Conversion of the M13 Viral Single Strand to the Double-stranded Replicative Forms by Purified Proteins*

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(Received for publication, December 26, 1973)

SUMMARY

Earlier studies showed that the M13 single-stranded circle is converted to duplex replicative forms (RF) in vivo and in vitro by a rifampicin-sensitive system that fails to act on \( \phi X174 \) DNA. In the absence of DNA polymerase I, the synthetic complementary strand produced by a crude cell extract contained a small gap at a unique position relative to the viral template strand.

In these studies we show that the conversion of viral M13 DNA to the covalently closed duplex form requires the action of six purified proteins: RNA polymerase, DNA unwinding protein of Escherichia coli, DNA polymerase III*, copolymerase III*, DNA polymerase I, and DNA ligase. In the absence of polymerase I, the location of the gap in the RF produced by the reconstituted protein system is the same as that produced by the crude cell extract. The functions of the several proteins, either demonstrated or inferred, are as follows: RNA polymerase to form a priming fragment, DNA unwinding protein to mask the single-stranded DNA in all but the promoter region for RNA polymerase and to support polymerase III*-copolymersase III* in carrying out most of the DNA chain growth, polymerase I to fill the small remaining gap, including that created by its 5'-3' exonucleolytic excision of the remaining RNA fragment, and ligase to join the polynucleotide ends to complete the synthetic complementary circle.

Essential functions of RNA polymerase, polymerase III*, and polymerase I in the crude system have been verified by studies of extracts of strains deficient in these proteins. The DNA unwinding protein coded by gene 5 of M13 and essential for synthesis of the viral strands later in infection, fails to replace the E. coli unwinding protein, but instead inhibits conversion of the single strand to RF.

Rifampicin inhibition of conversion of M13 SS to the duplex RF by Escherichia coli cells pointed to a function for RNA polymerase in this reaction (1). The hypothesis that RNA polymerase is required for synthesis of an RNA chain which acts as a primer for covalent extension by a DNA polymerase (1) was subsequently supported and extended by results with a crude enzyme extract (2-4). To answer many important questions about the reaction required that this crude system be resolved into its components and that the reaction be reconstituted using only highly purified components. This report describes our progress toward this objective.

EXPERIMENTAL PROCEDURE

Materials

Escherichia coli Strains

H1020 is F-, plai A, end 1, leu-, strR (5); H508 is F-, iwr A, end 1, and the parent strain of H512 (5). H1026 is H506 dna E (6); H1026 is H1026 pol Bl (7); E8400 is ddu E04, su II, lac A, strR, thyR, leuR, metR, lacR (8); E8408 is E8400 pol AI (8); PC79 is dtd ID, pol AI, his-, strR, mal A, xylR, milR, thiR (8); HM893 is pol AI, pol Bl, thyR, lacZ, lacR (9); R2-38 is end 1, lacR, refR, RPI*, thiR, xylR, strR, leuR, arg A, F-, mal-, xylR, milR. RPM is temperature-sensitive in RNA polymerase. We gratefully acknowledge gifts of these strains from the indicated authors and the generous provision of strain R2-38 by Dr. D. Dütting and Dr. F. Bonhoeffer of Tübingen.

Nucleotides

\( \alpha-\text{32P-labeled Deoxyribonucleotide Triphosphates} \) These were prepared according to Symons (10), except that the monophosphate was eluted from a small QAE-Sephadex column (formate form) with 0.1 m formic acid and the triphosphate was separated from monophosphate on DEAE-cellulose (bicarbonate form) with successive applications of 0.22 m and 0.5 m ammonium bicarbonate.

Bromodeoxyuridine Triphosphate—The monophosphate was prepared from the unprotected nucleoside with POC13 in trimethylphosphate (11) and phosphorylated to the triphosphate (12).

