RNA Synthesis Initiates *In Vitro* Conversion of M13 DNA to Its Replicative Form

(ϕX174 DNA/DNA initiation/DNA replication/rifampicin)

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ABSTRACT Soluble enzyme fractions from uninfected *Escherichia coli* convert M13 and ϕX174 viral single strands to their double-stranded replicative forms. Rifampicin, an inhibitor of RNA polymerase, blocks conversion of M13 single strands to the replicative forms *in vivo* and *in vitro*. However, rifampicin does *not* block synthesis of the replicative forms of ϕX174 either *in vivo* or in soluble extracts. The replicative form of M13 synthesized *in vitro* consists of a full-length, linear, complementary strand annealed to a viral strand. The conversion of single strands of M13 to the replicative form proceeds in two separate stages. The first stage requires enzymes, ribonucleoside triphosphates, and single-stranded DNA; the reaction is inhibited by rifampicin. The macromolecular product separated at this stage supports DNA synthesis with deoxyribonucleoside triphosphates and a fresh addition of enzymes; ribonucleoside triphosphates are not required in this second stage nor does rifampicin inhibit the reaction. We presume that in the first stage there is synthesis of a short RNA chain, which then primes the synthesis of a replicative form by a DNA polymerase.

RNA polymerase (2), unlike the known DNA polymerases (3, 4, and Kornberg, T. and Gefter, M., personal communication), can initiate as well as extend polynucleotide chains. It was this property of RNA polymerase that led us to inquire whether RNA polymerase might initiate the synthesis of a new DNA chain. We found (1) that the conversion of the single-stranded DNA circle (SS) of M13 phage into a double-stranded circle (replicative form, RF) in *Escherichia coli* was prevented by rifampicin, a specific inhibitor of RNA polymerase (5). Inasmuch as this effect on RNA polymerase was not related to protein synthesis, a role for RNA polymerase in synthesizing a primer for initiating a DNA chain seemed likely.

We now report the conversion of M13 SS to RF with soluble enzymes extracted from *E. coli* and show with this system that a stage of RNA synthesis must precede DNA replication. Although chemical characterization of the synthetic product is incomplete, our results strengthen the suggestion (1) that RNA polymerase serves an essential role in priming DNA synthesis. RNA polymerase may have a similar role in other instances of replication in which a transcriptional event has been implicated. Examples are phage λ (6), *E. coli* (7), colicinogenic factor (Col E1) (8), and M13 RF (1).

Silverstein and Billen (9); D. S. Ray, and L. McFadden and D. Denhardt (personal communications) have found that

conversion of ϕX174 SS to RF in *E. coli* is not inhibited by rifampicin. Their results indicate that RNA polymerase is not involved in formation of ϕX174 parental RF. Our enzyme studies show that there are distinctive mechanisms for conversion of the viral strands of M13 and ϕX174 to RF.

MATERIALS AND METHODS

Materials were from sources described previously (1). Rifampicin (Rifampin, Calbiochem) was used as before (1). [α-^{32}P]dTTP was the generous gift of Dr. Robert H. Symons. M13 and ϕX174 phage were labeled with [^{3}H]thymidine by the method of Forsheit and Ray (10) and Francke and Ray (11), respectively. SS were extracted by a minor modification of the method of Ray (12).

Growth of Cells. E. coli H560 (F+, PolA1$^-$, Endo I$^-$) (13) was grown in Hershey broth at 37° with rotary shaking to 2×10^8 cells/ml and then chilled. The cells were collected and resuspended in 50 mM Tris·HCl (pH 7.5)-10% sucrose at $5-8 \times 10^{10}$ cells/ml and frozen in liquid nitrogen. No loss of activity (conversion of SS to RF) was seen in lysates after the cells were stored for up to 1 month.

