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Replication of φX174 DNA by *Escherichia coli polA⁻ In Vitro*

(φX174 DNA/DNA replication/*E. coli polA⁻*)

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ABSTRACT **Lysates of an *Escherichia coli polA⁻* strain convert single-stranded DNA from φX174 virus to the double-stranded replicative form with high efficiency on cellophane discs. The initiation of synthesis of the complementary strand appears to be rate limiting; once initiation occurs, the chain is propagated rapidly. Under these conditions, the unsealed replicative form accumulates and is slowly converted to the sealed form; this conversion requires the activity of the DPN-dependent DNA ligase.**

Infection of *Escherichia coli* by phage φX174, which contains single-stranded, circular DNA, involves, as an early step in DNA synthesis, the conversion of the single strand to the double stranded or replicative form (RF). The double-stranded form may either contain a discontinuity in one strand (RF II) or may have intact circular DNA on both strands (RF I). The conversion of the single-stranded viral strand to the RF is known to require only host functions; no viral genes need be expressed to form the parental RF. However, for further replication to occur, viral genes are required (1).

The conversion of single-stranded circular DNA to a RF is of particular interest, since it probably represents one of the simplest cases of initiation of new DNA chains. As a first step toward understanding the enzymology of this process, the replication of φX174 DNA must be studied *in vitro*.

The studies of Mitra *et al.* (2) and Goulian and Kornberg (3) have shown that φX174 DNA could be converted to the RF by highly purified DNA polymerase I when primer DNA is supplied for initiation. RF I could be generated by the addition of DNA ligase and DPN. Recent experiments show that the conversion of single-stranded φX174 DNA to RF also occurs in polymerase I-deficient strains (4), an observation indicating that the enzyme involved in this replication *in vivo* might not be polymerase I. Thus, the problems of how replication is initiated and which enzyme is actually responsible for polymerization of the complementary DNA chain remain open questions. The system that seems most promising for the investigation of the *in vitro* replication of φX174 DNA is one that has been used for studies of bacterial DNA replication*. As we shall show in this paper, externally added φX174 DNA is converted to RF II and RF I in this *in vitro* system.

Certain features of the system described below lead us to believe that the components that perform the conversion of single-stranded DNA to the RF *in vitro* are the very components that are responsible for replication *in vivo*. It can be hoped that at least some of the components can be purified and characterized.

Abbreviations: RF, replicative (double-stranded) form.

* Schaller, H., B. Otto, V. Nüsslein, J. Huf, R. Herrmann, and F. Bonhoeffer, submitted to *J Mol. Biol.*

MATERIALS AND METHODS

Incubation Mixtures on Cellophane Discs. A culture of *E. coli* H560, a PolA⁻ Endo I⁻ strain (4), was grown at 37°C in Penassay broth, containing 2 μg/ml of [^{14}C]thymine (320 mCi/mol), to a cell density of 2×10^8 cells/ml. The cells were sedimented and resuspended in a Tris-buffered salt and glucose medium, supplemented with 2 μg/ml of thymine and 0.5% Brij 58, to a final concentration of 10^{11} cells/ml. The cells (10^8/cm^2) were then spread on a cellophane disc containing 1 μg/cm^2 of lysozyme (EC 3.2.1.17) that was placed on top of an agar plate containing 2% Bacto-agar, 20 mM morpholinopropane sulphonic acid buffer, 5 mM $MgCl_2$, 10 mM ethyleneglycol-bis (β-aminoethylether)-*N,N'*-tetra-acetic acid (EGTA) and 0.33 M sucrose, adjusted to pH 7.5 with NaOH. After 20 min, the cellophane disc was transferred to a second agar plate identical to the first except that it lacked sucrose and EGTA, and incubated for 10 more min. All the above operations were done between 0–4°C. A more detailed description of the lysis procedure will be published†. φX174[^{32}P]DNA (10^8 molecules/cm^2) was added to the membranes at the same time as the cells. When φX174 DNA was applied to the disc after the cells were lysed, results were generally more erratic. Such erratic results may have been due to the greater difficulty in spreading the DNA on the disc evenly once the cells have lysed.