Enzyme Preparations

Growth of cells and preparation of extracts have been described previously (13). DNA polymerase III* and copolymerase III* were isolated by the method of Wickner et al. (15) as described below. DNA unwinding protein of E. coli (unwinding protein) was isolated by a modified procedure of Sigal et al. (14). RNA polymerase I was isolated by the method of Sproul et al. (16). RNase H was isolated by the method of Nakamura et al. (17). DNA polymerase II is the product of gene 15 of M13 and was isolated by the method of Nakamura et al. (17). DNA polymerase I was isolated from E. coli K12 (18) and the parent strain of H506 (19).

The abbreviations used are: SS, single-stranded, circular DNA; RF, circular double-stranded DNA of replicative form; RF I, covalently closed RF; RF II, RF with a discontinuity in one strand.

* This work was supported in part by grants from the National Institutes of Health and the National Science Foundation (A. K.), and an International Fellowship of the National Institutes of Health (K. G.). This is the eighth paper of the series dealing with the initiation of DNA synthesis. The preceding paper is Ref. 19.

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polymerase was a gift of Dr. M. Chamberlin and Dr. W. Zillig. E. coli DNA ligase (15) (DNA ligase) was kindly provided by Dr. I. R. Lehman. Fraction VII of E. coli DNA polymerase I (16) was used. M13 gene 5 protein (17,18) was furnished by Dr. O. Westergaard, and Haemophilus influenzae (19) endonuclease (Hin d) by Dr. H. Tabak.

**Methods**

**Assays of DNA Synthesis**

Assays of DNA synthesis (13), were at 30° for 10 min in a 20-µl reaction mixture containing 25 µM each of dATP, dCTP, dGTP, and dTTP (α-32P label in dATP or dCTP, 100 to 700 cpm per pmole), 200 µM each of CTP, GTP, and UTP, 1 mM ATP, 5 mM MgCl₂, 20 mM Tris-HCl (pH 7.5), 80 mM NaCl, 20 mM mercaptoethanol, 0.2 mM EDTA, 0.2 µg ml of bovine serum albumin (19), 10% glycerol (omitted in assays of the crude extract), and 4 x 10⁶ airads of M13 DNA (400 pmoles of nucleotide residues). Protein was determined according to Groves et al. (20) or Büchler (21).

**Clearavage of RF with restriction endonuclease from H. influenzae** has been described (19).

**Fractionation of Crude Extracts for Components Required for M13 SS → RF Conversion (Scheme 1)**

The high speed supernatant (extract) of H560 cells (13) was subjected to phase partition. Polystyrene glycol 6000 (6 g), dextran T500 (4 g), and NaCl (23.2 g) were added to 100 ml of extract. The top phase was dialyzed for 7 hours against three changes of 2 liters of Buffer C25 (Buffer C with 25 mM NaCl). The dextran sulfate eluate was dialysed against tripolyphosphate (p-galactosidase on the Bio-Gel A-1.5m column). The enzyme was reprecipitated in trichloroacetic acid and counted on glass filters. The direction of sedimentation and the increase of density are plotted from right to left.

**RESULTS**

A Temperature-sensitive, RNA Polymerase Mutant Verifies the Requirement for RNA Polymerase in Conversion of SS to RF—Despite the strong indications from rifampicin-inhibition studies in vivo (1) and in vitro (2) that RNA polymerase participates in the initial events of M13 replication, a slight possibility remained that some other rifampicin-sensitive system might be responsible. The behavior of a mutant R2-38, known to be temperature-sensitive in RNA polymerase, has disposed of this possibility. An extract of R2-38 cells synthesized M13 SS to RF. RNA polymerase (3 µg) from wild type Escherichia coli was added to 100 µl of crude extract of strain R2-38 where indicated and assayed as under "Methods."
Table I

DNA polymerase III*, a dna E gene product, is required in the conversion of M13 SS to RF II

The extracts (at 5 to 20 mg of protein per ml) were frozen and thawed 3 to 5 times; 15 ¼ were used in each assay. M13 DNA was added and the reaction was at 30° under conditions described under "Methods." Additions were 5 ¼ of polymerase III* (Column b) or 5 ¼ of buffer (Column a).

