INFECTIVITY TO *ESCHERICHIA COLI* SPHEROPLASTS OF LINEAR φX174 DNA STRANDS DERIVED FROM THE REPLICATIVE FORM (RFII) OF φX DNA

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1. Introduction

Infection of *E. coli* spheroplasts with circular single-stranded DNA extracted from bacteriophage φX174 leads to the formation in the infected spheroplasts of a near-normal burst of progeny phage [1]. The infectivity of φX DNA has been shown to depend on the circular form of the DNA molecules [2]: if the DNA circles are opened artificially at random by a single hit by limited action of endonuclease the resulting linear DNA strands proved to be no longer infectious.

The replication during the infectious cycle of φX DNA is supposed to occur in three stages [3]: (1) Shortly after infection the single-stranded DNA ring of the φX174 phage is converted to a double-stranded DNA ring, the parental replicative form (RF). The RF is found in the infected cells: i) as supertwisted RFI in which both strands are covalently closed and ii) as RFII with one single strand break. At neutral pH RFI and RFII sediment in sucrose gradients with 21 S and 16 S, respectively. Upon alkaline denaturation at pH 12.6 the strands of RFII are separated. The resulting single-stranded DNA rings sediment with 16 S in alkaline sucrose gradients (pH 12.6) whereas the linear DNA strands sediment with 14 S under these conditions. (2) The parental RF replicates semiconservatively at a bacterial site whereby the parental viral DNA strand remains as a closed ring and persists at the site. The daughter RF molecules, appearing first as RFII bearing a single strand, break either in the viral or in the complementary strand and are released into the cytoplasm and partially converted to RFI. (3) After the formation of some 10–50 molecules of daughter RF per infected cell, RF replication ends. The progeny RF now participates in the synthesis of single-stranded viral DNA whereby the viral strands are displaced from the RF duplexes by concomitant synthesis of new viral strands on the persisting circular complementary strand templates [4].

Recently Schekman and Ray [5] reported that linear single-stranded φX DNA, formed in a DNA-ligase defect mutant host during stage III of φX DNA replication, as well as the linear component of stage III type RFII DNA, appeared to be infectious to *E. coli* spheroplasts to a low degree as compared to circular strands. We have isolated φX RFII DNA from DNA ligase wild type hosts during the period of RF replication (stage II) and found the linear component of these duplexes to be infectious.

2. Materials and methods

2.1. *Viruses*

φX174 wt; the DNA of the temperature-sensitive and host range extended mutant φX tsγh [6] served as a biological marker in sucrose gradients.

2.2. *Bacetrial strains*

E. coli H514 (F^- *uvRA*$^-$ *thy*$^-$ *arg*$^-$ *endol*$^-$ su^-_{amber}) was used as the host strain. Spheroplasts were prepared from strain W6 (ϕX, su^-_{amber}). φX tsγh phage, formed upon infection of spheroplasts, was plated on *E. coli* C1 which is insensitive for φX wt.

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2.3. *Media*

NP medium has been described earlier [7] and was supplied with 2 μg/ml each of thymine and arginine for the growth of H514.

2.4. *Mitomycin treatment*

Before φX infection H514 cells were grown to a density of 4×10^8 cells/ml and treated with mitomycin according to Lindqvist and Sinsheimer [8] in order to suppress, preferentially, the synthesis of host cell DNA.

2.5. *φX infection*

After the mitomycin treatment the host cells were infected with 2–5 pfu ^{32}P-labelled φX/cell in the presence of 100 μg/ml of chloramphenicol (CM) (Boehringer, Mannheim). Under these conditions RF synthesis ceases after formation of the parental RF [9]. Ten min later the cells were rapidly freed of the CM by filtration through a membrane filter, resuspended in fresh NP medium and aerated at 25° in order to reduce the rate of RF replication [10].

2.6. *Extraction of DNA*

At appropriate times the infected cells were pelleted, opened by lysozyme/EDTA at a concentration of about 8×10^9 cells/ml. 20 μg/ml ribonuclease was added to the lysate. After 10 min at 0° the lysate was brought to 0.5 M NaCl, cleared by addition of 0.4% Sarcosyl (Geigy, Basel) and digested with 150 μg/ml pronase (Serva-Entwicklungslabor, Heidelberg) for 4 hr at 37°. The lysates were centrifuged through 7–20% neutral sucrose gradients in a SW41 rotor at 23,000 rpm for 15 hr at 10°. Fractions containing RFII were collected and dialysed against 0.01 M Tris-HCl buffer pH 7.2, 0.1 M NaCl, 5×10^{-3} M EDTA, 0.1% Sarcosyl. Before treatment with exonuclease III the RFII samples were dialysed against 0.01 M Tris-HCl buffer pH 8.1, 0.02 M NaCl and 5×10^{-4} M EDTA.

