

## Initiation of DNA Synthesis: Synthesis of $\phi$ X174 Replicative Form Requires RNA Synthesis Resistant to Rifampicin\*

(dnaA gene product/dnaB gene product/M13 DNA/spermidine)

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**ABSTRACT** Conversion of single-stranded DNA of phage  $\phi$ X174 to the double-stranded replicative form in *Escherichia coli* uses enzymes essential for initiation and replication of the host chromosome. These enzymes can now be purified by the assay that this phage system provides. The  $\phi$ X174 conversion is distinct from that of M13. The reaction requires different host enzymes and is resistant to rifampicin and streptolydigin, inhibitors of RNA polymerase. However, RNA synthesis is essential for  $\phi$ X174 DNA synthesis; the reaction is inhibited by low concentrations of actinomycin D, all four ribonucleoside triphosphates are required, and an average of one phosphodiester bond links DNA to RNA in the isolated double-stranded circles. Thus, we presume that, as in the case of M13, synthesis of a short RNA chain primes the synthesis of a replicative form by DNA polymerase. Initiation of DNA synthesis by RNA priming is a mechanism of wide significance.

Conversion of the viral single strand of M13 DNA to its double-stranded replicative form is inhibited by rifampicin (1). Studies with enzyme fractions have shown that RNA synthesis by RNA polymerase is required in this reaction (2). Our findings indicate that the RNA synthesis provides a primer to initiate DNA synthesis (1, 2).

How general is this RNA priming mechanism for DNA-strand initiation? Inhibition of other DNA synthetic events by rifampicin suggests the involvement of RNA priming in multiplication of the M13 replicative forms (1) and in synthesis of a colicinogenic factor (3).

However, replication of  $\phi$ X174, a virus of base composition similar to that of M13, is not inhibited by rifampicin *in vivo* (4) or *in vitro* (2). Does this absence of rifampicin inhibition imply a different mechanism of DNA-strand initiation or is there an RNA synthetic system that, unlike RNA polymerase, is resistant to rifampicin?

We have found that *Escherichia coli* has distinctive enzymes for replication of  $\phi$ X and M13 DNA (2). We now report that the initiation of a  $\phi$ X DNA strand requires RNA synthesis, presumably in a primer role; the absence of rifampicin inhibition points to an RNA synthetic system that behaves differently from RNA polymerase. A remarkable feature of the

enzymes for  $\phi$ X replication is that they include two or more of the gene products (dnaA and B) that are known to be required for initiation and replication of the *E. coli* chromosome. The dna A and B proteins have been partially purified.

### MATERIALS AND METHODS

Materials were from sources described (1). Streptolydigin (Upjohn Company, Lot no. 10799) was a gift from Dr. Walter Mangel. Actinomycin D was obtained from Merck. [ $\alpha$ - $^{32}$ P]-deoxyribonucleoside triphosphates were synthesized by a modification of the procedure of Symons (5).

**Bacterial Strains.** *E. coli* H560 ( $F^+$ , *thy*<sup>-</sup>, *endo I*<sup>-</sup>, *pol A1*<sup>-</sup>) was provided by Dr. H. Hoffmann-Berling, H560 dnaB by Dr. F. Bonhoeffer, and CRT 4638 dnaA ( $F^-$ , *thy*<sup>-</sup>, *endo I*<sup>-</sup>, *pol A1*<sup>-</sup>) by Dr. R. Wickner (strain originally from Dr. Y. Hirota). Temperature-resistant revertants were selected by spreading about  $10^8$  cells on tryptone-supplemented agar plates; clones that formed overnight at 42° were selected.

**Growth of Cells and Preparation of Extract.** Cells were grown and collected as before (2), except that they were not chilled before harvest. Instead cells were suspended at room temperature (22-24°) and quickly frozen in liquid nitrogen. Extracts and supernatant fractions were prepared as before and used directly without addition of MgCl<sub>2</sub>. Extracts from temperature-sensitive strains were prepared in the same way except that the 1-min warming step was between 25 and 30° rather than at 37°. The supernatant fraction (Fraction I) was prepared in polycarbonate tubes in the type 40 or Ti 60 rotor of the Beckman L2-65 B centrifuged at 100,000 to 200,000  $\times g$  for 30-60 min. Fraction I has about 20 mg of protein per ml.

