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A New Form of DNA Polymerase III and a Copolymerase Replicate a Long, Single-Stranded Primer-Template†

(DNA replication/M13/ϕX174/spermidine/*dna*E mutant)

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ABSTRACT **A new form of DNA polymerase III, termed Pol III star (Pol III*), has been purified to homogeneity from *Escherichia coli*. Pol III* is temperature sensitive when isolated from a thermo-sensitive *dna*E mutant, as had been described for Pol III. Pol III* and Pol III are separable by gel filtration. Pol III* utilizes a duplex template containing short gaps with the same catalytic properties as Pol III. However, Pol III* is able to replicate long, single-stranded templates such as homopolymer chains and viral circles of M13 and ϕX174 if provided with the following: spermidine, a primer fragment, and a new protein, termed copolymerase III* (Copol III*). The latter, purified to homogeneity, has no known independent enzymatic activity and supports synthesis by Pol III* but not by Pol I, Pol II, or Pol III.**

Conversions of M13 and ϕX174 single-stranded, circular DNA (SS) to the double-stranded, replicative form (RF) in extracts of *Escherichia coli* were found to depend on distinctive host enzyme systems (1, 2). M13 replication required RNA polymerase to initiate synthesis (1), whereas ϕX required a novel RNA synthetic system and also involved the *dna* A, *dna* B, *dna* C–D, and *dna* G gene products (2, 3). Both M13 and ϕX replication were found to require the *dna* E gene product, identified by Gefter *et al.* as DNA polymerase (Pol) III (4).

Upon purification of the enzymes responsible for the replication of M13 and ϕX DNA, we found purified Pol III (5) to be inactive. Instead a novel and presumably more complex form of the enzyme, here called Pol III*, was responsible for chain growth. An additional protein, copolymerase (Copol) III*, is essential for Pol III* action. In this report, we describe the purification and properties of the components of this new Pol III* replicative system.

MATERIALS AND METHODS

Materials were from previously described sources (2).

Preparation of Templates. Activated calf-thymus DNA was prepared by a minor modification of the method of T. Kornberg and Gefter (5). To prepare "RNA-primed SS", 7×10^{13} ϕX174 SS [purified by the method of Francke and Ray (6) without Pronase], 1.3 μmol of ATP, 0.17 μmol each of CTP, GTP, and UTP, 3.3 μmol of $MgCl_2$, 290 μmol of sucrose, 16.6 μmol each of dithiothreitol, NaCl, and Tris·HCl (pH 7.5), 67 μg of bovine-serum albumin, and 15 μg of *E. coli* RNA polymerase were incubated in 1 ml for 2 min at 30°. The product was precipitated with 2 volumes of ethanol and filtered through a Biogel A5m column [equilibrated with 10 mM Tris·HCl (pH 7.5)–1 mM EDTA] to separate RNA-primed SS from protein.

Assays. DNA polymerase activity on activated, calf-thymus DNA was assayed by the method of Kornberg and Gefter (5). Pol III* and Copol III* were assayed in a 25-μl reaction mixture at 30° containing 3 μl of triphosphate mixture (40 mM $MgCl_2$, 0.15 mM [α-^{32}P]dCTP at 10^3 cpm/pmol, 0.4 mM each of dATP, dGTP, dTTP), 2 μl of 40 mM spermidine·HCl, template (20 nmol of nucleotide), and 5 μl of assay buffer [10% sucrose–50 mM Tris·HCl (pH 7.5)–50 mM NaCl–50 mM dithiothreitol–0.2 mg/ml of bovine-serum albumin]. Acid-insoluble nucleotide was determined as described (7). One unit of Pol III* is defined as 1 nmol of nucleotide incorporated per min at 30°. Either Pol III* or Copol III* was assayed in the presence of saturating amounts of the other enzyme.

