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DNA Polymerase III Star Requires ATP to Start Synthesis on a Primed DNA†

(spermidine–copolymerase III star–pol III* complex)

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ABSTRACT **DNA polymerase III star replicates a φX174 single-stranded, circular DNA primed with a fragment of RNA. This reaction proceeds in two stages. In stage I, a complex is formed requiring DNA polymerase III star, ATP, spermidine, copolymerase III*, and RNA-primed φX174 single-stranded, circular DNA. The complex, isolated by gel filtration, contains ADP and inorganic phosphate (the products of a specific ATP cleavage) as well as spermidine, polymerase III star, and copolymerase III star. In stage II, the chain grows upon addition of deoxynucleoside triphosphates; ADP and inorganic phosphate are discharged and chain elongation is resistant to antibody to copolymerase III star. Thus ATP and copolymerase III star are required to initiate chain growth but not to sustain it.**

Conversion of φX174 and M13 viral single-stranded circular DNA (SS) to the double-stranded replicative form (RF) involves synthesis of an RNA priming fragment that is covalently extended by a growing DNA chain (1, 2). For chain growth, DNA polymerase III star (pol III*) and copolymerase III star (copol III*) are required (3).

Pol III* is a complex form of DNA polymerase III (pol III) (3, 4). "Pol III*" is a provisional designation until such time as the structural differences between pol III* and pol III are better understood. Both enzymes are products of the *dna* E gene (3, 5) and have similar salt, ethanol, and deoxynucleoside triphosphate optima on a DNA template with short gaps. The enzymes are readily separated physically and are distinguished by the ability of pol III*, but not pol III, to replicate long, single-stranded templates for which spermidine and copol III* are specifically required (3).

Closer studies of the mechanism of starting DNA chain growth by the pol III* system has uncovered an absolute requirement for ATP. ATP and copol III* participate in the formation of a complex between pol III*, spermidine, and primer-template which can be isolated and shown to support rapid DNA synthesis upon addition of deoxynucleoside triphosphates. The complex contains RNA-primed SS, spermidine, pol III*, copol III*, and ADP and inorganic phosphate (P_i), the products of ATP cleavage. This structure is referred to as pol III* initiation complex or simply as the complex. The bound ADP and P_i are released when the complex is exposed to deoxynucleoside triphosphates.

Abbreviations: φX, φX174; SS, (phage) single-stranded, circular DNA; RF, replicative form; pol III*, DNA polymerase III star; pol III, DNA polymerase III; copol III*, DNA copolymerase III star.

† This paper is no. 6 in a series on "Initiation of DNA Synthesis"; paper 5 in this series is ref. 3.

Formation of pol III* initiation complex occurs on templates as dissimilar as RNA-primed SS and $(dT)_9 \cdot (dA)_{1500}$. The experiments reported here concern initiation events that occur after primer has been synthesized and do not consider the template specificities of priming enzymes. Although RNA polymerase is not the enzyme responsible for priming φX174 SS→RF in the cell, its convenience and availability recommended its use for priming in these studies.

MATERIALS AND METHODS

Materials were from previously described sources (3). [*Adenine*-^{3}H]ATP, [^{3}H]TTP, and [^{14}C]spermidine were purchased from New England Nuclear Corp. $(dA)_{1500}$ and $(dT)_9$ were the products of PL Biochemicals. Freund's adjuvant, complete and incomplete, was purchased from Difco Laboratories. Preparation of RNA-primed φX SS and purification and assay of pol III* and copol III* were described (3). Assay buffer contained 10% sucrose, 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 40 mM dithiothreitol, and 0.2 mg/ml of bovine-serum albumin.

Rapid isolation of pol III* was by a modification of the published procedure (3). Pol III* was purified through fraction III (200 ml), mixed with diluent (400 ml of 20% glycerol–20 mM dithiothreitol–1mM EDTA), and applied to a P11 phosphocellulose column (3 × 4 cm) equilibrated with 25 mM NaCl buffer [30% glycerol, 50 mM Tris·HCl (pH 8.5), 25 mM NaCl, 20 mM dithiothreitol, 1 mM EDTA). After the column was washed with 25 mM NaCl buffer (30 ml) and 100 mM NaCl buffer (50 ml), the enzyme was eluted with 175 mM NaCl buffer.