<table>
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<tr>
<th>Strain</th>
<th>Polymerase I (pol A)</th>
<th>Polymerase II (pol B)</th>
<th>Polymerase III (dna E)</th>
<th>No polymerase III* addition (a)</th>
<th>Polymerase III* addition (b)</th>
<th>(b)/(a)</th>
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<tr>
<td>H508</td>
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DNA Polymerase III*, a dna E Gene Product, Is Required in Conversion of SS to RF II—In vivo studies with dna E mutants have led to the conclusion that its gene product, polymerase III, is not required for conversion of M13 SS to parental RF (23) as it is for replication of the host chromosome (6, 8). Cells, whose extracts lack polymerase II due to a mutation in the pol B gene, have not been found defective in M13 or any other replicative activity (9). Nevertheless, in vivo studies of mutants cannot exclude that sufficient enzyme activity persists for M13 SS to RF conversion under the restrictive conditions.

In order to determine which of the known DNA polymerases is responsible for conversion of M13 SS to RF II, extracts of cells with mutations in the pol A, pol B, and dna E genes were examined. Extracts of mutants in dna D, defective in X174 parental RF formation (24), were also studied. Extracts which contained 5 to 20 mg of protein per ml were as active when prepared from any of the mutants as those from wild type cells. However, upon freezing and thawing more dilute extracts, only those from dna E mutants were inactivated (Table I). Combination of these inactive extracts with a purified preparation of polymerase III*, a complex form of polymerase III (13), stimulated M13 DNA replication strikingly (Table I); an earlier study (13) showed that this result with polymerase III* was not obtained with polymerase I, polymerase II, or polymerase III.

Reconstitution of SS to RF II Conversion with Purified Proteins—Four proteins are required (Fig. 2): RNA polymerase, polymerase III*, copolymerase III*, and DNA unwinding protein of E. coli. The need for the first two was already evident in the crude extract (Fig. 1 and Table I); dependence on RNA polymerase (Fig. 2A) and polymerase III* (data not shown) was absolute. The importance of copolymerase III* for polymerase III* function (Fig. 2B) has been reported (13). The requirement for unwinding protein (Fig. 2C) is novel and more complex. It appears to serve in two stages of the over-all reaction: in limiting and directing the RNA initiation, as described below, and in the extension of an RNA-primed template (Fig. 3). The optimal concentration of the unwinding protein represents a molar ratio relative to the added M13 DNA, of about 1 per 5 to 10 nucleotide residues, a value near that expected for complete binding of the DNA (14). However, the optimal concentrations are rather narrow, presumably due to inhibitory effects upon template function especially in RNA polymerase initiation.

Spermidine appears to replace the unwinding protein (Fig. 2D) in supporting the rate and extent of DNA synthesis but not in producing the nearly full length complementary strand whose missing segment has a unique location relative to the template (see below). DNA unwinding protein and spermidine in the same reaction mixture appear to be additive in their stimulation of DNA synthesis (data not shown).

X174 DNA replaced M13 DNA as a template in this purified system and was converted to RF II. Although the product resembles in size that produced by the crude extract (3), the reconstituted system lacks a specific factor in the extract that prevents the use of the X174 template and directs it to the rifampicin-resistant, multicomponent system responsible for its conversion to RF (3). As with M13 DNA, spermidine could replace the unwinding protein in sustaining the rate of synthesis but not for producing the long product strands.

Besides M13 and X174 DNA, a mini-form of M13, about one-third the length of M13 DNA (25), was also efficiently replicated with purified components. The product sedimented in alkaline sucrose gradients at a value about 4 S slower than that of full length M13 linear; this sedimentation value is consistent with the small size of the mini-template strand.