2.7. *Alkali denaturation of RFII*

The samples were brought to pH 12.6 with 1 M NaOH and layered on 5–20% alkaline (pH 12.6) sucrose gradients containing 0.1 M NaCl and 5×10^{-3} M EDTA. The gradients were spun for 15 hr in a SW41 rotor at 28,000 rpm, 10°, or in a SW27 rotor for 17 hr at 26,000 rpm at 10° in a Spinco centrifuge.

2.8. *Infection of spheroplasts*

Was performed according to Guthrie and Sinsheimer [1]. Infection assays were performed independently with at least two different batches of *E. coli* spheroplasts.

2.9. *Exonuclease I-digestion of single-stranded DNA*

The DNA samples were brought to 7×10^{-3} M Mg^{2+} and to pH 9.2 by addition of glycin buffer to 0.07 M. 2 units [11] of *E. coli* exonuclease I (a kind gift of Dr. Schaller, Tübingen) was added per 0.3 ml assay mixture. After 20 min incubation at 30° the reaction was stopped by adding 10^{-2} M EDTA.

2.10. *Digestion with exonuclease III of φX RFII*

The reaction mixtures contained per ml: 66 μmoles Tris-HCl, pH 8.1; 0.66 μmoles Mg-acetate, 5 μmoles mercaptoethanol, 30 units of purified [12] *E. coli* exonuclease III [13], and about 0.1 μg RFII. The assays were incubated for 60 min at 37°. The reaction was stopped by addition of 0.02 M EDTA and 0.4% Sarcosyl.

3. Results

Fig. 1 shows the distribution in a neutral sucrose gradient of φX RF extracted from infected cells after 10 min of RF replication at 25°. RFII was saved from fraction no. 23 of the gradient and, after denaturation with NaOH, sedimented through alkaline sucrose gradients. The distribution of the parental radioactivity and the DNA infectivity profile, determined as described in the *E. coli* spheroplast system, of the alkaline sucrose gradients are plotted in fig. 2a. The coincidence of the ^{32}P-radioactivity peak with the 16 S φX tsγh reference suggests that the majority of the parental φX DNA had persisted as closed rings during RF replication in accordance with the concept described above. Infective φX wild-type DNA was found in two peaks sedimenting with 16 S and with 14 S, the established sedimentation rates of circular and linear φX DNA single strands, respectively [3].

The suggestion that the 14 S infective material consisted of linear φX DNA was supported by its sensitivity to exonuclease I. Exonuclease I degrades linear single DNA strands in the $3' \rightarrow 5'$ direction and does not attack DNA rings. Fig. 2b shows the infectious capacity