Cell Extract. Frozen cells (1 ml) were removed from liquid nitrogen, placed on ice for 20 min, then thawed in a 20° bath and immediately placed at 0°. Lysozyme (0.1 ml of 2 mg/ml in 0.25 M Tris·HCl, pH 7.5) and 25 μl of 4 M NaCl were added. After 45 min, the cells were warmed to 37° for 1 min, then chilled to 0°. The lysate was centrifuged for 15 min at 50,000 $\times$ g. The clear amber supernatant was made 10 mM in MgCl$_2$ (by addition 10 μl of a 1 M solution) and stored at −20° (fraction A).

Ammonium Sulfate Precipitation. 1 ml of fraction A was poured through a 0.6 $\times$ 1 cm DEAE cellulose column (DE52, H. Reeve Angel Inc., Clifton, N.J.) equilibrated with buffer A (50 mM Tris·HCl, pH 7.5, 10 mM MgCl$_2$, 10% sucrose, 0.1 M NaCl). Saturated ammonium sulfate (3 ml, neutralized with Tris base) was added at 0° to 1 ml of the material that had been treated with DEAE cellulose. After 30 min, the precipitate was collected and redissolved in 1 ml of buffer A. After dialysis near 0° for 4 hr against four 100-ml volumes of buffer A, the solution (fraction B) was stored at −20°.

Assay of Conversion of SS to RF. The triphosphate mixture contained the four deoxyribonucleoside triphosphates, each at 150 μM, and ATP at 6 mM. In some experiments, dTTP or dGTP was labeled in the α position with ^{32}P at a specific activity of 1 Ci/mmol. The four ribonucleoside triphosphates (1 mM each) were added to the mixture in some experiments.
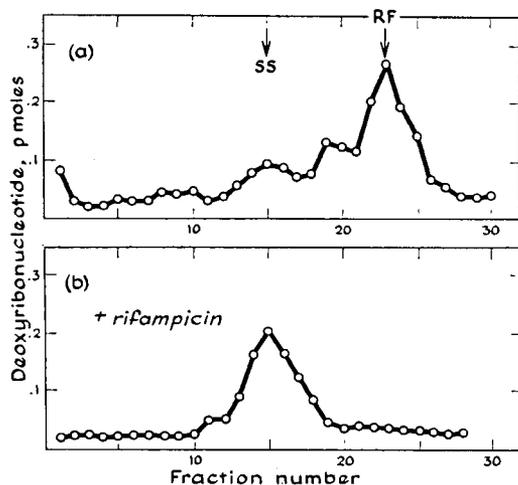
FIG. 1. Conversion of M13 SS to RF in soluble extracts is blocked by rifampicin. Fraction A (100 μl) and unlabeled triphosphate mixture was used in (a) and (b); in (b), rifampicin (5 μl, 0.5 mg/ml) was added to fraction A 1 min before addition of ³²P-SS.

The incubation mixture (120 μl) contained fraction A or B (100 μl), SS (273 pmol nucleotide in 10 μl of 10 mM Tris·HCl, pH 7.5), and triphosphate mixture (10 μl). Incubation was at 37°. The reaction was stopped by addition of sodium dodecyl sulfate (100 μl of a 10% solution). After 15 min at 60°, 240 μl of water was added and the mixture was layered on a 5-ml sucrose gradient (5–20% in 10 mM Tris·HCl, pH 8, –1 mM EDTA–1 M NaCl) and centrifuged at 4° for 2.5 hr at 65,000 rpm in a Beckman SW 65 rotor; 0.1-ml fractions were collected. Scintillation fluid (5 ml of Triton X-100-toluene base) was added, and the samples were counted in a Nuclear Chicago scintillation counter. In some experiments, 10-μl aliquots of the reaction were added to 100 μl of 10% trichloroacetic acid; incorporation into the acid-insoluble fraction was measured (14).

RESULTS

Soluble-Enzyme Fraction Converts SS to RF. A soluble-enzyme fraction from gently lysed *E. coli* (fraction A) catalyzed the conversion of SS to a form that co-sedimented with RF II (Fig. 1). Rifampicin, an inhibitor of RNA polymerase that blocks the conversion of SS to RF in intact cells at 200 μg/ml, prevented the *in vitro* reaction at 5 μg/ml (Fig. 1). RF synthesis similar to that shown in Fig. 1a was catalyzed by extracts of rifampicin-resistant *E. coli* in the presence of 5 μg/ml rifampicin.