Glycerol kinase (the generous gift of Dr. J. Thorner and Dr. H. Paulus) was used to remove ATP that contaminated the deoxynucleoside triphosphates. The procedure used was: glycerol kinase (50 μg), assay buffer (0.3 ml), glycerol (0.1 ml), and deoxynucleoside triphosphates (0.12 μmol of each including [α-^{32}P]dCTP) were mixed (0.8 ml final volume), incubated for 10 min at 30°, and then heated at 100° for 2 min.

For preparation of pol III* initiation complex, a preincubation mixture (120 μl) of 10 μl of assay buffer, 5 μl of 10 mg/ml of bovine-serum albumin, 2 μl of 0.25 M $MgCl_2$, 30 μl of RNA-primed φX SS (A_{260} = 6), 10 μl of water, 4 μl of 0.1 M spermidine·HCl, 2 μl of 2 mM ATP, 30 μl of 40 μg/ml of copol III*, and 20 μl of pol III* (4 units) was incubated 5 min at 30°, then filtered through a Biogel A5m column (0.6 × 8 cm, equilibrated at 4° with assay buffer containing 4 mM $MgCl_2$). Void-volume fractions (80 μl) were assayed for DNA synthesis by incubation for 5 min at 30° with de-

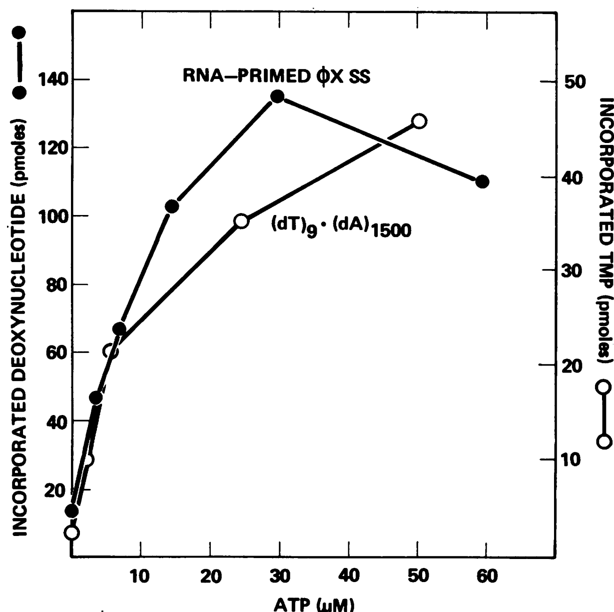


FIG. 1. ATP is required for synthesis on RNA-primed ϕX SS and on $(dT)_9 \cdot (dA)_{1500}$. Assays of pol III* (0.2 unit) and copol III* (0.05 μg) were performed on RNA-primed ϕX SS (○——○) as described (3), or on $(dT)_9 \cdot (dA)_{150}$ (●——●). for 5 min. Assays with the homopolymer were in an incubation mixture (25 μl) containing [^{3}H]TTP (1 μl, 400 μM, 10^3 cpm/pmol), $MgCl_2$ (1 μl, 120 mM), $(dA)_{1500}$ (2 μl, A_{260} = 5), $(dT)_9$ (1 μl, A_{260} = 5), spermidine·HCl (1 μl, 0.1 M), and assay buffer (10 μl). Deoxynucleoside triphosphates (8 μl of a dNTP mixture per 25-μl assay) were freed of ATP as described in *Methods*. ATP was added in the indicated amounts.

oxynucleoside triphosphates (20 μM of each, including [α-^{32}P] dCTP).