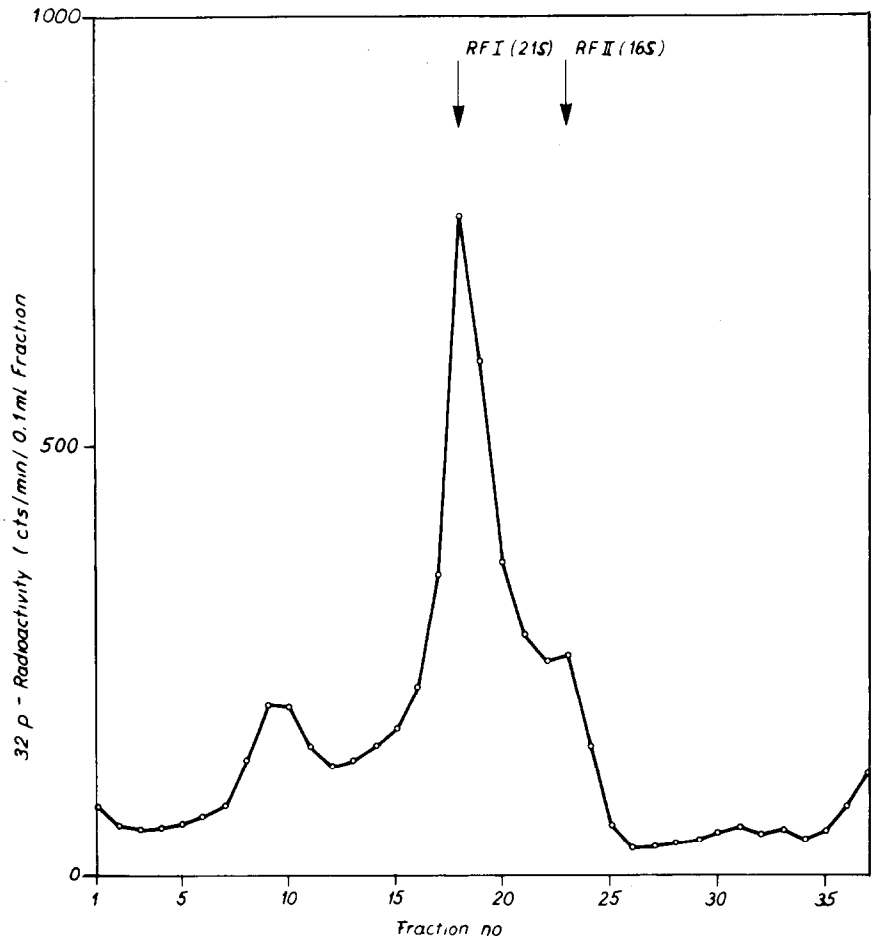

Fig. 1. Distribution in a neutral sucrose density gradient of ϕX174 replicative form DNA. Experimental details are given in Methods. 4×10^{10} H514 cells (grown in 100 ml NP-medium to 4×10^{8}/ml) were treated with mitomycin, infected at 37° with 3 pfu ^{32}P-labelled ϕX per cell in presence of 100 μg/ml CM. After 10 min aeration in fresh NP-medium at 25° the cells were harvested and extracted. The distribution of the parental ^{32}P-label in a 7–20% neutral sucrose gradient was determined in 0.1 ml aliquots of the gradients fraction. o——o: ^{32}P-radioactivity (cpm).

left in the same gradient fractions after exonuclease I digestion: this capacity of the 14 S material appeared to be completely destroyed whereas the majority of the DNA rings remained infectious.

In another series of experiments ^{32}P-labelled ϕX RFII was treated with *E. coli* exonuclease III as detailed in Methods. This enzyme attacks double-stranded DNA at single strand nicks, hydrolyzing the nicked strand in the 3′ → 5′ direction. The enzyme reaction was stopped by addition of EDTA, the RFII digest denatured with NaOH and spun through an alkaline sucrose gradient. Parental ^{32}P-radioactivity and infectious DNA were determined in aliquots of the gradient fractions (fig. 3b). No infectious 14 S material could be detected in the alkaline sucrose gradient of the exo-III digest as compared to an untreated control (fig. 3a). This confirms the view that the 14 S infective DNA represents the linear component of RFII duplexes.

Similar degrees of infectivity associated with the linear component of RFII as shown in fig. 2 were observed in some 20% of studied RFII preparations