**Assay of DNA Replication.** The assay measured incorporation of a labeled nucleotide into an acid-insoluble fraction as before (2). The assay mixture (25  $\mu$ l) contained 2.5 mM MgCl<sub>2</sub>, 20  $\mu$ M each of the four deoxyribonucleoside triphosphates, 3 mM spermidine·HCl, 6% sucrose, 30 mM Tris·HCl (pH 7.5), 60 mM NaCl, 700  $\mu$ M ATP, 100  $\mu$ M each of CTP, GTP, and UTP, and 20  $\mu$ M  $\phi$ X174 single-stranded DNA (nucleotide residues). The enzyme fraction (1-15  $\mu$ l) was added last. [ $\alpha$ - $^{32}$ P]deoxyribonucleoside triphosphates had a specific activity of 1 Ci/mmol (about  $2 \times 10^9$  cpm/ $\mu$ mol). One unit of activity is defined as 1 pmol of deoxyribonucleotide incorporated per min at 30°. Fraction I has a specific activity of 5-10 units/mg protein.

Abbreviations:  $\phi$ X,  $\phi$ X174; SS, (phage) single-stranded circular DNA; RF, (phage) double-stranded DNA of circular replicative form; RF II, (phage) RF with a discontinuity in at least one strand.

\* This is the third paper in a series on Initiation of DNA Synthesis; papers I and II in this series are refs. 1 and 2.

TABLE 1. Polyamines stimulate conversion of  $\phi$ X SS to RF

|                | Polyamine concentration                 |        |
|----------------|---|--------|
|                | 1.7 mM                                  | 5.1 mM |
|                | Deoxyribonucleotide incorporated (pmol) |        |
| Spermidine     | 2.7                                     | 8.5    |
| Spermine       | 3.2                                     | 3.2    |
| Cadaverine     | 0.6                                     | 1.0    |
| Putrescine     | 0.8                                     | 1.4    |
| Diaminopropane | 1.0                                     | 1.5    |
| None           | 0.5                                     |        |

Standard assays (30 min at 30°) were performed with 10  $\mu$ l of Fraction I enzyme.

## RESULTS

### Soluble enzymes convert $\phi$ X SS to RF

Olivera and Bonhoeffer (6) showed that an *E. coli* lysate supported on a cellophane disc converted  $\phi$ X SS to RF. We developed a high-speed supernatant enzyme fraction capable of performing the conversion of an M13 template to its RF (2). This crude fraction also proved capable, under comparable conditions (see below), of sustaining the  $\phi$ X SS to RF reaction (2). As with M13 (2), the  $\phi$ X product sediments in a neutral sucrose velocity gradient as RF II; of the input viral DNA (with 2  $\mu$ M  $\phi$ X DNA) 80% was in the RF II product and the remainder appeared as single strands. The product was analyzed further by subsection of denatured RF II to velocity sedimentation in alkaline sucrose gradients or by equilibrium sedimentation in alkaline CsCl. The labeled product appeared as a full-length linear complementary strand, and the template appeared as an intact, circular, viral strand (see ref. 2 for conditions).

### Spermidine stimulates conversion of $\phi$ X SS to RF

In some experiments the rate of DNA synthesis on a  $\phi$ X single-stranded template was stimulated by spermidine 5- to 20-fold (Table 1). Although the stimulation was not always observed with different Fraction-I preparations, more purified fractions showed an invariable and near absolute requirement for spermidine (data not shown). Other polyamines were less effective. In the presence of spermidine, a rifampicin-sensitive reaction occurs at low concentrations of Fraction-I enzyme (1-5  $\mu$ l in a 25- $\mu$ l assay). The product of

TABLE 2. Effect of inhibitors of RNA synthesis on conversion of M13 and  $\phi$ X174 SS to RF in vitro

| Inhibitor                      | Single-strand template |          |
|--------------------------------|------------------------|----------|
|                                | M13                    | $\phi$ X |
|                                | % of control           |          |
| Rifampicin, 5 $\mu$ g/ml       | 10                     | 80       |
| Streptolydigin, 600 $\mu$ g/ml | 10                     | 90       |
| Actinomycin D, 5 $\mu$ g/ml    | 7                      | 11       |

Standard assays with 15  $\mu$ l of Fraction I were for 10 min at 30°; 10- $\mu$ l samples were removed and precipitated. Control values in the rifampicin, streptolydigin, and actinomycin experiments were, respectively, as follows (in pmol): 25, 29, and 15 for M13; 25, 31, and 15 for  $\phi$ X.