The RF product was characterized as RF II by velocity sedimentation in neutral and alkaline sucrose and by equilibrium banding in alkaline CsCl as follows: (i) molar equivalence of the viral and newly synthesized DNA (Fig. 2a), (ii) Sedimentation of the new DNA as full-length linear strands and the viral strands mainly as circles (Fig. 2b), and (iii) Banding of the new DNA at the density of M13 complementary strands (12) (Fig. 2c). On the basis of these findings, we conclude that the *in vitro* product is RF II in which a newly synthesized, full-length linear complementary strand is annealed to a circular viral strand.

With fraction A, from which much of the cellular DNA has been removed, assay of the conversion of SS to RF could be measured directly by incorporation of a labeled nucleotide into an acid-insoluble material. Total nulceotide incorporation into the acid-insoluble material was identical to that identified in the RF region of sucrose velocity gradients (Table 1). Over 80% of the acid-insoluble nucleotide was RF. Conversion of M13 SS to RF as a function of time showed a requirement for added M13 DNA and inhibition by rifampicin (5 μg/ml) (Fig. 3).

Conversion of φX174 SS to RF followed a time course similar to that of M13 but was not inhibited by rifampicin (Fig. 3).

RNA Synthesis Initiates Conversion of SS to RF. Enzyme treated with DEAE cellulose and precipitated with ammonium sulfate (fraction B) required the addition of ribonucleoside triphosphates for conversion of M13 SS to RF (Fig. 4). A strong, but less complete, dependence was also seen in
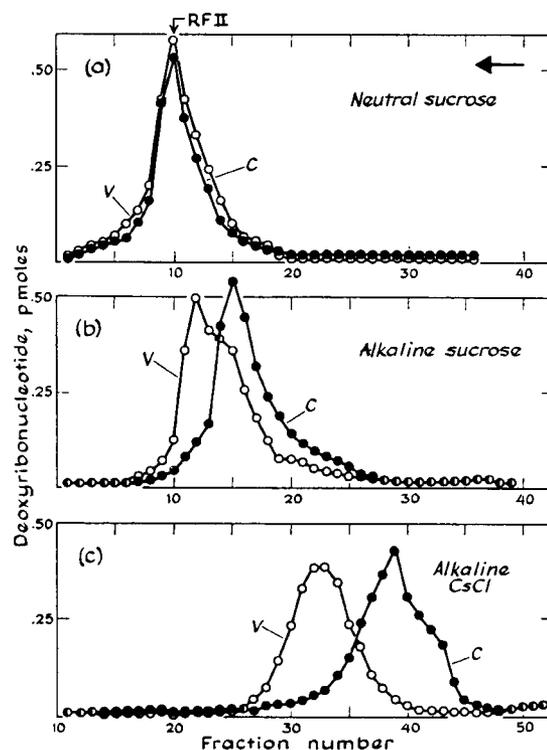

FIG. 2. Characterization of RF synthesized *in vitro*. Triphosphate mixture (40 μl with [³H]TTP, 2900 cpm/pmol), ³²P-labeled M13 SS (10 μl, 260 pmol, 1280 cpm/pmol), and fraction A (200 μl) were incubated at 37° for 5 min. Sodium dodecyl sulfate was added (25 μl, 5%), and the RF was purified by sucrose gradient centrifugation and ethanol precipitation (1). 51 pmol of ³²P-labeled viral DNA, *V*, and 53 pmol of ³H-labeled complementary DNA, *C*, were recovered as RF. (a) Centrifugation of product DNA (5 pmol) for 4.5 hr at 4° through a neutral sucrose gradient [5–20% in 1.0 M NaCl, 1.0 mM EDTA, and 10 mM Tris·HCl (pH 8.0) in an SW56 rotor]. (b) Centrifugation of product DNA (5 pmol) for 4.5 hr at 4° through an alkaline sucrose gradient (5–20% in 0.8 M NaCl, 0.2 M NaOH, 1 mM EDTA in an SW 56 rotor). (c) Equilibrium alkaline CsCl sedimentation of product DNA (5 pmol) as described by Ray (12).