The disc containing the lysed cells and the viral DNA was transferred to a warm drop of incorporation mixture. After incubation (generally 5–10 min), the reaction was stopped by immersion in 0.1 M Tris (pH 8.0)–5 mM EDTA–0.5% Sarkosyl ("stop-mix"). The lysate was eluted from the disc into "stop-mix" with the aid of a very fine glass rod, and the cellophane was removed from the "stop-mix". The "stop-mix" eluate was heated at 60°C for 10 min and then subjected either to alkaline sucrose gradient sedimentation or CsCl density gradient analysis.

Sedimentation Analysis of Alkaline Sucrose Gradients. Sedimentation was on alkaline sucrose gradients (5–20% sucrose) containing 0.2 M NaOH–0.5 M NaCl–0.05 M Tris–3 mM EDTA at 50,000 rpm and 20°C with the SB 283 rotor of the International centrifuge for 60–90 min. Gradients were separated into 22 fractions by collection of drops directly on 2.4-cm discs of Whatman 3MM filter paper. These discs were treated with acid and prepared for scintillation counting as

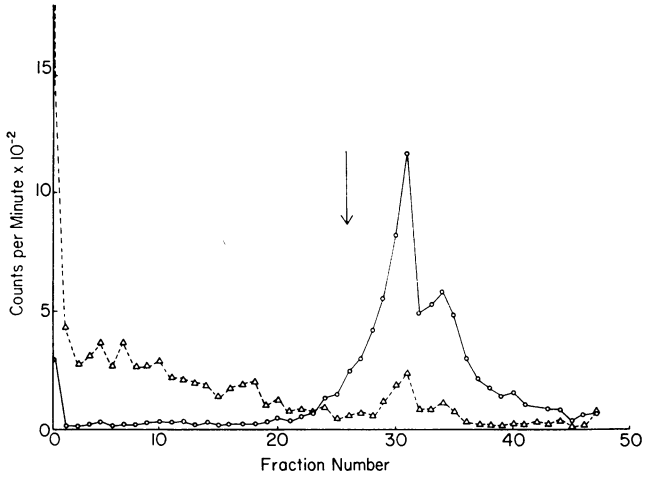

FIG. 1. Sedimentation of reaction products in a neutral sucrose gradient. A cellophane disc (6 cm in diameter) containing 2.5×10^9 bacteria and 3.5×10^9 molecules of ϕX174[^{32}P]DNA (10^3 cpm/10^8 DNA molecules) was incubated for 30 min on top of 1 ml of [^{3}H]dTTP incubation mixture at 37°C. The reaction was stopped by immersion of the disc in 0.5 ml of stop-mix (*Methods*). The eluate was heated at 60°C for 10 min and then sedimented through a 5–20% sucrose gradient containing 0.5 M NaCl–0.05 M Tris (pH 8.0)–3 mM EDTA–0.1% Sarkosyl at 20,000 rpm for 14 hr at 20°C in the SB 283 rotor of the B50 centrifuge, International Centrifuge Co. Samples were collected dropwise, 0.2 mg of salmon-sperm DNA was added as a carrier, and 1.0 ml of cold 10% trichloroacetic acid dissolved in 50% ethanol was added. The precipitated samples were filtered on a glass-fiber filter (Whatman GF/C, 2.4-cm diameter) and washed with 1.0 M HCl containing 0.1 M pyrophosphate, and then with 95% ethanol. The filters were dried briefly at 75°C and counted on a Beckman LS200 scintillation counter. The spillover of ^{32}P into the narrow ^{3}H channel has been corrected for. The *solid line* is the ϕX174[^{32}P]-DNA radioactivity and the *dotted line* is the incorporated ^{3}H derived from [^{3}H]TTP. The *arrow* marks the position of a single-stranded marker of ϕX174 DNA. From the ratio of ^{3}H/^{32}P in the faster sedimenting ^{32}P peak, a stoichiometry of about 1500 dTTP molecules incorporated per ϕX174 viral DNA equivalent can be calculated.

described by Barnett and Jacobson (5), then counted in a Beckman scintillation counter with a toluene-based scintillation fluid.