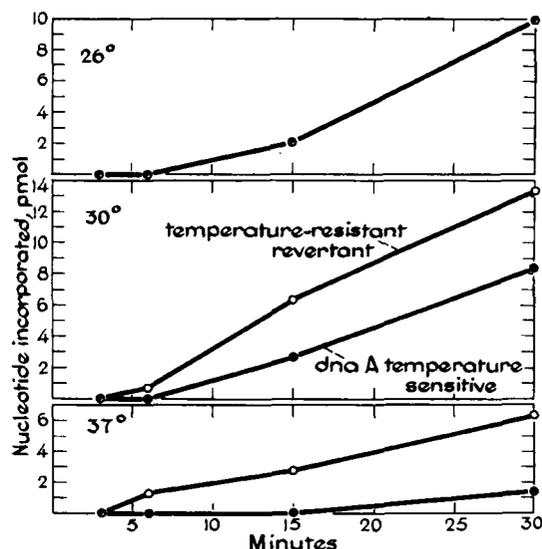


FIG. 1. Mutant dnaA protein is thermolabile in catalyzing the conversion of  $\phi$ X174 SS to RF. CRT 4368 dnaA and a temperature-resistant revertant were grown at 30° in H-broth (2) to an optical density (595 nm) of 0.25. Fraction I (10  $\mu$ l) was assayed except that spermidine was omitted and 2  $\mu$ M  $\phi$ X DNA was used. Parallel assays without  $\phi$ X DNA yielded values between 0 and 10% of those with  $\phi$ X DNA added; the values plotted are corrected for this background.

such a rifampicin-sensitive reaction is not RF II, as judged by alkaline velocity sedimentation and by equilibrium sedimentation in neutral CsCl. Under such circumstances RNA polymerase may act nonspecifically to provide a primer for DNA synthesis. All assays presented here were done under conditions of rifampicin resistance.

### Replication proteins from *E. coli* function in $\phi$ X DNA synthesis

*E. coli* mutants that fail to duplicate their chromosomes at higher temperatures have been grouped in two classes: dnaA and C mutants that continue DNA synthesis at 42° but fail to initiate a new chromosome, and dnaB, D, E, F, and G mutants that stop DNA synthesis immediately at 42°. In extracts (Fraction I) of the thermosensitive dnaA and dnaB mutants there was little or no synthesis of  $\phi$ X DNA at 37°, although the rates at 25° were at or near those of extracts of wild-type or temperature-resistant revertants (Figs. 1 and 2). When equal amounts of enzyme fractions from the dnaA and dnaB mutants were combined,  $\phi$ X DNA synthesis was at the wild-type level (Fig. 3). The rate of M13 DNA synthesis was the same in extracts of mutant cells as in those of wild-type cells whether measured at 25° or 37° (data not shown).

### dnaA protein participates in early phase of the reaction

Since the dnaA mutation affects initiation of chromosome replication in *E. coli*, we tried to see whether the dnaA gene product acts at a stage before DNA synthesis. An aliquot of Fraction I from the dnaA mutant was incubated first with  $\phi$ X SS in the absence of deoxyribonucleoside triphosphates at either 25° or 37° for 8 min. At this point, the DNA precursors were added and synthesis was followed in both cases at