Antibody to copol III* was prepared by injecting 0.25 mg of copol III* in complete Freund's adjuvant into the footpads of a white, male rabbit, followed by 0.25 mg of copol III* in incomplete adjuvant 2 weeks later. Blood was removed from ear veins and serum was collected. The gamma globulin was purified by Na_2SO_4 precipitation (25% at 25°), dialysis [60 mM potassium phosphate (pH 6.3)], and passage through DEAE-Sephadex [equilibrated with 60 mM potassium phosphate (pH 6.3) at 4°]. We gratefully acknowledge the help and guidance of Dr. Paul Gottlieb in this procedure.

[γ-^{32}P]ATP (6) was purified by Norit adsorption followed by filtration through G-150 Sephadex [in 0.1 M NH_4HCO_3 (pH 8.0)] to remove trace, high-molecular-weight contaminants.

TABLE 1. *Formation of a complex between pol III* and primer–template*

Preincubation reaction	Deoxynucleotide incorporated by isolated pol III* initiation complex (pmol)
Complete	56
−ATP	6
− pol III*	3
− copol III*	4
− sperimidine	<0.1
− primed ϕX SS	<0.1
− Mg^{++}	1

The complex was isolated as described in *Methods*. In each case, the factor omitted in the preincubation reaction was added for the assay of pol III* initiation complex.

RESULTS

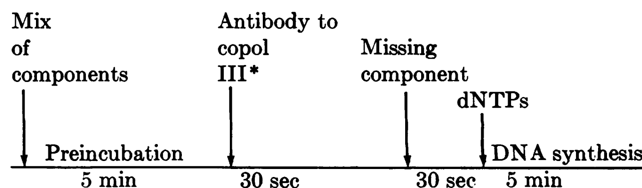
ATP Requirement. Pol III* required ATP for synthesis of the complementary strand on an RNA-primed ϕX viral SS (Fig. 1). The K_m for ATP was 8 μM; CTP, GTP, and UTP showed no detectable stimulation of the reaction (<10%) and could not replace ATP. ADP inhibited the reaction with a K_i of 400 μM. P_i at 5 mM was without effect. ATP was also required (with a similar K_m) for DNA synthesis on a $(dT)_9 \cdot (dA)_{1500}$ primer-template (Fig. 1). ATP was not required by pol III* or pol III for DNA synthesis on calf-thymus DNA with short gaps.

Isolation of Pol III Initiation Complex.* Pol III*, copol III*, ATP, spermidine, RNA-primed ϕX SS, and Mg^{++} were mixed, incubated for 5 min at 30°, and filtered through a column of Biogel A5m. This resin separates RNA-primed SS in the void volume from all other components of the preincubation mixture (1). Fractions in the void volume were able to support DNA synthesis upon addition of deoxynucleoside triphosphates (Table 1, first line). The components required

TABLE 2. *Requirements for forming a pol III* initiation complex resistant to antibody to copol III**

Preincubation	DNA synthesis resistant to antibody to copol III* (pmol of deoxynucleotide)
Complete	12.0
− RNA-primed ϕX SS	0.3
− spermidine	1.1
− ATP	0.2
− copol III*	1.2
− pol III*	<0.1
− $MgCl_2$	0.1
+ antibody to copol III*	0.5

Complete preincubation mixture (20 μl) contained 4 μl of assay buffer, 1 μl of 10 mg/ml of bovine-serum albumin, 5 μl of RNA-primed ϕX SS (A_{260} = 6), 1 μl of 0.1 M spermidine·HCl, 2 μl of 0.4 mM ATP, 1 μl of 0.05 M $MgCl_2$, 1 μl of 0.04 mg/ml of copol III*, and 3 μl of pol III* (0.6 unit). In one tube excess antibody to copol III* was present during the preincubation. Preincubation (5 min) was terminated with excess antibody to copol III*, followed in 30 sec by addition of the component omitted from the preincubation. Deoxynucleoside triphosphates (5 μl containing 400 μM of each, including [α-^{32}P]dCTP) were added 30 sec later. Acid-insoluble nucleotides were measured after 5 min. All incubations were at 30°.



to form the complex in the void-volume fraction were determined by omitting them one at a time. Omission of any one of the listed components yielded void-volume fractions that failed to support DNA synthesis, even though in each instance (Table 1) the missing component was added subsequently along with the deoxynucleoside triphosphates. Thus, each component of the preincubation mixture is essential to produce the pol III* initiation complex.