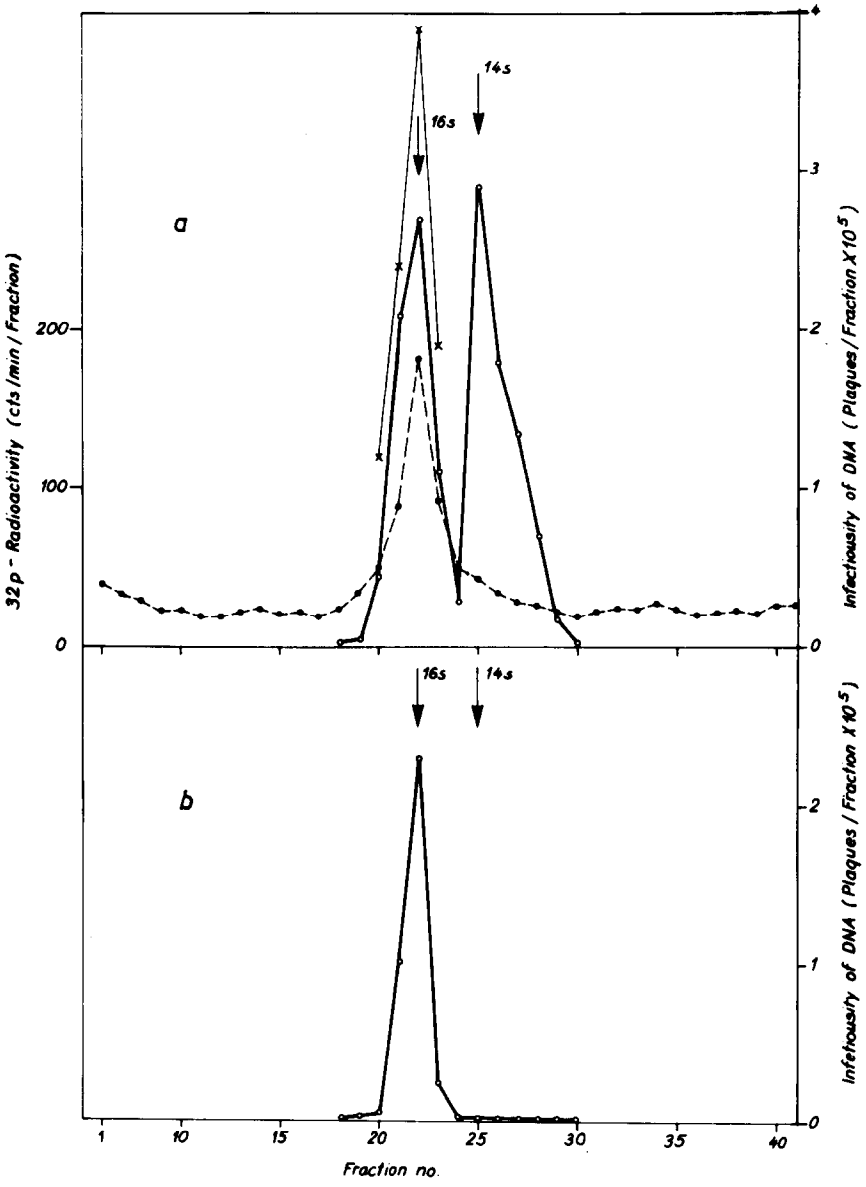

Fig. 2. Sedimentation analysis of alkali-denatured φX RFII. RFII contained in fraction 23 of the neutral sucrose gradient shown in fig. 1 was denatured with alkali and spun through an alkaline 5–20% sucrose gradient as detailed in Methods. In appropriate aliquots of the gradient fractions the parental ^{32}P-radioactivity was measured. Other aliquots, after 1:10 dilution into 0.05 M Tris-HCl buffer pH 7, served for the determination of infectious single-stranded φX DNA in a *E. coli* W6 spheroplast system. a: •- - -•, ^{32}P-radioactivity; o——o, infectious φX wt DNA; x——x, infectious φX tsγh DNA which served as a marker for the position in the gradient of φX DNA circles. b: o——o, infectious single-stranded φX wt DNA left in the same fractions after exonuclease I digestion as described in Methods.