Density Analysis on a CsCl Gradient. Isopycnic analysis of the products of the reactions containing either BrdUTP- or BrdU-labeled DNA was done by resuspension of the lysate in 0.9 ml of 0.1 M Tris (pH 8.0)–5 mM EDTA–0.5% Sarkosyl, and addition to this solution of saturated CsCl to yield a refractive index of 1.4015 (generally about 3.3 ml). The gradients were centrifuged in Beckman 5/8″ × 3″ polyallomer tubes, in either the Beckman 50 Ti rotor in a Spinco L2 50 centrifuge or the International 321 rotor in the International B50 centrifuge. In both cases, centrifugation was for at least 40 hr at 20°C. Gradients were collected dropwise in about 40 fractions, acid precipitated, and counted as were the fractions from alkaline sucrose gradients.

Preparation of ϕX174[^{32}P] DNA. ϕX-174[^{32}]P DNA was prepared according to the method of Lindqvist and Sinsheimer (6). Most preparations had specific activities in excess of 10^3 cpm/10^8 phage-DNA equivalents.

Incorporation Mixture. The standard [^{3}H]TTP incorporation mixture contained 20 mM morpholinopropane sulphonic acid (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 20 μM (each) of dATP, dCTP, dGTP, and [^{3}H]dTTP (500 Ci/mol), and 170 μM thymidine. For density-label experiments ("BrdUTP mixes"), the dTTP was replaced by 20 μM BrdUTP plus 1 μM [^{3}H]dTTP (10,000 Ci/mol). Cellophane discs were incubated on about 50 μl of incorporation mixture per cm^2 of disc area.

RESULTS

Evidence for conversion of single-stranded DNA to double-stranded form

In all the experiments described here, *E. coli* cells are lysed by conversion of the bacteria to spheroplasts on a cellophane disc, and then subjecting the resultant spheroplasts to osmotic shock. The disc is then transferred onto a drop of incorporation mixture and incubated at 37°C for the desired length of time (see *Methods*).

When ϕX174[^{32}P]DNA is present on a cellophane membrane containing lysed *E. coli* cells, there is an efficient conversion of the single-stranded to the double-stranded form. This is shown by three types of analysis.

If the membrane containing the lysate and the [^{32}P]DNA is eluted after incubation and the eluate is sedimented in a neutral sucrose gradient, all of the [^{32}P]DNA sediments more slowly than the single-stranded, circular viral DNA (Fig. 1). Two distinct DNA peaks can be detected, both of which contain [^{3}H]TTP incorporated from the incubation mixture. These peaks sediment at a velocity corresponding to that expected for double-stranded RF I and RF II.

The formation of RF I is shown more convincingly by sedimentation of the membrane eluate in an alkaline sucrose gradient. It is known that in alkali, the closed-circular RF I sediments extremely rapidly, since the two strands are topologically locked into each other (1). As shown in Fig. 2, after incubation of the phage DNA with the lysate for 30 min, the fast-sedimenting form, characteristic of RF I, appears on an

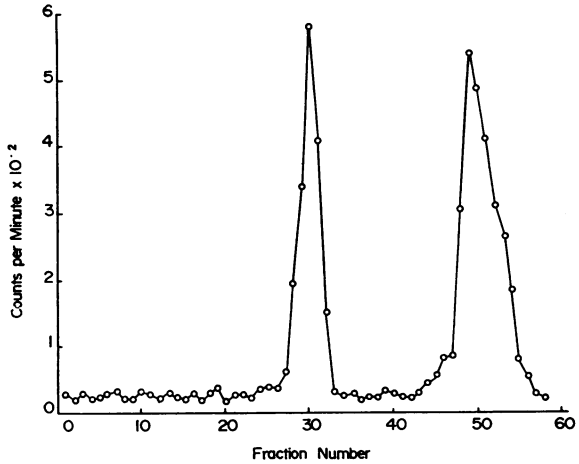

FIG. 2. Alkaline sedimentation of reaction products. The membrane eluate described in the legend to Fig. 1 was subjected to alkaline sucrose gradient sedimentation analysis. Sedimentation was at 37,000 rpm for 2.5 hr at 20°C. Sedimentation direction is from *right* to *left*; the slow-sedimenting peak is single-stranded DNA, while the fast-sedimenting peak is the denatured RF I.

alkaline sucrose gradient. In this experiment, 38% of the DNA has been converted to the fast-sedimenting form.