Pol III* initiation complex can be isolated with $(dT)_9 \cdot (dA)_{1500}$ as primer-template as well as with RNA-primed ϕX SS. ATP, copol III*, and spermidine are necessary to form either complex.

[^{14}C] Spermidine (2mM, 10 Ci/mol) was mixed with RNA-primed SS (2 mM in nucleotide) in assay buffer. Although 2 mM spermidine was optimal for pol III* activity in assays with 0.2–2.0 mM template nucleotide (3), a spermidine–DNA complex, separated from unbound spermidine by gel filtration, contained only 1 mol of spermidine per 10^3 mol of nucleotide. This spermidine–DNA complex supported DNA synthesis upon addition of pol III*, copol III*, ATP, and deoxynucleoside triphosphates (data not shown). DNA replication of the spermidine–DNA complex was not stimulated by further addition of spermidine.

Pol III Initiation Complex Contains Copol III**. Copol III*, which is ordinarily included in Biogel A5m, was isolated from the Biogel void-volume fractions which contain pol III* initiation complex (see *Methods*) by adsorption of the complex to DEAE–cellulose followed by elution with 0.4 M

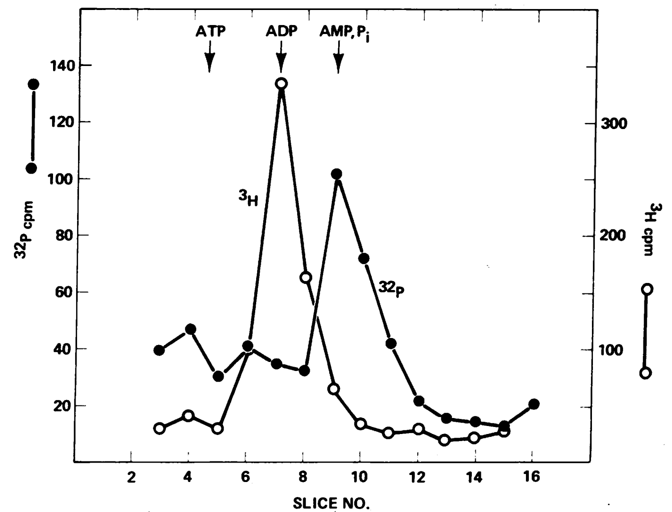


FIG. 3. ADP and P_i, products of ATP cleavage, are bound to pol III* initiation complex. The complex, doubly labeled with ^{3}H and ^{32}P, was isolated from a preincubation mixture (200 μl, as described in *Methods*) containing [^{3}H]ATP (50 μCi, 1.5 nmol) and [γ-^{32}P]ATP (2 μCi, 1.6 nmol) and dried as a band on a 4 × 10-cm strip of polyethyleneimine cellulose (Polygram cel 300 PEI, Brinkmann Instruments, Inc.) with ATP, ADP, and AMP markers. After ascending chromatography in NH_4HCO_3 (0.4 M), markers were located with UV light. The chromatogram was then sliced and assayed for ^{3}H and ^{32}P.