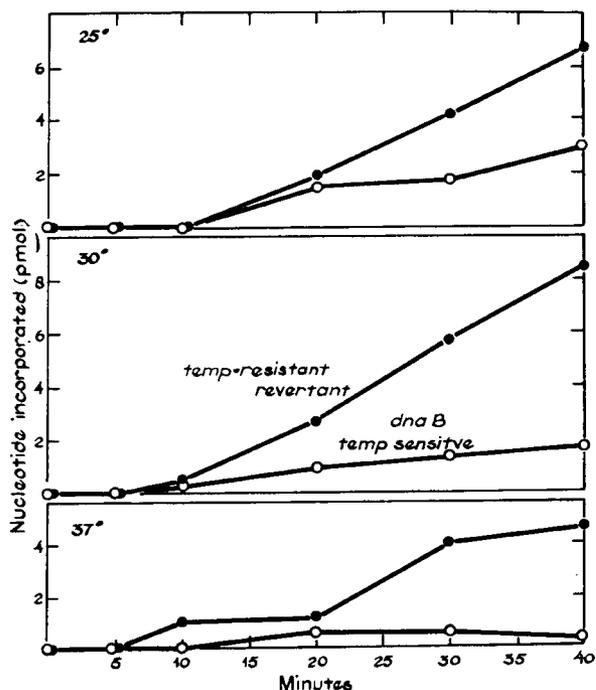


FIG. 2. Mutant *dnaB* protein is thermolabile in catalyzing the conversion of  $\phi$ X174 SS to RF. H560 *dnaB* and a temperature-resistant revertant were grown at 30° in H-broth to an optical density (595 nm) of 0.5. Fraction I was assayed as in Fig. 1.

37°. When incubations were performed at 37° throughout, no synthesis occurred; however preincubation at 25° enabled extensive DNA synthesis at 37° (Fig. 4). Thus, the *dnaA* gene product acts in the initiation phase of the reaction or is stabilized to a subsequent 37° exposure by an early interaction.

#### Partial purification of the *dnaA* and *dnaB* proteins

Complementation at 37° of Fraction I of the *dnaB* mutant provided a linear assay to determine amounts of the *dnaB* gene product in extracts of wild-type cells. A similar complementation assay for amounts of the *dnaA* gene product was feasible by use of Fraction I of the *dnaA* mutant. Complementation reactions were rifampicin-resistant, and the product was RF II. By fractionation procedures, to be reported in detail elsewhere, the *dnaA* and *dnaB* proteins have been partially purified.

#### RNA synthesis is required for conversion of $\phi$ X SS to RF

Rifampicin, which prevents RNA polymerase from initiating synthesis (7), inhibits replication of M13 but not of  $\phi$ X (2) (Table 2). The same result was found with streptolydigin, which prevents RNA polymerase from propagating a chain (8) (Table 2). From these results, one could conclude that RNA synthesis is not required for conversion of  $\phi$ X SS to RF or that RNA is synthesized by a system distinguishable from RNA polymerase. Involvement of RNA synthesis is indicated by three lines of evidence: (i) inhibition by low amounts of actinomycin D, (ii) requirement for all four ribonucleoside triphosphates, and (iii) covalent linkage of DNA to RNA in the product.

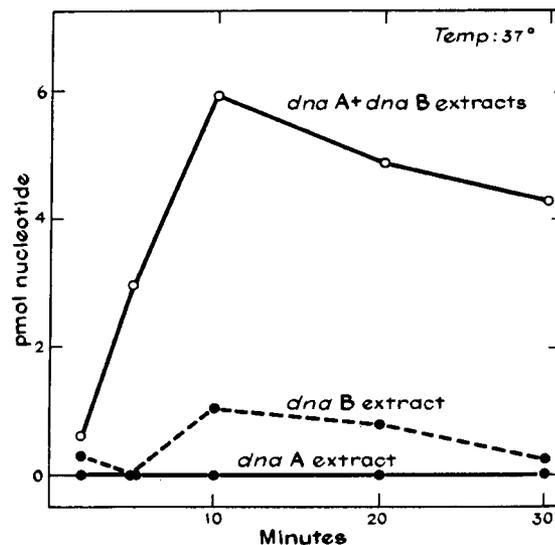


FIG. 3. Thermolabile *dnaA* and *dnaB* mutant extracts complement at the nonpermissive temperature. Fraction I from *dnaA* and *dnaB* mutant strains were assayed together (5  $\mu$ l of each) or separately (10  $\mu$ l of each) at 37° as in Figs. 1 and 2.