Synthetic RF II Contains a Nearly Full Length Complementary Strand and a Gap at a Unique Location—The RF II produced by a crude extract contains a complementary strand of nearly full genome length (2) and a gap in a specific region relative to...
the viral template (19). The purified system reconstituted from four proteins produced an RF II with the same features. The complementary strand, of approximately 16 S, was as long as the product of the crude system as shown by sedimentation in an alkaline velocity gradient (Fig. 4A). After cleavage of the RF II samples by the Hind d restriction endonuclease, a small fragment was produced in each case (Fig. 4B). Their identical, distinctive size indicates the same, unique location of the gap in both RF II products (19).

The importance of the DNA unwinding protein in directing what appears to be an initiation of replication to a unique region of the template, became manifest by substituting spermidine. The synthetic strand of the RF II in this case consisted of short pieces (Fig. 5A). Because synthesis under these conditions was equivalent to only about 10 to 20% of the template, it was uncertain whether replication utilized all the circles partially or some completely. That the short pieces completely fill viral templates was indicated by an experiment using the bromouracil analog of thymidinc triphosphate. The bromouracil product possessed the expected buoyant density increase (Fig. 5B). In order to be assured that the density shift was attributable to bromouracil and not to RNA in the RF II, the product was treated with pancreatic RNase (25 µg per ml for 30 min at 37°); a shift to a lower buoyant density in CsCl was not observed.

When DNA unwinding protein and spermidine were both present and at optimal concentrations, short DNA products were more numerous than the near full length lines. Of the recovered radioactivity in the products, over 50% was in molecules sedimenting slower than 16 S, suggesting that spermidine, even in the presence of unwinding protein, causes nonspecific initiation events.

These results indicate that in the absence of DNA unwinding protein, many starts are made on the template circle and are then extended by polymerase III* molecules. When limited in number, the latter may move from one nascent fragment to the next to make the short chains which comprise the complementary strand.

**Gene 5 Protein Inhibits Rather than Stimulates SS to RF Conversion**—Synthesis of single strands for assembly into M13 filaments requires that they be enveloped by a protein coded for by gene 5 of M13 (28). The gene 5-protein-DNA complexes accumulate in the cell (29) while awaiting packaging of the DNA into virus particles by capsid protein stored in the inner membrane (30). The function of gene 5 protein may be to inhibit RF synthesis by masking the SS as templates (31). In vivo studies (32) have shown that a contrived accumulation of gene 5 protein before the parental RF is multiplied results in a bypass of the RF multiplication stage and an immediate synthesis of viral strands.

An in vitro effect consistent with these in vivo findings has now been observed with the purified proteins responsible for SS to RF conversion. The gene 5 protein, despite its high affinity for DNA, not only failed to replace the DNA unwinding protein of E. coli in facilitating replication of the viral strand but strongly antagonized its action in a cooperative manner, when the protein...
Effects of M13-gene 5 protein on M13 SS to RF conversion. Incubations were as in Fig. 2C. When RNA-primed M13-DNA was used, the concentration was raised from 400 to 3000 pmoles. Assays were as in Fig. 3.

FIG. 6 (left). Conversion of M13-SS to RF by DNA polymerase I inhibited by unwinding protein. The incubation was for 10 min at 30°C in 50 mM and contained 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.5), 0.3 μg of RNA polymerase, 0.2 μg of DNA polymerase I, and additional assay components as described under “Methods.”

Polymerase I Fails to Replace Polymerase III* in SS to RF II Conversion—Polymerase I could not be substituted for inactivated polymerase III* in a crude extract (13) nor was the deficiency of polymerase I in extracts of pol A cells manifested in the SS to RF II conversion (Table 1). Furthermore, polymerase I failed to replace polymerase III* in the purified protein system that converts SS to RF II (Fig. 7). At the level of unwinding protein, optimal for producing an RF II possessing a complementary strand of nearly full genome length, synthesis with polymerase I in place of polymerase III* was almost completely inhibited. This level approximates 1 molecule of unwinding protein per 5 to 10 DNA nucleotides and corresponds with that required to saturate the DNA (14) and to support optimal synthesis by polymerase III* (Fig. 3). Stimulation by spermidine may be due either to provision by RNA polymerase of more priming sites for polymerase I or to facilitation of DNA chain growth as in the case of polymerase III* (Fig. 3). The rapid rate of synthesis by polymerase III* in the presence of unwinding protein despite only a single priming start per template circle may be ascribed to an inherently higher turnover number or to some specific and favorable interaction with the unwinding protein.