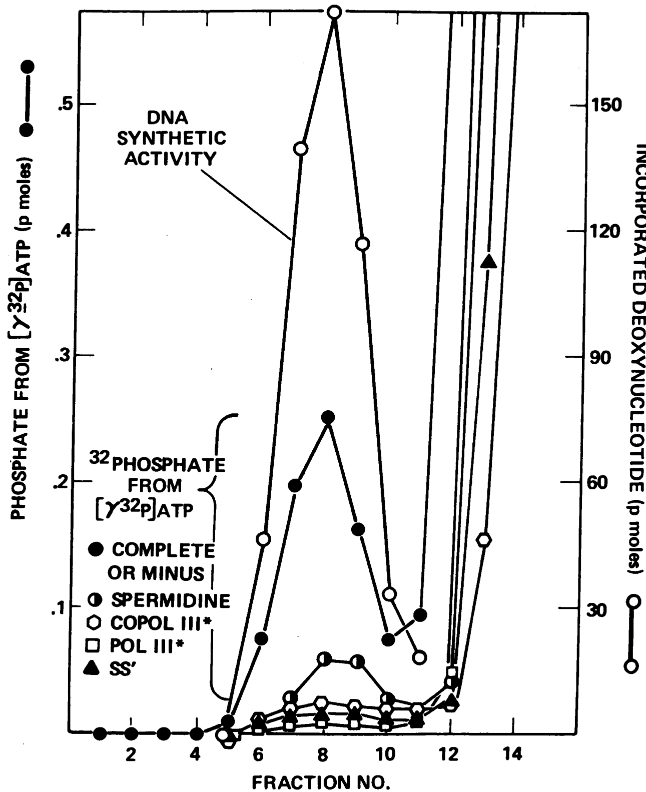


FIG. 2. Transfer of label from [γ-^{32}P]ATP to a pol III* initiation complex. [γ-^{32}P]ATP (50 μCi, 40 nmol) was added in place of unlabeled ATP to a preincubation mixture (200 μl). Preincubation, isolation of complex, and assay of DNA synthetic activity were as described in *Methods*. Components were omitted one at a time, as indicated, from the preincubation mixture.

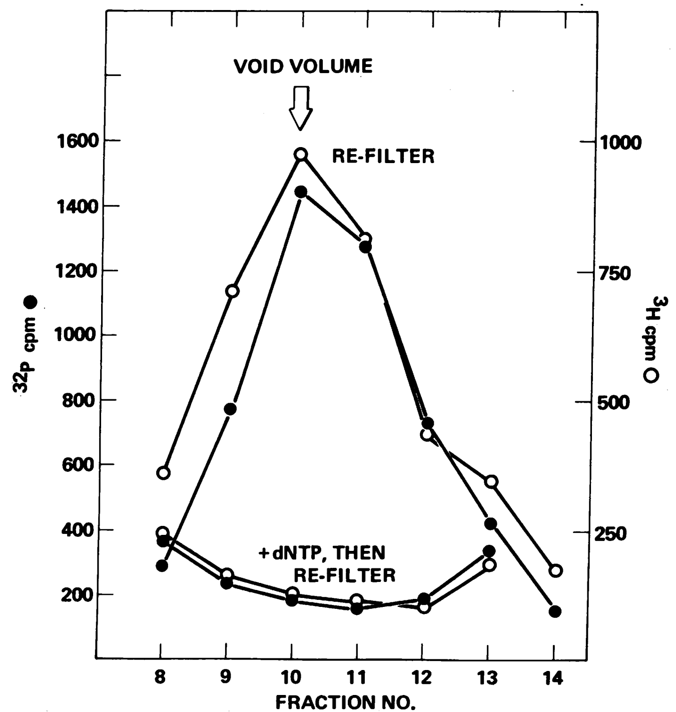


FIG. 4. Deoxynucleoside triphosphates release ADP and P_i from the pol III* initiation complex. Portions of isolated doubly labeled complex ([^{3}H]ADP and $^{32}P_i$; see Fig. 2) were incubated for 5 min at 4° with or without the four deoxynucleoside triphosphates (20 μM each). Each portion was then filtered through a Biogel A5m column (0.6 × 8 cm, equilibrated with assay buffer plus 4 mM $MgCl_2$, 4°), and void-volume fractions were assayed for ^{3}H and ^{32}P.