Actinomycin D inhibits RNA synthesis by virtually all RNA polymerases (bacterial and animal) by intercalating into a duplex DNA template and binding to deoxyguanosine (9). Since  $\phi$ X synthesis was inhibited by actinomycin D almost completely (as was M13) at concentrations that do not profoundly affect DNA replication (Table 2), an RNA synthetic event is clearly indicated.

Fraction I enzyme had a strong requirement for ATP for maximal  $\phi$ X DNA synthesis. Although a dependence on added CTP, GTP, and UTP was not apparent in this fraction, it was clearly demonstrable with an enzyme fraction precipitated with ammonium sulfate (at 40% saturation).

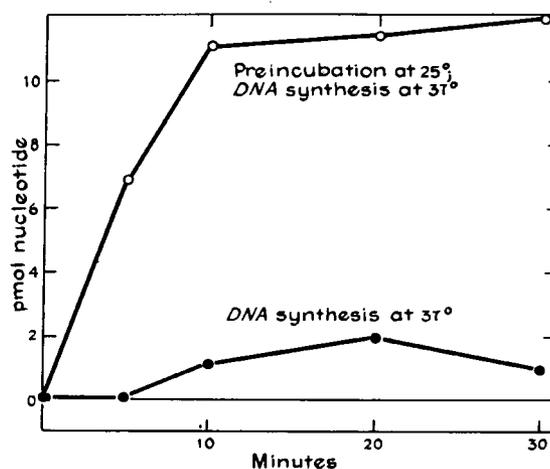


FIG. 4. *dnaA* protein is required for an early step of the  $\phi$ X174 SS to RF reaction. Fraction I was prepared from CRT 4638 *dnaA* and assayed (10  $\mu$ l enzyme in each assay) for  $\phi$ X template activity. Prior incubation denotes an 8-min incubation of the entire reaction mixture minus the four deoxyribonucleoside triphosphates; the latter were added at 8 min.

TABLE 3. Requirements for ribonucleoside triphosphates in the conversion of  $\phi X$  SS  $\rightarrow$  RF

| Ribonucleoside triphosphate additions | Deoxyribonucleotide incorporated (pmol) |             |
|---------------------------------------|---|-------------|
|                                       | -rifampicin                             | +rifampicin |
| A                                     | 8.5                                     | 9.5         |
| A, G                                  | 17.0                                    | 8.1         |
| A, C                                  | 9.8                                     | 7.6         |
| A, U                                  | 8.8                                     | 7.7         |
| A, C, U                               | 12.7                                    | 13.7        |
| A, G, C                               | 14.4                                    | 17.1        |
| A, G, U                               | 14.4                                    | 11.6        |
| A, G, C, U*                           | 52.5                                    | 38.8        |

Fraction I (3 ml) was put onto a 0.6-ml DEAE-cellulose column (DE 52, H. Reeve Angel Inc., Clifton, N.J.) equilibrated with buffer A [50 mM Tris·HCl (pH 7.5)–10% sucrose–0.1 M NaCl]. Ammonium sulfate (0.72 g, 40% of saturation) was added to the DEAE pass-through and left for 30 min at 0°. The precipitate was collected by centrifugation at  $17,000 \times g$  for 15 min. The drained pellet was redissolved in 2 ml of buffer A (with 0.2 mM dithiothreitol), and dialyzed for 5 hr with three changes of 200 ml of buffer A. After 8 hr at 0°, the fraction was used in standard assays (15  $\mu$ l of enzyme, 30-min incubation at 30°), except that only the indicated ribonucleoside triphosphates were added (100  $\mu$ M each). Assays were performed with or without rifampicin (10  $\mu$ g/ml).

\* The value without  $\phi X$  added was 3.2 pmol.

All four ribonucleoside triphosphates were required for maximal synthesis of  $\phi X$  DNA; 2- to 5-fold lower DNA synthesis with  $\phi X$  SS was observed when any one of the ribonucleoside triphosphates was omitted (Table 3).

