 if the DNA-cytochrome *c* mixture is allowed to stand at pH 5 before spreading.

RF preparations: The procedure described as "Method A" in the previous report[5] was used in the initial stages of all preparations. P^{32}-labeled RF was prepared as described by Pouwels *et al.*[7] Dephosphorylated casamino acids prepared by the method of Astrachan and Volkin[8] were employed. When 50 mc P^{32} was added per liter of infected cells, the isolated RF had a specific activity of 300,000 cpm/μg of DNA.

Infectivity assay: RF preparations were assayed for infectivity on spheroplasts of *E. coli* K12W6 as described by Guthrie and Sinsheimer[9] except that the spheroplasts were lysed with chloroform. DNA concentrations were demonstrated to be within the linear range of the assay.

Results.—Purification of multiple-length rings: Previous attempts[5] to separate multiple- from single-length rings by the use of sucrose gradients indicated the presence in the multiple-length fraction of many linear forms. Most of these had long contour lengths and therefore appeared to be fragments of host-cell DNA. They were not satisfactorily eliminated by repeating the alkali denaturation step at pH 11.8 for 1 minute. Such linear forms have been removed after the steps of Method A by employing a cesium chloride density gradient centrifugation in the presence of ethidium bromide.[5, 10] This is illustrated in Figure 1 for an experiment in which 20 μg of P^{32}-labeled RF was added to 40 μg of an unlabeled preparation. Fractions containing covalently closed rings (form I) were separated from those containing a mixture of single-nicked rings and linear forms. Ethidium bromide was removed with Dowex-50 resin (Na^+ form). The purification of multiple-length rings was accomplished by selection and rerunning of rapidly sedimenting material

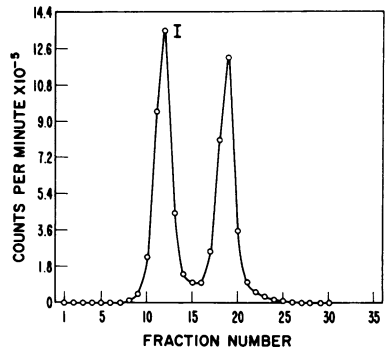

FIG. 1.—Sedimentation equilibrium of purified RF in CsCl containing ethidium bromide. The solution contained 700 μg ethidium bromide, 60 μg RF, and 7 mmoles Tris-HCl, pH 7.5 in 7.0 ml CsCl, density 1.59 by refractometry. Fractions were collected after 36 hr centrifugation at 50,000 rpm and 20°. The peak corresponding to the double-stranded, covalently closed circles is identified as form I. The lighter component consists of circles with one or more single-strand scissions and also contains a small percentage of linear forms.

in three successive sucrose gradients. Figure 2 shows a series of such gradients in which the initial material was the combined form I fractions from the CsCl–ethidium bromide gradient shown in Figure 1. When the 21*S* position is used as a reference, the average sedimentation coefficient of the rapidly sedimenting component is 29*S*.

Electron microscopy: Fractions 2 and 4 from the sucrose gradient shown in Figure 2*C* were combined and examined by electron microscopy. No long linear forms were evident, and all of the circular molecules were of multiple length, predominantly double. A small percentage of triple-length rings, and an even smaller percentage of short linear forms, were also present. Selected micrographs are shown in Figure 3.