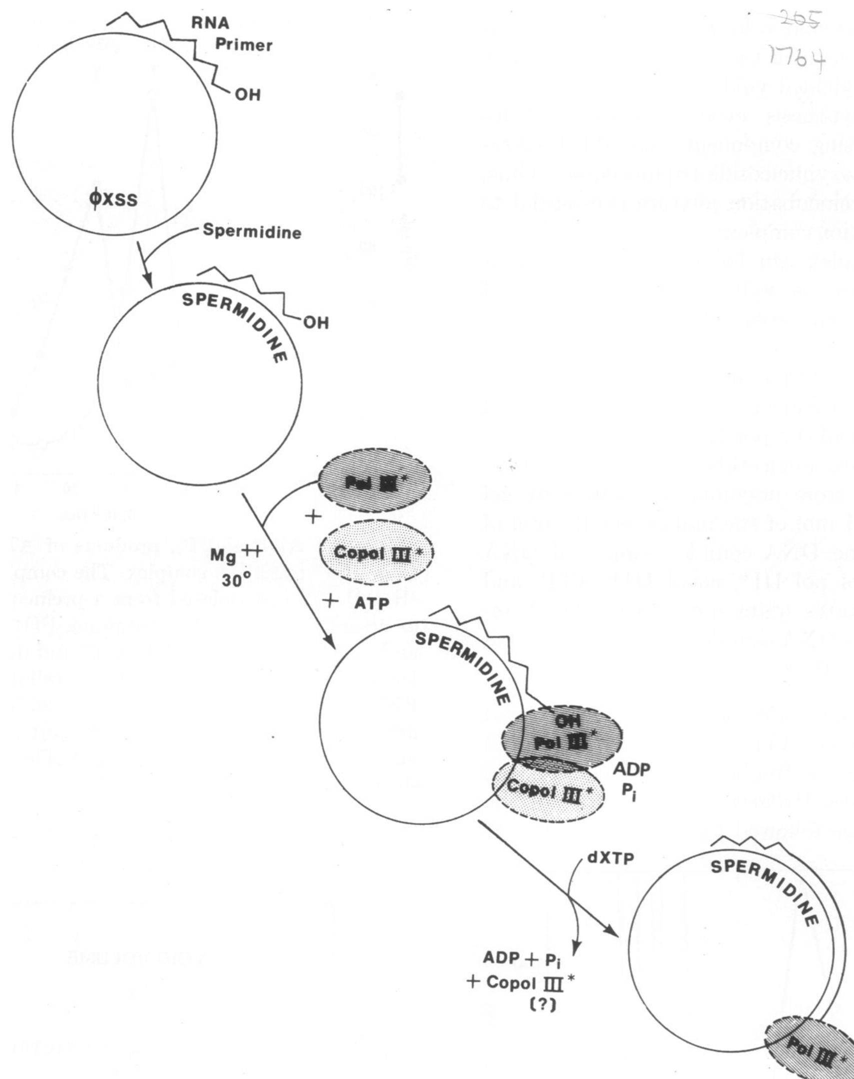


Fig. 5. Steps in a scheme proposed for the start of chain growth on a primed template.

NaCl. In an experiment in which all components, including 120 units of copol III*, were present during the preincubation, 43 units of copol III* were contained in fractions excluded from Biogel. In parallel experiments in which RNA-primed SS, pol III*, or ATP were singly omitted during preincubation, no active pol III* initiation complex was formed and no copol III* was found in the fractions excluded from Biogel. These results indicate that copol III* is a part of the pol III* initiation complex.

Antibody to Copolymerase III.* Antibody to copol III* inhibited the DNA synthesis reaction supported by copol III* (Table 2, last line). A γ-globulin preparation from a nonimmunized rabbit was without effect. Preincubation of the reaction components (without deoxynucleoside triphosphates) protected the subsequent DNA synthetic reaction from inhibition by antibody to copol III* (Table 2). Protection required that each reaction component be present in the preincubation stage (before addition of antibody to copol III*) rather than only during the DNA synthesis stage (after addition of antibody to copol III* and deoxynucleoside triphosphates). Copol III* is therefore required in an early stage of the reaction, presumably in the formation of pol III* initiation complex, and is either not directly involved in DNA chain growth or is in an antibody-resistant state during DNA synthesis.