Growth of Cells and Preparation of Extracts. E. coli H560 was grown in Hershey broth to an optical density (590 nm) of 0.5 (2×10^8 cells per ml) in a Fermacell Fermentor (New Brunswick Scientific Co.) at 37° with aeration and without agitation or antifoam. Cells were harvested in a Sharples continuous flow centrifuge at room temperature (25°), suspended at 8×10^{10} cells per ml in 10% sucrose–50 mM Tris·HCl (pH 7.5) and frozen in liquid N_2. They were stored at −20° and remained stable for at least 1 year.

Cells (400 ml) were thawed in a 4° bath. Lysozyme [10 ml, 4 mg/ml in 10% sucrose–50 mM Tris·HCl (pH 7.5)] and NaCl (10 ml, 4 M) were added. Cells were incubated 30 min in polycarbonate tubes on ice, warmed for 2 min in a 37° bath, then centrifuged 90 min at 56,000 rpm at 4° in a 60 Ti rotor (Beckman Instruments Co.), and the supernatant was decanted (Fraction I).

Protein was determined by the method of Lowry *et al.* (8), and in later purification stages by comparison of electrophoretic band intensity to bovine-serum albumin standards.

RESULTS

Purification of Pol III and Copol III**. Procedures for purification of the components of the Pol III* system from Fraction I are summarized in Table 1.

Abbreviations: ϕX, ϕX174; SS, (phage) single-stranded, circular DNA; RF, (phage) double-stranded DNA of circular, replicative form; DNA polymerase I, Pol I; DNA polymerase II, Pol II; DNA polymerase III, Pol III.

† This paper is no. 5 in a series on "Initiation of DNA Synthesis"; paper 4 in this series is ref. 17.

Homogeneity of Pol III and Copol III**. Pol III* was judged to be essentially homogeneous by these criteria: single band in either sodium dodecyl sulfate or nondenaturing disc-gel electrophoresis (Fig. 1) and coincidence of protein and activity in a glycerol gradient. The single polypeptide seen in the sodium dodecyl sulfate–gel has a molecular weight of 90,000 (Fig. 1). Copol III* also appears to be pure as indicated by disc-gel electrophoresis (Fig. 2); it migrates as a single polypeptide chain of 77,000 daltons (data not shown).

TABLE 1. *Purification of Pol III* and Copol III**

Fraction		Pol III* Units	Pol III* Specific activity (units/mg)	Copol III* Units	Copol III* Specific activity (units/mg)
I	Supernatant	240	0.045	1760	0.33
II	Ammonium sulfate	180	1.6	520	4.55
III	Gel filtration†	32	1.2	271	10.0
IV	Phosphocellulose	31	720		
V	Glycerol gradient	15	1800		
VI	DEAE-cellulose			225	66.2
VII	Sephadex G-150			117	167

All operations were performed at 0–4°. Fraction I (390 ml) was stirred for 5 min with 50 ml (settled volume) of DEAE–cellulose [equilibrated with 10% sucrose–0.1 M NaCl–50 mM Tris·HCl (pH 7.5)]. DEAE–cellulose was then removed by filtration. Ammonium sulfate (67 g) was added to the filtrate (276 ml). The precipitate, collected by centrifugation, was resuspended in 140 ml of buffer A [20% glycerol, 50 mM Tris·HCl (pH 8.5), 0.1 M NaCl, 1 mM dithiothreitol, 1 mM EDTA] containing ammonium sulfate to 35% saturation and centrifuged. The pellet was resuspended in buffer A (14 ml). This solution (Fraction II) was clarified by centrifugation and filtered through a Biogel A5m column (3.4 × 34 cm) equilibrated with buffer A. Peak fractions were pooled (Fraction III, 68 ml) and adjusted to pH 7.5 with 1 N HCl. An equal volume of diluent (20% glycerol, 20 mM dithiothreitol, 1 mM EDTA) was added and the sample was applied to a column of phosphocellulose (1.5 × 9 cm, equilibrated with buffer B [20% glycerol, 20 mM dithiothreitol, 40 mM Tris·HCl (pH 7.5), 25 mM NaCl, 1 mM EDTA]). Copol III* was not adsorbed. Pol III* (Fraction IV) was eluted with a linear gradient of NaCl (120 ml, 25–275 mM NaCl in buffer B); assays required addition of saturating amounts of Copol III* (or phosphocellulose-flow-through fraction). Fraction IV (24 ml) was mixed with two volumes of dilution buffer, adsorbed to a column of phosphocellulose (1 × 1.5 cm, equilibrated with buffer B), and eluted in 0.9 ml with a NaCl step (0.2 M in buffer B). This was purified in 0.1-ml aliquots in glycerol gradients [3.6 ml, 25–40% in 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 1 mM EDTA in Beckman SW56 tubes]; centrifugation for 9 hr at −5° at 55,000 rpm.