Conversion of RF II to RF I Requires Polymerase I Action—With crude extracts from pol A cells or with the purified-protein system lacking polymerase I, the principal product of SS conversion was RF II (Fig. 8A). Complementation of the four proteins with polymerase I and ligase led to a substantial conversion of SS to the fully covalent duplex RF I as indicated by banding in a CsCl-ethidium bromide gradient (Fig. 8B). Of the radioactively labeled synthetic strands, 40% were recovered as RF I when polymerase I was present as compared to only 8% in its absence. Similar results were found when the RF products of synthesis by crude extracts of pol A and pol+ cells were compared. Extracts (supplemented with M13 DNA, DNA ligase, and DPN, and incubated for 10 min at 30°C) yielded RF I as 7% of the products of synthesis with pol A (H560) cells compared to 42% with pol+ (H508). The requirement for polymerase I to produce RF I, and the failure of polymerase III* to replace it, is consistent with in vivo findings (33). This may be explained by a need for the unique 5'-3' exonuclease activity linked to polymerase I activity to remove the RNA priming fragment at the 5' end before closure by E. coli DNA ligase can be effected.

The higher buoyant density of the RF I produced in the purified system and also in crude extracts compared to RF I isolated from cells (Fig. 8B) is presumably due to a relative lack of negative supercoiling in the in vitro form and a consequent decrease in the amount of ethidium bromide bound at the high dye concentrations used (34).

DISCUSSION

The first stage in replication of phage M13 in E. coli is the simultaneous uncoating and conversion of the viral single-stranded circle to the duplex form, called the parental RF I.
This stage has been duplicated in cell-free extracts (2) using viral DNA artificially freed of its coat, and now as described here, has been reconstituted with purified proteins isolated from these extracts (Fig. 8). The functions of these proteins will be discussed according to a hypothetical sequence (Fig. 9) of four discrete steps: (a) preinitiation, in which a DNA-binding protein prepares the template for a specific start; (b) initiation, in which RNA polymerase transcribes a small specific region; (c) chain growth, in which the DNA polymerase III* system extends the RNA priming fragment to a nearly full genome length to form an RF II with a small gap in a unique location relative to the template; and (d) gap filling and closure, in which the RNA fragment is excised and the gap in the RF II is filled by DNA polymerase I, and closure by ligase produces the parental RF I.

Preinitiation—RNA polymerase is thought to bind nonspecifically to single-stranded DNA but more selectively to certain duplex regions. With the single-stranded M13 DNA as template, some special means is needed to prevent RNA polymerase from making multiple initiations and to direct it to a specific site. The unwinding protein with its strong affinity for single-stranded DNA (14) appears to serve that function. When present at a level sufficient to bind the available template, RNA starts are minimized and the synthetic strand appears to start at a particular place as suggested by the unique location of the small gap in the RF II product (Fig. 4B).

When the unwinding protein is replaced by spermidine, the rate and extent of synthesis are maintained but the product strand consists of many short pieces indicating multiple starts (Fig. 5). Such short replication fragments characterize the nascent state of SV40 (35) and polyoma (36) DNA replication in animal cells and have been observed as intermediates in ΦX174 replication in ether-treated E. coli (37, 38).