Experiments in which the DNA is incubated with the lysate in a reaction mixture in which 95% of the dTTP was replaced by BrdUTP provide additional evidence that the single-stranded viral DNA is being replicated. A shift in the density of the [^{32}P]DNA is observed from the density expected of denatured light DNA to that of double-stranded "hybrid" DNA that contains thymine in one strand and bromouracil in the other (Fig. 3). The reciprocal experiment, with ^{32}P-labeled, BrdU-containing phage DNA and a reaction mixture with TTP, has also been performed. Under these conditions, a shift from the denatured, "heavy" position to the hybrid density is observed.

In these experiments, the single-stranded, circular DNA strand is efficiently converted into the closed-circular RF I and the unsealed, double-stranded RF II. This conversion is presumably mediated by some component other than *E. coli* DNA polymerase I, since all the experiments were done with a PolA$^-$ strain (which contains no detectable polymerase I). In this respect, the present experiments differ from previous *in vitro* experiments (2, 3). The possibility that a minor amount of contamination by linear DNA is present cannot be excluded, although we believe that any such contamination is quite minor, since at these concentrations linear, single-stranded DNA appears to be rapidly degraded by the lysate.

Conversion of single-stranded DNA to RF DNA as a function of the multiplicity of viral DNA to lysed cells

The experiments described in the previous section were done at a ratio of φX174 DNA to lysed cells of about 1 (we will refer to this ratio as the multiplicity). The conversion of single-stranded light DNA to double-stranded hybrid DNA (in a reaction mixture containing BrdUTP) as a function of the multiplicity is shown on Fig. 4. It is clear that there are two distinct regions on the curve. At low multiplicities, a constant fraction of the DNA is converted to the RF, and the total

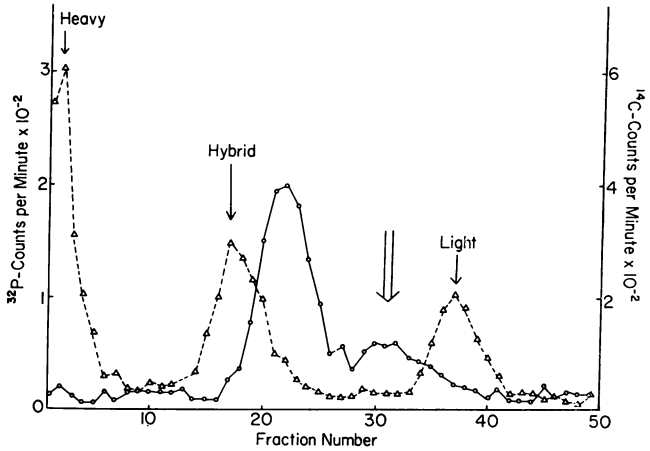

FIG. 3. φX174[^{32}P]DNA and a bacterial lysate were incubated on a cellophane disc for 15 min on a drop of BrdUTP incubation mixture (*Methods*). The eluted DNA was centrifuged for 40 hr at 20°C in a CsCl density gradient. [^{14}C]BrdU and hybrid *E. coli* DNA were added as markers; the light, *E. coli* [^{14}C]DNA was derived from the labeled cells. O——O, ^{32}P; Δ- - -Δ, ^{14}C; *arrows* mark the position of *light*, *hybrid*, and *heavy E. coli* DNA; the *double arrow* marks the position of single-stranded, light, φX174 DNA.

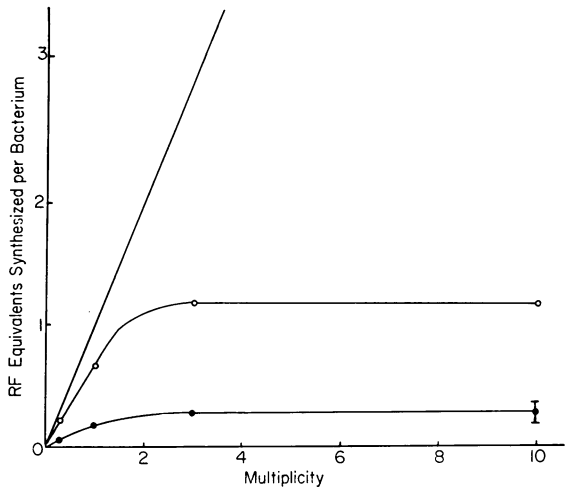

FIG. 4. A plot of the total conversion to hybrid DNA and to RF I, as a function of the multiplicity (see *text*) of the DNA to the bacterial lysate. The total amount of hybrid is calculated from the fraction of radioactivity at the hybrid density after incubation of the ^{32}P-labeled light DNA with lysate in a reaction mixture containing BrdUTP. The amount of RF I is calculated from the fraction of fast-sedimenting radioactivity in an alkaline sucrose gradient. O——O, total RF; ●—●, RF I. The *unsymboled line* corresponds to the expected slope were all the phage DNA converted to RF.

conversion is therefore a linear function of the multiplicity. At very high multiplicities, however, an increase in the amount of the input DNA does not result in more of the DNA being converted to the RF. Thus, while at multiplicities below 1, 70% of the DNA is converted to RF, at a multiplicity of 10, less than 20% of the DNA is converted to RF after a 15-min incubation.