Purification of Copol III*. Protein unadsorbed to phosphocellulose was mixed with 2 volumes of diluent and adsorbed to a column of DEAE–cellulose (1.5 × 13 cm, equilibrated with buffer B lacking NaCl). Copol III* was eluted with a linear salt gradient (200 ml, 0–0.25 M NaCl in buffer B); assays required addition of saturating amounts of Pol III*. The pool of peak fractions was diluted with 3 volumes of diluent, applied to a DEAE–cellulose column (1.5 × 2.5 cm, equilibrated in buffer B lacking NaCl), and step-eluted (buffer B with 0.2 M NaCl) in 1.5 ml. Enzyme was filtered through a column of G-150 Sephadex [1.3 × 22 cm, equilibrated with 20% glycerol, 0.1 M NaCl, 50 mM Tris·HCl (pH 7.5), 10 mM dithiothreitol, 1 mM EDTA]. Fractions containing homogeneous Copol III* (as judged by electrophoresis, see Fig. 2) were pooled. The enzyme remained stable at 4° after 3 months.

† The yield at this step was lower than usual and is also reduced by removal of stimulatory factors.

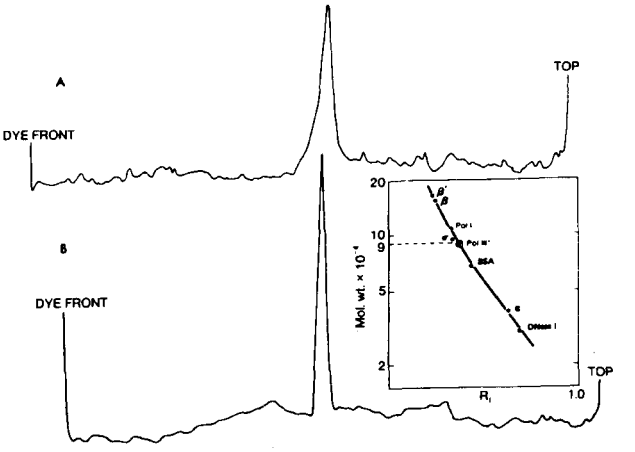

FIG. 1. Homogeneity of Pol III* and molecular weight of the Pol III* polypeptide. (*A*) Gel electrophoresis of Fraction V was in the Tris system of Jovin *et al.* (9) for 4 hr at 4° at 200 V and stained by the Weber and Osborn method (10). (*B*) Sodium dodecyl sulfate–gel electrophoresis was in the same Tris system with 0.1% sodium dodecyl sulfate in the gel and in the reservoir buffer; gels contained 10% acrylamide and 0.25% bisacrylamide. α, β, β', and σ represent subunits of RNA polymerase; BSA is bovine-serum albumin. R_f is the distance of migration relative to a bromphenol blue marker.