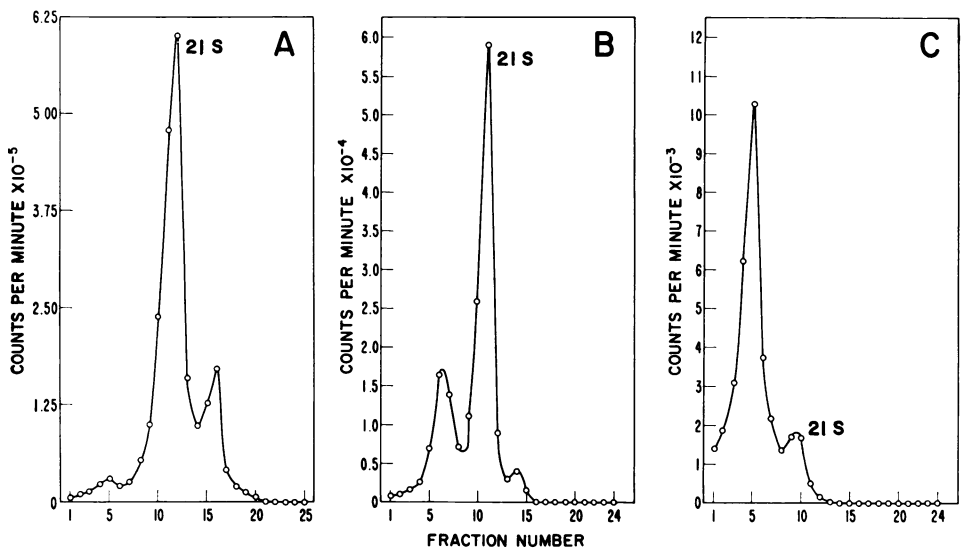

FIG. 2.—Sucrose-gradient sedimentation of P^{32}-labeled RF. Thirteen-ml linear gradients prepared from 5 to 23% (w/v) sucrose in SSC were centrifuged at 40,000 rpm; 24 fractions were collected. The 21*S* peak corresponding to the sedimentation of form I is identified on each graph. (*A*) Thirty μg of RF from the heavy ethidium peak, I, in Fig. 1, after 7 hr centrifugation at 8°. Fractions 1–9 were pooled, dialyzed, and concentrated to a volume of 200 μliters. (*B*) Combined fractions 1–9 from (*A*) after 7.5 hr centrifugation at 5° through another gradient. Fractions 1–8 were pooled, dialyzed, and concentrated. (*C*) Combined fractions 1–8 from (*B*) after 8 hr centrifugation at 4°. The ordinates are the total cpm in each fraction.

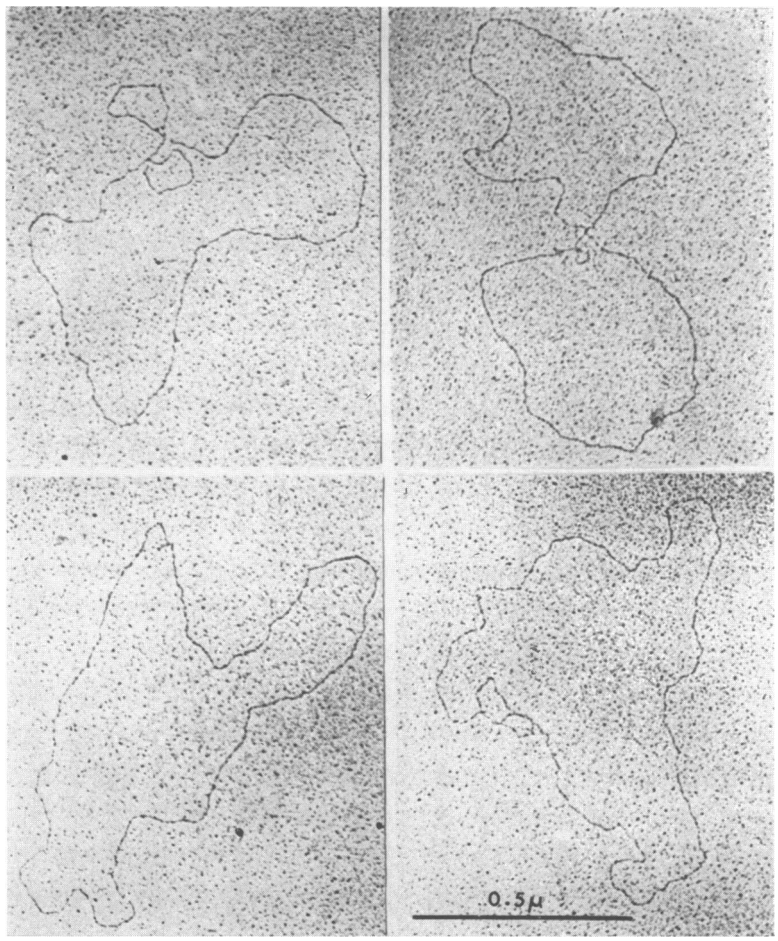

FIG. 3.—Electron micrographs of selected double-length rings. Magnification ×58,000. DNA and cytochrome *c* were mixed in pH 5 acetate buffer and, after standing at 4° for 14 hr, were spread on a pH 5 subphase. The preparations were stained with uranyl acetate in acetone.[6] The mean contour length of 35 double-length rings was 3.69 ± 0.16 μ.