The behavior of gene 5 protein coded by M13 and produced late in infection for synthesis of viral single strands is instructive with respect to that function as well as the specificity of the unwinding protein in parental RF formation. Despite the strong affinity of the gene 5 protein for single-stranded DNA, it not only fails to substitute for the unwinding protein in replication of the viral DNA template but inhibits its action (Fig. 6). This capacity of the gene 5 protein to prevent a viral strand from being replicated to form RF may, as has already been suggested (31), represent its principal function in the cell.

Although the unwinding protein is an essential component of the reconstituted purified protein system that forms the parental RF, there are several important reservations to accepting it as part of the in vivo system. One is that it is unknown where on the viral template the replication origin of the RF II is located and whether it corresponds to the one observed in vivo. Another is that the stripped viral DNA being furnished as a template to the purified enzyme system does not appear as such in the cell. Instead all indications are that uncoating of the viral DNA is dependent upon concurrent replication. Thus, in vivo, the viral cap protein may partially replace the unwinding protein by masking all but the promoter region of the DNA and dissociate as replication proceeds to form the duplex. Finally it is not known whether spermidine, which is invariably present in the cell and can support RF II synthesis in vitro, may serve a physiological role.

Initiation—Transcription of a small segment of the viral DNA by RNA polymerase is clearly the principal event in initiation but few details about it are known. The presence in M13 DNA of a duplex region of about 40 nucleotides (39), coupled to the RNA polymerase recognition of duplex regions for initiation suggest the plausible hypothesis that this hairpin region of the DNA is the promoter site for starting replication. In unpublished experiments, DNA unwinding protein at optimal concentrations for replication of single-stranded M13 DNA was found to reduce RNA synthesis by more than 80%. Because newly synthesized double-stranded DNA may immediately serve as a template for transcription, rifampicin was added to inhibit transcription of the duplex after initiation had occurred. An improved, but still gross estimate of RNA synthesis tied to DNA synthesis suggests a primer length of less than 50 ribonucleotides per circle of M13 template. However, the exact size and composition of the primer are still unknown. A persistent background of synthesized RNA which is not covalently linked to DNA has confounded our attempts to make an accurate determination.

Chain Growth—Extension of the RNA priming fragment requires polymerase III* and copolymerase III*, presumably as the natural holoenzyme (40). Initially a complex with the primer terminus is formed for which ATP is needed and cleaved to ADP and P_i (40). Thereafter replication may depend only on polymerase III*. The failure of polymerase I, polymerase II, or polymerase III to substitute for polymerase III* may be localized at one or more of the several components of this step: displacement of RNA polymerase, complex formation with the primer terminus, interaction with the unwinding protein, and rapid replication of the template.

The explicit dependence on polymerase III*, the dnaE gene product, for parental RF formation in vitro conflicts with conclusions drawn from in vivo experiments that this protein is not involved (23). Conclusions about in vivo biochemical events based on the use of temperature-sensitive mutants must be guarded because one does not know how much of an enzyme is needed for a particular job and how labile it is in its particular location at the restrictive temperature. A reasonable supposition in this instance is that dnaE mutants, while unable to sustain M13 RF or host chromosomal replication at 42°, retain enough polymerase III* activity to produce the few parental M13 RF molecules observed in vivo normally.
Gap Filling and Closure—The last step leading to RF I requires polymerase action to fill a small gap in the synthetic strand in order that apposed 5’-phosphoryl and 3’-hydroxy termini are made available for DNA ligase action. It was evident both in the crude and purified-protein systems that only polymerase I could do this job (Fig. 8). The strong presumption that removal of an RNA priming fragment from the 5’ end is essential for action by E. coli DNA ligase (4), leads directly to the suggestion that it is the unique 5’-3’ exonuclease property of polymerase I that qualifies it for this function. The facility with which polymerase I carries out replication concomitant with 5’43 excisions readily describes how a gap created by removal of RNA can be filled. What is not explained is the reason for the gap in the RF II to begin with and whether special features it may possess might also dictate a preference for polymerase I over the other polymerases.

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