Specificity of Copol III for Pol III**. Copol III* was essential for the action of Pol III* on a long, single-stranded template, such as an RNA-primed ϕX SS (Table 2), but not for replication of duplex DNA with short gaps. As anticipated

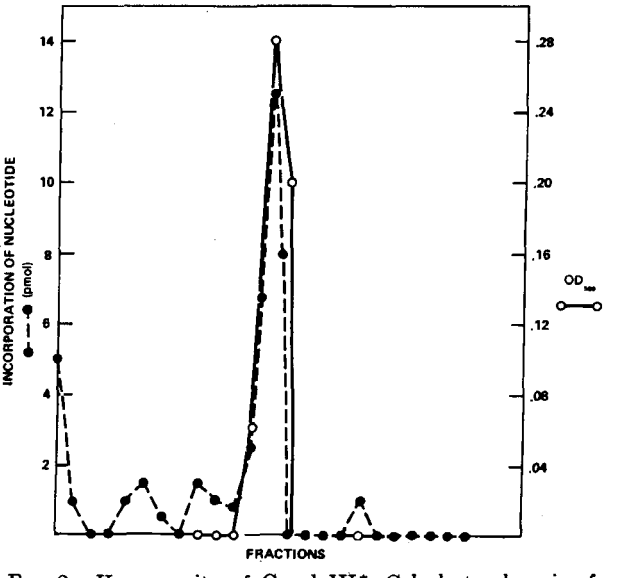

FIG. 2. Homogeneity of Copol III*. Gel electrophoresis of Fraction VII was as in Fig. 1. Gels were either stained (as in Fig. 1) *or* sliced with a razor into small discs from which enzyme was eluted by 12-hr incubation at 4° in buffer [20% glycerol–20 mM dithiothreitol–0.1 M Tris·HCl (pH 7.5)–1 mM EDTA] and assayed on RNA-primed ϕX SS in the presence of Pol III* (Fraction IV).

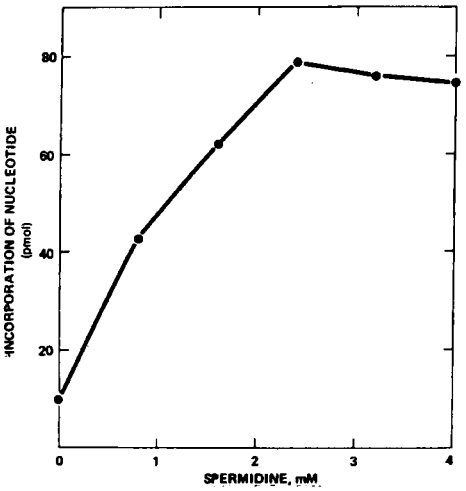

FIG. 3. Spermidine is required for replication of RNA-primed ϕX SS by Pol III* and Copol III*. Pol III* [Fraction IV, 0.2 unit (5) on activated, calf-thymus DNA] and Copol III* (Fraction VII, 0.7 μg) were assayed on RNA-primed ϕX SS at the concentrations of spermidine indicated.

from the studies of Kornberg and Gefter (5), Pol III was inactive with the single-stranded template and Copol III* had no influence (Table 2). Copol III* had no effect either on the action of Pol I or Pol II.

Copol III* was without effect when incubated with the template and then inactivated before addition of Pol III* to the assay. This observation argues against a modification of the template by Copol III*, as by a nuclease or a phosphatase. Although the mechanism of its action is still unknown, there is clearly a specificity for Pol III* in its replication of a long, single-stranded chain.

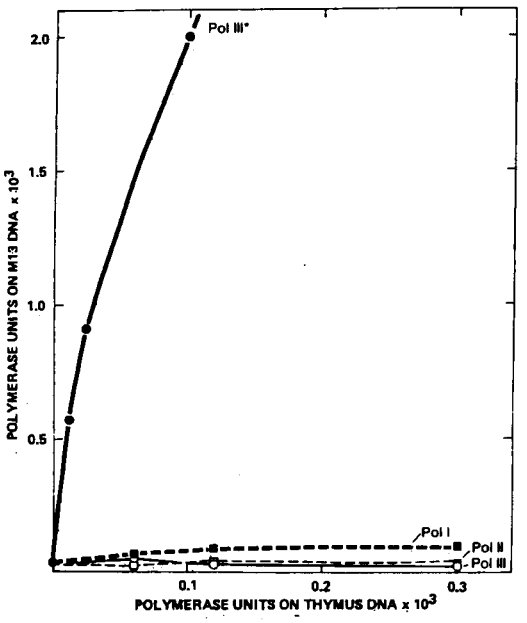

FIG. 4. Replication of M13 DNA in extracts of *dna* E mutants requires Pol III*. Extracts from the temperature-sensitive *dna* E mutant H10261 at 5 mg of protein per ml were frozen and thawed five times and assayed as described (1); in assays of Pol III and Pol III* (Fraction IV) on the activated, calf-thymus DNA, NaCl was replaced by 5 mM spermidine·HCl.