TABLE 1. *Assays of conversion of M13SS to RF**

| SS added per cell equivalent† | Deoxyribonucleotide incorporated | |
	RF (pmol)	Acid-insoluble fraction (pmol)
1	4.7	4.8
5	20.2	23.3

* Isolation of RF compared to a direct assay of nucleotide incorporation into the acid-insoluble fraction. Fraction A (80 μl) was used for conversion of SS to RF. With 40 μl of the 100-μl reaction mixture, total acid-insoluble nucleotide was determined; with another portion (40 μl), RF was isolated by sucrose velocity sedimentation. The values cited for RF are acid-insoluble nucleotide in the RF region of a sucrose gradient.

† 1 mg of protein = 3×10^9 cell equivalents.

dialyzed or diluted extracts (data not shown). No single ribonucleoside triphosphate sufficed for efficient RF synthesis (Table 2); maximal activity was seen only when all four were present.

Does RNA synthesis precede DNA synthesis? Fraction B was incubated in a first-stage reaction with SS and the four ribonucleoside triphosphates in the absence of deoxyribonucleoside triphosphates. The mixture was filtered through Sephadex G-200 to remove the ribonucleoside triphosphates (less than 0.01% remained in the filtrate). In the second stage, the filtrate was treated with fresh fraction B and deoxyribonucleoside triphosphates. Formation of RF from SS required that the ribonucleoside triphosphates be present during the first, but not the second, stage (Table 3, compare Exps. 1 and 4). Rifampicin inhibited the first stage of the reaction but had a relatively small effect on the second (Table 3).

DISCUSSION

The conversion of M13 viral single strands (SS) to the double-stranded replicative forms (RF) upon infection of *E. coli* requires the action of RNA polymerase (1). This observation, based on the inhibitory effect of rifampicin, has now been

TABLE 2. *Requirements for ribonucleoside triphosphates in the conversion of SS to RF*

Ribonucleoside triphosphates added	Deoxyribonucleotide incorporated (pmol)
ATP, GTP, UTP, CTP	7.8
ATP	0.4
GTP	0.7
UTP	0.0
CTP	0.0
None	0.1

The complete assay mixture contained fraction B (20 μl), deoxyribonucleoside triphosphates (2 μl containing dTTP, dCTP, dATP, and dGTP, each at 150 μM, with 0.1 mCi/ml of [α-^{32}P]dTTP), and all four ribonucleoside triphosphates (1 μl each of 2.5 mM solutions of GTP, ATP, CTP, and UTP). After 15 min at 37°, RF synthesis was measured as incorporation of nucleotide into the acid-insoluble fraction.
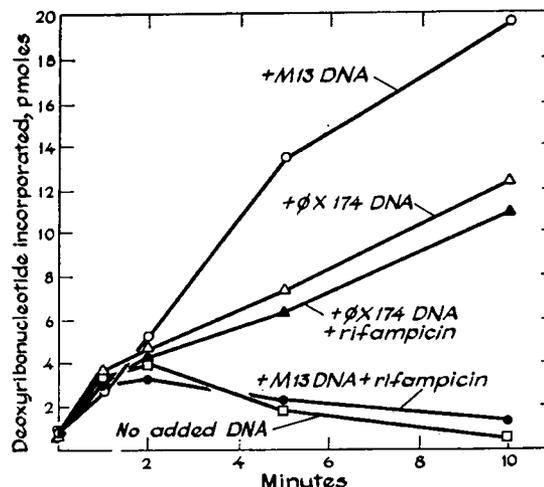

FIG. 3. Conversion of SS to RF: rifampicin inhibits M13 but not φX174. Incubations contained fraction A, the four deoxyribonucleoside triphosphates (30 μM each), ATP (1 mM), buffer (Tris·HCl, pH 7.5, 4 mM), MgCl₂ (5 mM), and one of the following: no DNA □; M13 DNA + rifampicin, ●; φX174 DNA + rifampicin, ▲; φX174 DNA, △; or M13 DNA, ○. The added DNA was 100 pmol nucleotide/mg protein: 3 SS/cell equivalent.

strengthened and extended by our studies with a soluble enzyme preparation from uninfected cells.