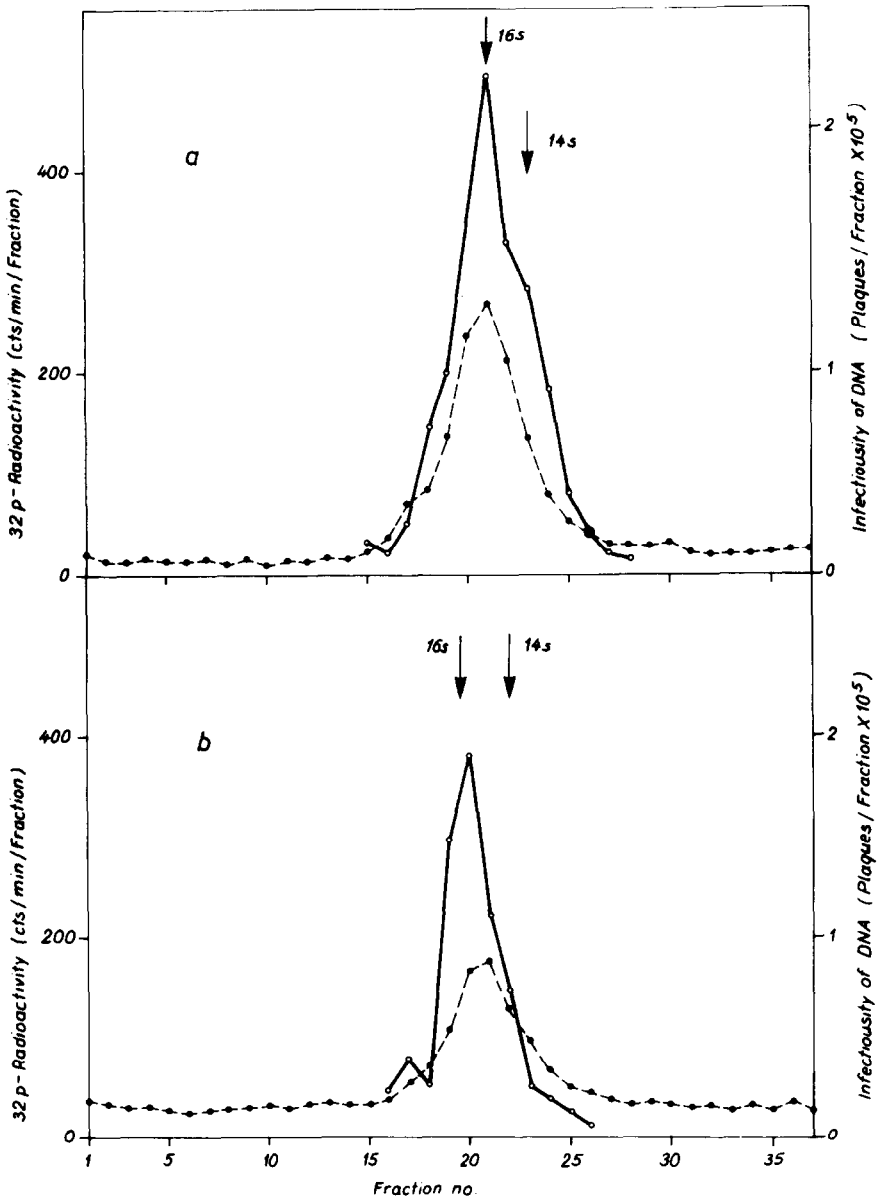

Fig. 3. Sedimentation analysis of an alkali denatured exonuclease III digest of φX RFII. RFII was extensively digested with *E. coli* exonuclease III. The digest was denatured with alkali and analysed in an alkaline 5–20% sucrose gradient (b). 3a shows the sedimentation pattern of a denatured aliquot of the same RFII preparation which had not been treated with exonuclease III before denaturation. •- - -•, ^{32}P-radioactivity (cpm); ○——○, infective DNA.

only. In other cases the infectious capacity in the 14 S position of alkaline gradients accounted for about 5–20% relative to the infectivity found in the area of the corresponding circles. Possible reasons of this finding are discussed below.

4. Discussion

The infectivity to *E. coli* spheroplasts associated with linear φX DNA strands derived from native φX replicative form II is not easy to explain if one considers that single breaks introduced into φX DNA rings lead to the loss of infectious capacity of the phage genomes. If the initiation of the infectious cycle in the host cells would strongly depend on the ring form of the φX DNA molecules one must assume that the incoming linear strands first have to be closed to rings, presumably by a host specific DNA-joining enzyme. *E. coli* DNA ligase, however, is known to repair single strand breaks (displaying a 3′ OH and a 5′ phosphate group) exclusively in double helical DNA [14].

One possibility how linear φX strands could be closed to rings by *E. coli* ligase would be the following: φX174 DNA has been supposed to contain a small self-complementary base sequence allowing a local bihelical hairpin structure [2]. If the physiological single strand interruption in native φX RFII would be located within this special region, circles could originate from the isolated linear component of the duplexes by *E. coli* DNA ligase action. A similar mechanism has been proposed by Knippers et al. [4] for the formation of circular single-stranded φX progeny DNA and has been supported by the results of Schekman and Ray [5].

It has been pointed out that the infectious capacity associated with the linear components of different φX RFII preparations varies relative to that due to the corresponding circular DNA strands. Since it could be assumed from the given results (fig. 2) that the specific infectivity of "native" linear φX DNA would not greatly differ from that of the circles, the variable degree of infectivity could reflect a physiological meaning: Schekman et al. [15] have reported that among the population of native RFII molecules there are only about 10% on the average in which the linear component has exactly the full length of a φX genome ("nicked RFII"), whereas 30% appear to be "gapped" RFII with an incomplete linear strand and that 50% of the RFII molecules probably carry a little more than one φX equivalent in their nicked strand ("tailed RFII").

Assuming a special nick within the above discussed self-complementary region the ratio of "nicked" RFII could correspond to the amount of infectious linear φX DNA strands in our experiments. On the other hand linear strands longer than one φX equivalent also could be envisaged to be infectious: after completion to double helices, the terminal redundance of these linear duplexes could allow the formation of φX RF rings by recombination. Considering that RFII duplexes were revealed to be 20 times less infective than single-stranded φX DNA circles [16], some loss of infectivity in the 14 S area of alkaline sucrose gradients of RFII either could be explained by occasional re-annealing of linear viral and complementary strands or of linear strands and overlapping 16 S circles.

For better understanding of the infectivity of linear φX DNA, experiments should now be done to decide definitely whether or not native φX RFII displays a special nick and to determine its position within the molecule. This information moreover appears desirable with respect to the mechanism of φX RF replication.

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