Replication of Single-Stranded Templates by the Pol III System.* Pol III* with Copol III* replicates ϕX and M13 single-stranded circles when provided with a short primer produced by RNA transcription; the newly synthesized DNA ranged from partial to nearly full length, as judged by alkaline sucrose velocity sedimentation and electron microscopic measurements of duplex lengths. Primed, homopolymer templates, such as poly(dA)$_{4000}$:oligo(dT)$_{10}$, served as efficiently as did the RNA-primed viral strands.

Polymerization required spermidine for optimal activity (Fig. 3). Spermidine stimulated rates of replication of both single-stranded and gapped-duplex templates by about 10-fold. The stimulatory effect of spermidine superficially resembles that produced by DNA-unwinding proteins (14, 15).

Pol III is the dna E Gene Product.* Fraction I prepared from a temperature-sensitive *dna* E mutant was unstable in its capacity to replicate ϕX or M13 single-stranded circles. The capacity could be restored by addition of Pol III* but not by Pol I, Pol II, or Pol III (Fig. 4). Pol III*, purified from the *dna* E mutant to the stage of Fraction IV, showed a marked temperature lability like that reported for Pol III (4): replicative activities at 30° and 37° compared to that at 25° were *reduced* by 10 and 30%, respectively, whereas the activity of the enzyme from wild-type cells was *increased* by 80 and 170%.

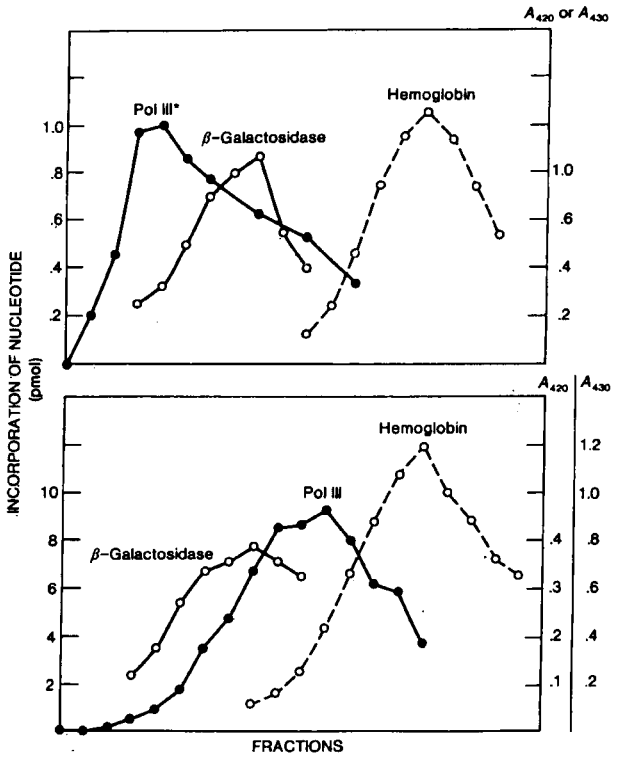

FIG. 5. Pol III* and Pol III are distinguished by gel filtration. Pol III* (Fraction IV, 40 units as assayed on activated, calf-thymus DNA) or Pol III [20 units (5)] was mixed with human hemoglobin (0.2 mg) and β-galactosidase (5 μg) and filtered through a column of Biogel A5m [1.16 cm, equilibrated with 30% glycerol, 0.5 mg/ml of bovine-serum albumin, 50 mM Tris·HCl (pH 7.5), 20 mM dithiothreitol, 0.1 M NaCl, 1 mM EDTA]. Pol III was assayed on activated, calf-thymus DNA and Pol III* was assayed on RNA-primed ϕX SS in the presence of Copol III*. Hemoglobin was minitored by absorbance (430 nm) and β-galactosidase by the method of Craven *et al.* (16).