Soluble enzymes catalyze the formation of full-length linear strands on an M13 circular template (Fig. 2). The production of this replicative form (RF II) can be separated into two stages (Table 3): an initial RNA synthesis followed
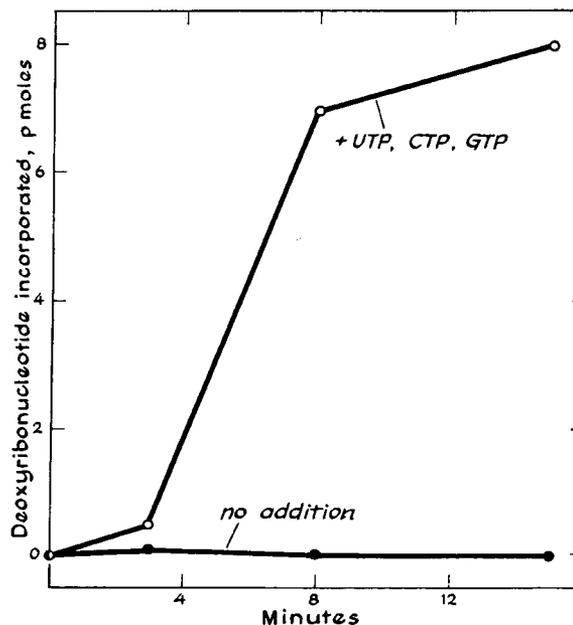

FIG. 4. Conversion of M13 SS to RF requires ribonucleoside triphosphates. Fraction B (100 μl) was incubated with, ○, or without, ●, ribonucleoside triphosphates (100 μM each of GTP CTP, and UTP); ATP (1 mM) was present in both experiments.

TABLE 3. *Distinct stages in the conversion of SS to RF*

Experiment	1st Stage (RNA synthesis)		2nd Stage (DNA synthesis)		Deoxyribonucleotide incorporated (pmol)
	Rifampicin added	UTP, CTP, GTP added	Rifampicin added	UTP, CTP, GTP added	
1	0	+	0	0	76
2	0	+	+	0	66
3	+	+	0	0	11
4	0	0	0	0	0

The incubation mixture (0.22 ml) contained fraction B (0.2 ml), SS (6 × 10¹⁰ in 10 µl of 10 mM Tris·HCl, pH 7.5), ribonucleoside triphosphates (8 µl of a solution containing UTP, CTP, GTP, and ATP, each of 2.5 mM) and, where indicated, rifampicin (2 µl of a 0.5 mg/ml solution in 0.1 M Tris·HCl, pH 7.5). After 6 min at 37° (1st stage reaction), the solution was chilled and filtered through a column (0.6 × 7 cm) of Sephadex G-200 at 4°. Fractions were assayed at once because the first stage reaction product was unstable. An aliquot (20 µl) of the void-volume fraction was mixed with [α-³²P]dTTP triphosphate mixture (5 µl), fraction B (20 µl) and, where indicated, rifampicin (1 µl of 0.25 mg/ml). The solution was incubated at 37° for 15 min (2nd stage reaction) and precipitated with acid. In control experiments, there was no detectable contamination (<0.01%) of void-volume fractions with ribonucleoside triphosphates.

by DNA synthesis. The first requires the four ribonucleoside triphosphates and is inhibited by rifampicin. A macromolecular product separated after this stage supports the synthesis of the complementary DNA strand in the absence of ribonucleoside triphosphates and in the presence of rifampicin.