Infectivity: Figure 4 represents the radioactivity and infectivity profiles of a P^{32}-labeled multiple-length preparation after the third sucrose-gradient centrifugation. In contrast to Figure 2, the P^{32}-labeled RF was undiluted. The peaks of PFU show exact coincidence with those of the P^{32} distribution. Therefore, infectivity in the 29*S* region cannot be due to contamination from poorly resolved single-length rings. Analysis of five independent infectivity profiles on the basis of specific infectivity (PFU/cpm) has shown that the 21*S* region is 1.5–2.1 times more infectious on a P^{32} basis than the region at 29*S*. If we assume that the 29*S* rings are twice the mass of those at 21*S*, the specific infectivities calculated on a molecular basis (PFU/molecule) are approximately equal. An infectivity profile through the gradient shown in Figure 2*C* yielded indentical results. Exact coincidence of the P^{32} and PFU peaks was found and the 21*S* region had a PFU/cpm ratio approximately twice that of the 29*S* fractions.

Virus particles released from spheroplasts infected with multiple-length RF rings were purified on a CsCl density gradient and examined by electron microscopy. This spheroplast lysate was also used to infect cultures of *E. coli* C and the progeny virus was similarly examined. Only small spherical virus particles with the same appearance as those in control preparations of normal virus were observed.

Discussion.—Purified multiple-length rings of RF-ϕX174 have been found to have an infectivity approximately equal to that of normal length rings. The multiple-length preparations were characterized by a sharply sedimenting, 29*S*, zone in a sucrose-density gradient. A preparation obtained in large enough quantity for electron microscopic examination was found to consist almost entirely of double-length rings. The conclusion that single and double-length molecules are equally infectious is based on the assumption that P^{32} labeling was uniform.

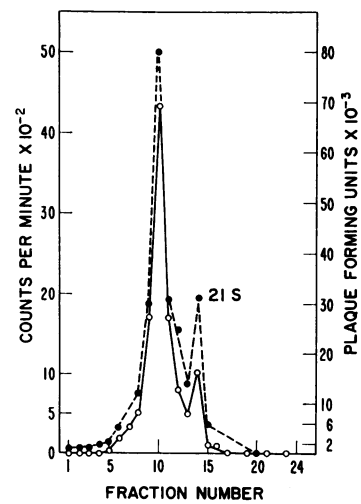

FIG. 4.—Infectivity of multiple-length rings. Multiple-length rings of P^{32}-labeled RF (3×10^5 cpm/μg) were prepared by successive sucrose-gradient sedimentation. The final gradient, shown above, was run at 5° for 6 hr and is comparable to that shown in Fig. 2*C*. Aliquots (50 μl) of fractions (500 μl) from this gradient were used for determination of radioactivity and for assay of infectivity. ○, Total cpm in fractions; ●, PFU/ml in the spheroplast assay tube. The specific infectivities (PFU/cpm) of fractions 9, 10, and 11 were 176, 180, and 179, respectively, while those of fractions 12, 13, and 14 were 310, 260, and 310.

The possibility that the observed infectivity in the 29*S* zone was due to contaminants and not to double-length rings must be considered. Single-stranded viral DNA and irreversibly denatured RF are highly infectious.[11] Single-nicked RF present during the alkali denaturation step of Method A would give rise to the former. The latter would be expected only if the conditions in the alkali step produced some irreversible denaturation. Both the nitrocellulose column, which employs a large excess of adsorbing capacity over the total single-stranded DNA, and the ethidium bromide separation are effective in removing these forms. However, the strongest argument against ascribing infectivity to them is the exact coincidence of infectivity and radioactivity peaks following sucrose-gradient sedimentation, and the constancy of specific infectivity through 29*S* fractions. Single-stranded viral DNA should have a sedimentation coefficient of about 24*S* under the conditions of salt concentration employed in our gradients.[11, 12] It would thus be strongly discriminated against in the selection of fractions to be rerun, and its infectivity peak should fall closer to 21*S* than to 29*S*. The possibility that there is infectivity due to denatured RF is eliminated by similar considerations. The sedimentation coefficient of the compact alkali-denatured RF-ϕX or of the similar

sized polyoma virus DNA has not been carefully examined at neutral pH. It is reported to be about twice that of form I and in alkaline media to be 33*S* to 54*S* depending on the conditions.[13-15]

We conclude that infectivity is a property of the double-length ring. When this structure infects a spheroplast, normal viral DNA is presumed to form. Without specifying the precise sequence of events in relation to single or double-strandedness, it is thus clear that double-length rings can be formed from single-length, and single-length from double-length. If the existence of multiple-length rings is related to the events of genetic recombination, a cyclic process of this nature is indicated. This possibility is being investigated.

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[1] Abbreviations used: RF, replicative form; PFU, plaque-forming unit. Other abbreviations are those defined by *J. Biol. Chem.*

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