#### DNA is covalently linked to RNA in $\phi X$ and M13 SS conversions to RF

A phosphodiester bridge between a deoxyribonucleotide and a ribonucleotide in the isolated RF product was reported earlier in the M13 reaction (2). The experiment entails alkaline hydrolysis of the RF II synthesized in the presence of four [ $\alpha$ - $^{32}$ P]deoxyribonucleoside triphosphates and isolation of the ribonucleotides from the alkaline digest. With the  $\phi X$  RF II products, as in the case of M13 (repeated in this study), about 1 mol of [ $^{32}$ P]ribonucleoside monophosphate was isolated for each mole of RF produced (Table 4). The mixtures of 2' and 3' ribonucleotides to which  $^{32}$ P was transferred contained largely AMP in the case of M13, but included significant amounts of all four ribonucleotides with AMP and GMP predominating in the case of  $\phi X$ .

#### DISCUSSION

DNA polymerases extend chains when provided with a DNA-primer strand and template but cannot initiate a chain. The unique capacity of RNA polymerases to initiate chains on DNA templates and the knowledge that DNA polymerase extends a primer, even when terminated with a ribonucleotide, suggested to us that these two polymerases might act cooperatively *in vivo* for starting a DNA strand. This expectation proved to be correct. In the initiation of a DNA strand on a phage M13 template, *E. coli* uses RNA polymerase; the reaction is inhibited by rifampicin both *in vivo* and *in vitro* (1, 2). RNA priming of DNA-strand initiation

on duplex DNA templates has also been inferred, from rifampicin sensitivity, with M13 RF replication (1) and colicinogenic factor  $E_1$  duplication (3).

DNA synthesis that is unaffected by rifampicin implies a mechanism of strand initiations without RNA involvement or else RNA synthesis catalyzed by an enzyme system with properties different from RNA polymerase. The ongoing synthesis of an *E. coli* chromosome (10) and the replication of  $\phi X$  DNA (4) are such rifampicin-resistant processes. We chose to investigate the replication of  $\phi X$  DNA because we could analyze it with a soluble enzyme system (2). Our studies of this system now show that  $\phi X$  strands are initiated by an RNA primer synthesized either by an enzyme distinct from RNA polymerase, or some rifampicin-resistant modified form of the classic enzyme. The evidence is of three kinds: (i) action of specific inhibitors (Table 2), (ii) requirement for all four ribonucleoside triphosphates (Table 3), and (iii) covalent linkage of DNA to RNA (Table 4). The action of inhibitors requires extra comment.

Actinomycin inhibits RNA polymerase action (bacterial and animal) by intercalation into a DNA helix and specific binding to deoxyguanosine residues (9). By contrast, rifampicin inhibits the initiation of RNA chains, and streptolydigin their propagation, by binding the core polymerase directly.

TABLE 4. The *in vitro* product contains RNA covalently attached to DNA

| Labeled ribonucleotide released by alkaline hydrolysis | M13 RF* $\phi X$ RF*  |        |        |        |
|--|-----------------------|--------|--------|--------|
|  | mol nucleotide/mol RF |        |        |        |
|  | Exp. 1                | Exp. 2 | Exp. 1 | Exp. 2 |
| Ap   | 1.2                   | 1.2    | 0.40   | 0.40   |
| Gp   | <0.1                  | <0.2   | 0.40   | 0.35   |
| Cp   | <0.1                  | <0.2   | 0.10   | 0.20   |
| Up   | <0.1                  | <0.1   | 0.20   | 0.10   |

RF was synthesized with all four [ $\alpha$ - $^{32}$ P]deoxyribonucleoside triphosphates added; 400–1500 pmol of DNA was obtained in a 1–1.5 ml, 10-min incubation containing 0.6–0.9 ml of Fraction I. RF isolated by neutral velocity sedimentation was adjusted with KOH to 0.3 M and incubated at 37° for 18 hr. Yeast tRNA (20  $\mu$ g) and calf-thymus DNA (12.5  $\mu$ g) were added as carriers to assist the precipitation of the alkaline-resistant DNA with perchloric acid (0.9 M). The acid-soluble supernatant was neutralized with KOH and reduced to 50  $\mu$ l. The solution was clarified and applied to DEAE paper for electrophoresis (17) [pyridine-acetate 0.5%: 5.0% (pH 3.5); 2.5–3.5 hr; 30 V/cm]. Radioactive nucleotides, located by autoradiography, were measured by strip-chart radiochromatogram scanning. Nucleotide assignments were made by comparison with a hydrolyzed 5S mRNA standard run in parallel and by published mobilities relative to marker dyes (17). In addition, all nucleotide spots were eluted and reelectrophoresed on Whatman 3 MM paper with appropriate markers to confirm identification.