We presume that a short segment of RNA synthesized in the first stage of RF formation serves as a primer for the extensive DNA synthesis in the second. However, our attempts to determine the size and composition of this initiating RNA segment have been frustrated by the synthesis of nonspecific RNA by these relatively crude enzyme fractions. Unlike the DNA synthesis, this RNA synthesis (70 pmol/mg protein) is independent of the presence of M13 DNA; the RNA resists pancreatic RNase (25% persists) and contaminates the RF II product in density gradient separation. Further purification of the enzymes involved is required to clarify this problem.

Even though the RNA priming segment has not been identified, its existence is supported by evidence in addition to that already cited or reported here. The isolated RF II contains a 5' → 3' phosphodiester link of a deoxyribonucleotide to a ribonucleotide. Alkaline hydrolysis of the RF II synthesized in the presence of four [α-³²P]deoxyribonucleoside triphosphates, yielded ³²P-labeled ribonucleotide (1.47 mol/mol RF II) of which 84% was a mixture of 2'- and 3'-rAMP (Brutlag and Kornberg, unpublished data).

alkali cleavages
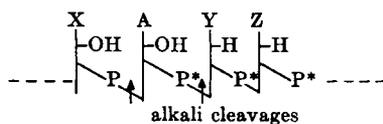
Another line of evidence suggesting the role of an RNA primer is the presence at the 5' end of the synthesized DNA

of a nucleotide structure, conceivably a 5'-ribonucleoside triphosphate derivative, which cannot be sealed by ligase. Attempts to convert the RF II to RF I by ligase, in the presence of DNA polymerase and deoxyribonucleoside triphosphates, succeeded with *E. coli* DNA polymerase I but not with T4 DNA polymerase. The *E. coli* enzyme, whose 5' → 3' exonuclease function can excise nucleotides terminating in 5'-triphosphate and mismatched regions at the 5' end, converted 35% of the RF II to RF I, whereas the T4 enzyme, which lacks this excision function, was inert in this regard (Brutlag and Kornberg, unpublished data). These data may also explain the failure of these extracts that are deficient in DNA polymerase I to seal covalently the RF synthesized *in vitro*.

Finally, model experiments with pure RNA and DNA polymerases show that the synthesis of a small piece of RNA primes the use of an otherwise inactive M13 DNA template (Bertsch and Kornberg, unpublished experiments). The capacity of DNA polymerases to extend a variety of oligoribonucleotides annealed to templates has been thoroughly explored by Wells and coworkers (15).

Is RNA synthesis involved in the initiation of M13 DNA synthesis in the cell as it is in these extracts? The identical behavior of rifampicin *in vivo* (1) and *in vitro* suggests that it is. Even more persuasive is the evidence showing the specificity of the enzymes catalyzing M13 and φX174 replication. In the same bacterial strain (H560), rifampicin inhibits conversion of M13 SS to RF *in vivo* but not that of the rather similar φX174 SS; extracts of this strain show the very same behavior (Fig. 3). Furthermore, certain *E. coli* mutants (*dna B*), which fail to synthesize host DNA at an elevated temperature and which support M13 but not φX174 parental RF formation (16, and Ray, personal communication) at the restrictive temperature, show an analogous behavior with enzyme fractions from this mutant (Schekman and Kornberg, unpublished experiments). Purification of the *dna B* gene product should reveal whether initiation of φX174 SS replication involves a rifampicin-resistant RNA synthesis or some other mechanism.

There are strong indications that RNA polymerase action is also involved in the replication of double-stranded DNA. Multiplication of M13 RF is immediately and profoundly inhibited by rifampicin (1); so is the replication of the RF-like colicinogenic factor Col E1 (8). Furthermore, Blair *et al.* (8) have found that alkaline or RNase digestion introduces breaks in the supercoiled Col E1, suggesting that a segment of RNA is present in the supercoiled DNA. Dove has implicated a transcriptional event in the initiation of phage λ DNA replication (6) and Lark has observed rifampicin sensitivity of the start of *E. coli* chromosome replication that he also attributes to an RNA synthetic action (7). Although the nature of the RNA polymerase action is uncertain in each of these instances, a primer function for DNA initiation appears to us as the most attractive explanation.

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