These experiments demonstrate that the lysate has a limited capacity to transform the single-stranded DNA to the RF. In 15 min, only a few complements of the viral DNA can be made for every lysed cell. The most likely explanation for these results is that the conversion to RF requires some component(s) that is present in a limited number of copies per cell.

The conclusions above are supported by experiments in which the labeled DNA is diluted by unlabeled φX DNA. At a 10-fold dilution with unlabeled DNA, a striking drop in the conversion to RF is observed, an expected result since an increase in the multiplicity results in a decrease in the fraction of the DNA converted to the RF. An interesting sidelight to such a competition experiment is that the ^{32}P-labeled φX174 DNA can also be diluted out by linear, broken φX174 DNA molecules that are formed by sonication of circular φX DNA.

Rate-limiting steps in the conversion of single-stranded DNA to the closed-circular form

In the conversion of the single-stranded viral DNA to the closed-circular form, three stages may be envisaged: the initiation of a chain, the propagation of a chain until the complementary strand has been completely, or almost completely, synthesized, and a finishing or sealing step, in which the synthesized strand is converted to a circular form. Our studies indicate that initiation and sealing are probably relatively slow in comparison to propagation.

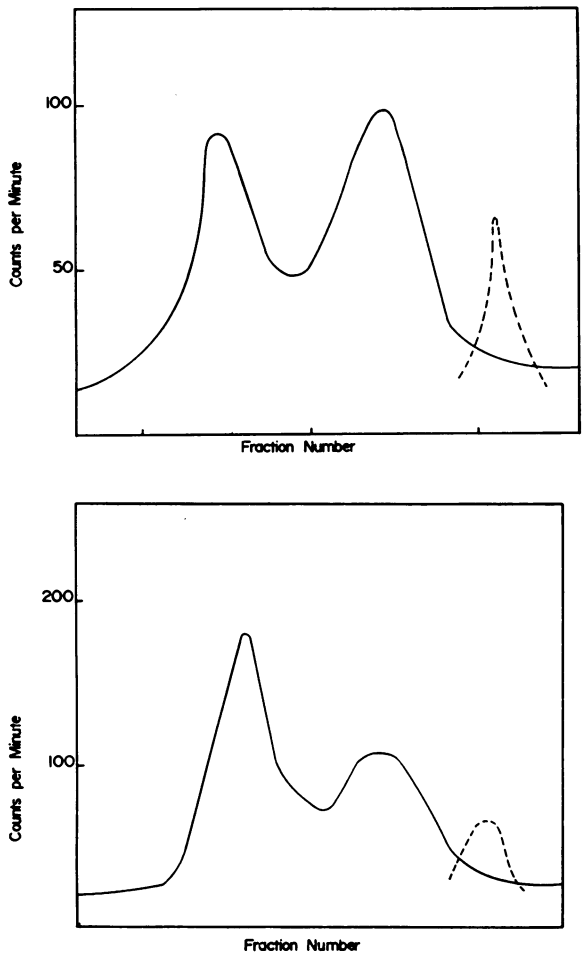

FIG. 5. Density distribution of reaction products as a function of time. Cellophane discs containing ϕX174 [^{32}P]DNA and *E. coli* lysate that had been incubated on top of a drop of BrdUTP incubation mix for 3 min (*top*) and for 15 min (*bottom*) were eluted and subjected to CsCl density analysis. The *dotted lines* represent the profile of the labeled [^{14}C]DNA, which bands at the double-stranded, light position. The *solid line* shows the ^{32}P profile, the leftmost peak of which corresponds to hybrid.