In some runs, an unidentified nucleotide was released by hydrolysis in variable amounts (<0.1–0.5 mol of  $^{32}$ P per mol of RF) and migrated as a nucleoside diphosphate in two different electrophoretic systems. We are grateful for the advice of Dr. Moshe Yaniv in this experiment.

\* <0.1 mol nucleotide/mol RF was found in otherwise identical, unhydrolyzed samples.

Whereas M13 DNA synthesis was inhibited by all three antibiotics,  $\phi$ X DNA synthesis was inhibited only by actinomycin. These results indicate an RNA synthesis catalyzed by a new or modified form of RNA polymerase and point to a duplex template region within the single-stranded, circular  $\phi$ X DNA. Such duplex or hairpin structures have been demonstrated in  $\phi$ X and M13 single-strands (13, 14). Some years ago, Sekiguchi and Iida (11) examined an *E. coli* mutant whose altered permeability rendered it susceptible to actinomycin. One of the interesting characteristics was the profound inhibition of DNA as well as RNA synthesis by low concentrations of the antibiotic. A possible explanation now for the actinomycin effect of DNA synthesis is the interruption of RNA-primed DNA initiations.

Most recently, Sugino, Hirose, and Okazaki (12) have made the important discovery that the newly synthesized fragments of DNA in the nascent part of the chromosome contain covalently linked RNA sections that are subsequently removed when the fragments become linked to the main body of the chromosome. This finding strengthens the validity of Okazaki's hypothesis that DNA growth is discontinuous (15) and the generality of our proposal for RNA priming as the mechanism of DNA initiation.

There are many unanswered questions regarding the initiation event: the specificity and number of starts, the size of the primer, the specificity of termination, and the nature of the excision. Regarding the nature of the excision, the 5'  $\rightarrow$  3' exonuclease of DNA polymerase I is a strong candidate. This enzyme readily excises RNA at the 5' terminal regions of DNA chains in model experiments (unpublished data), and high 5'  $\rightarrow$  3' activity is present even in Pol A mutants (personal communication, I. R. Lehman). For an accurate answer to all these questions, purification of the synthetic system is absolutely essential.

We have embarked on the purification of each of the enzymes involved in  $\phi$ X DNA replication. Inasmuch as  $\phi$ X synthesis exploits some of the enzymes that the host uses for replication of its own chromosome, we have the prospect of gaining insights into this system as well. Thus far, we have been able to partially purify the dnaA and dnaB proteins. In addition to our own work on the dna mutants, current studies by R. Wickner, M. Wright, S. Wickner, and J. Hurwitz (personal communication) indicate involvement of proteins specified by dnaC, dnaD, and dnaG genes. DNA polymerase III may be involved in the  $\phi$ X and M13 reactions. Under certain conditions extracts of dnaE mutants (defective in DNA polymerase III, refs. 16, 17) appear to be defective in the  $\phi$ X and M13 SS to RF reactions. Addition of purified DNA polymerase I or II did not restore activity, but addition of partially purified polymerase III did.

The *E. coli* enzyme system that M13 uses for its replication is distinct from that of  $\phi$ X in that the dnaA and dnaB proteins do not participate. Beyond an involvement of RNA polymerase, current purification efforts have shown that a DNA-binding (unwinding) protein (isolated by N. Sigal and B. Alberts, manuscript in preparation) is clearly required as are an enzyme fraction that contains DNA polymerase III activity, and additional factors. It is an intriguing question why  $\phi$ X and M13, so similar in DNA structure, exploit two different synthetic systems and, beyond that, what specific roles these two systems serve in the replication of *E. coli* DNA.

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