ATP Cleavage during Formation of the Pol III Initiation Complex.* The complex was isolated from a preincubation reaction containing [α-^{32}P]ATP and [adenine-^{3}H]ATP. The ^{32}P was found with the complex only when the preincubation contained all the components necessary for complex formation (Fig. 2); similar results were obtained with ^{3}H label (data not shown). The ^{32}P label in complex was identified as P_i and the ^{3}H as ADP by polyethyleneimine-cellulose chromatography (Fig. 3) and Norit-adsorbability. Addition of 50 μM ADP or 5 mM K_2HPO_4 to the preincubation reaction did not dilute the ^{3}H or ^{32}P in the isolated pol III* initiation complex.

Release of ADP and P_i from the Pol III Initiation Complex.* Upon incubation of the isolated complex at 4° for 5 min with deoxynucleoside triphosphates, both $^{32}P_i$ and [^{3}H]ADP were released (Fig. 4). The complex reisolated by filtration through a second column of Biogel was still fully active in the growth

of a DNA chain upon a further addition of deoxynucleoside triphosphate.

DISCUSSION

Pol III* is a complex, possibly dimeric, form of pol III (3). In the presence of another protein, copol III*, it has the capacity to copy primed, single-strand templates which pol III cannot replicate (3). Copol III* has now been shown to function in the formation of a primed-DNA complex that sets the stage for DNA chain growth. In addition to copol III*, spermidine and ATP are required to form the complex with primed-DNA. The order of events is suggested in the scheme in Fig. 5. Spermidine may react with DNA first; then pol III*, copol III*, and ATP interact to form the complex. The only chemical change that accompanies complex formation is cleavage of ATP to ADP and P_i. Measurements of the stoichiometric relationships of the components of the complex have been hampered by the instability of the isolated complex.

Beyond the base specificity and very low K_m (8 μM) for ATP in forming the pol III*–primed-DNA complex, nothing is known of its function. Conjectures that it serves in altering the structure of pol III* to a form suitable for replication of the primed-single-strand ϕX174, M13, and $(dT)_9 \cdot (dA)_{1500}$ templates require more information about pol III* than is now available. Studies of the enzyme are complicated by its lability and irreversible conversion to pol III. The function of copol III* is also uncertain and its study is likewise dependent on a better understanding of pol III*. The dispensability of copol III*, once the pol III* complex with primed DNA is formed, may be analogous to the participation of the σ subunit in assisting the core RNA polymerase in forming its initiation complex (7).

The need for ATP in the operation of the pol III* system and an earlier appreciation of its participation in synthesis of an RNA-priming fragment (1, 2), account for two functions that ATP performs in DNA synthesis. Whether these functions account for the ATP requirement seen in DNA synthesis is permeabilized cells, lysates, and soluble fractions (8–12; Milewski, E. & Kohiyama, M., personal communication) is not certain. Inasmuch as neither the reactions involved in conversion of ϕX SS to duplex form nor those responsible for replication of the host chromosome have been fully resolved, still more functions of ATP may emerge.

Although spermidine served an essential role in formation of pol III* initiation complex (Table 1, Fig. 1), it does not follow, as might be inferred from the scheme in Fig. 5, that it functions this way *in vivo*. It may be augmented, or even replaced, by other components in the cell such as the DNA unwinding protein (13). In reconstitution of the system for converting the M13 single strands to the replicative form, the unwinding protein is more effective than spermidine. DNA unwinding protein enables the RNA-polymerase–copol III*–pol III*-catalyzed synthesis to produce a full-length complementary strand rather than short pieces obtained with spermidine (Geider, K. & Kornberg, A., unpublished results). Further work is needed to explain exactly how the apparently dissimilar effects of unwinding proteins and spermidine on DNA structure can stimulate the rate and extent of DNA replication.

Note added in proof

We have recently purified pol III* and copol III* as a single polymerase, termed holoenzyme, which is separable into its components by phosphocellulose. The holoenzyme activity is completely dependent on ATP.

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