Fig. 5 shows the CsCl density profile of ϕX174[^{32}P]DNA that has been incubated with the lysate in a BrdUTP-containing incubation mixture for 3 min and for 15 min. It is clear that in both cases the distribution of densities is bimodal between material in the fully hybrid and in the single-stranded light position; however, the amount of hybrid material increases with increased incubation time. Since the distribution is bimodal at all times, the amount of intermediate-density material that is present must always be small, i.e., the fraction of molecules that is in the process of propagation at any time is very low. These results suggest that once a complementary strand is initiated, it is completed quite rapidly. Additional support for this concept comes from the observation that with 30-sec incubation periods, some fully hybrid material is already detectable. It must, therefore, take less than 30 sec to polymerize the complementary strand once an initiation has occurred under these experimental conditions.

Fig. 6 shows the rate of RF I and RF II formation. The RF I conversion was determined by sedimentation in an alkaline sucrose gradient, while the total conversion to RF I and RF II was determined from the amount of material shifted to a hybrid density in a reaction mixture containing BrdUTP. As expected, there is a fairly rapid conversion to RF II. Surprisingly, however, there is a lag of several minutes before any RF I appears. Such a lag is apparent even if DPN is added at a high concentration (1 mM) to the incubation mixture. Thus, the availability of the cofactor for DNA ligase is not the limiting factor in the conversion of RF II to RF I.

Since RF I can be formed even when the acid-soluble pool of nucleotides is dialyzed away and no DPN is added to the reaction mixture, the question is raised whether DNA ligase is involved in the conversion of RF II to RF I at all. We have, therefore, added nicotinamide mononucleotide (NMN) to the reaction mixture. NMN discharges the active, adenylylated form, of DNA ligase to the free enzyme, which is inactive in joining. Under these conditions, there is a complete inhibition of the formation of RF I, although RF II is formed at the normal rate. We postulate, therefore, that DNA ligase is involved in the conversion of RF II to RF I, and that the addition of DPN is not required in the reaction mixture because a sufficient amount of adenylylated DNA ligase is present in the extracts to provide the required joining activity. If the endogenous charged DNA ligase is discharged, then no RF I is formed. The DPN-dependent activity appears to be the only DNA ligase active in this system.

DISCUSSION

The experiments described here clearly show that the cellophane disc system is capable of the replication of exogenously added single-stranded DNA to both RF II and RF I forms. It may provide a useful assay for purification of the components involved in replication.

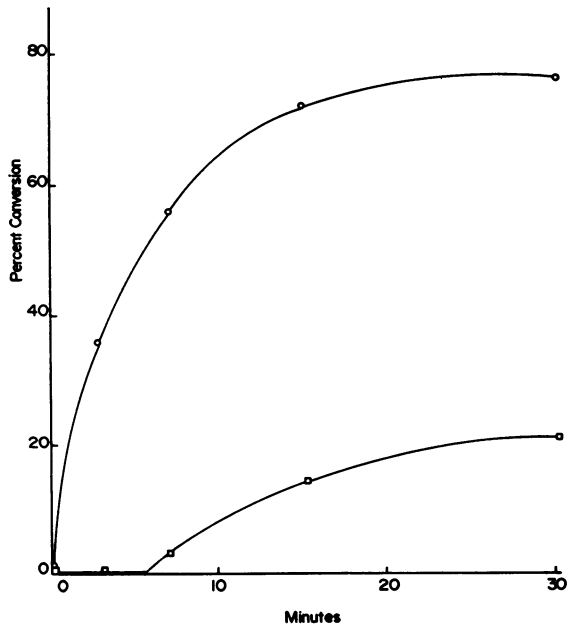

FIG. 6. Conversion of ϕX174 [^{32}P]DNA to RF I and total RF, as a function of time. The percent conversion to RF and to RF I is determined by CsCl density centrifugation and alkaline sucrose gradient sedimentation, respectively, as described in the legend to Fig. 4 and *Methods*. □—□, RF I; O—O, total RF.

Our experiments indicate that the DPN-linked DNA ligase is involved in the completion or sealing stage of the reaction (in which RF II is converted to RF I). There appear to be no other DNA joining activities present. What our experiments do not reveal is whether DNA ligase is the only component necessary for sealing the RF II molecules formed early during the reaction. The fact that such a marked lag exists between the formation of RF II and RF I is consistent with there being one or more other steps, apart from the actual joining event, that may be involved in the sealing process.

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