Comparison of Pol III with Pol III.* Pol III*, replicating a gapped, duplex template, displays many of the distinctive features described for Pol III: inhibition by salt (90% by 0.13 M KCl), stimulation by 10% ethanol, and a high K_m for deoxyribonucleoside triphosphates. Pol III* can be separated from Pol III by gel filtration (Fig. 5) and by chromatography on phosphocellulose. The skewing of Pol III* on gel filtration suggests a protomer–multimer interconversion. Even though Pol III* enters the agarose gel less well than β-galactosidase, a protein of 540,000 molecular weight, its true size may be smaller. The sedimentation behavior of Pol III* in glycerol gradients resembles that of Pol III and suggests that Pol III* may be asymmetric.

Pol III* purified from wild-type cells lost more than 80% of its activity on single-stranded templates after 30 min at 39° but retained full activity on a gapped duplex. This conversion of Pol III* behavior to that of Pol III was accompanied by a concomitant alteration in a physical property, filtration on agarose.

DISCUSSION

Our studies of the enzymatic conversion of single-stranded viral circles of M13 and φX to the duplex replicative form (RF) have revealed a new form of DNA polymerase III as the enzyme responsible for this replication. The new enzyme, called Pol III*, is clearly related to Pol III (5) in these ways: (*a*) product of *dna* E gene, (*b*) same characteristics (inhibition by salt, stimulation by ethanol, high K_m for deoxyribonucleoside triphosphates) in replication of a duplex template with short gaps, and (*c*) conversion to Pol III upon heating. Pol III* is distinguished from Pol III by: (*a*) its capacity to replicate long, single-stranded templates when another protein, Copol III*, is also present, and (*b*) physical features that separate the enzymes on agarose gels and on phosphocellulose chromatography (unpublished results).

Both Pol III* and Copol III* have now been isolated in a near-homogeneous state, and it should be possible to determine the physical distinctions between Pol III* and Pol III on the one hand and the interaction of Pol III* and Copol III* on the other. Pol III* may be a holoenzyme composed of subunits of identical size including a core unit of Pol III or it may simply be a multimeric and asymmetric form of Pol III.

What might have appeared to be a simple, replicative operation of converting a single-stranded circle to a duplex form must now be considered as a relatively complex, multistage operation. Initiation of the M13 chain involves synthesis by RNA polymerase of a short RNA fragment (1, 2, 17) that serves as a primer for the Pol III* system. Initiation of the φX chain involves proteins other than RNA polymerase, including the *dna* A gene product and, very likely, additional proteins (2, 3). Chain growth on viral templates by the Pol III* system requires spermidine. Yet it remains uncertain whether spermidine performs such a replicative function in the cell.

Beyond the events of initiation and DNA chain growth, several additional operations must now be considered: (*a*) interruption of the initiation event by the Pol III* replicative system, (*b*) excision of the initiating RNA primer, and (*c*) replicative gap filling that enables the 3′-hydroxyl and 5′-phosphoryl DNA termini to be joined by ligase. Preliminary enzymatic studies indicate that an early displacement of the RNA polymerase transcriptional system is not spontaneous; Pol III*–Copol III* can effect this more readily than Pol I.

TABLE 2. *Template and enzyme specificity of Copol III**

Polymerase (units)		Copol III* (μg)	RNA-primed, φX single strand (pmol)	Activated thymus
Pol I	0.05	0.00	4	35
	0.05	0.08	4	38
Pol II	0.01	0.00	0	5
	0.01	0.08	0	5
Pol III	0.03	0.00	0	14
	0.03	0.08	0	17
Pol III*	0.15	0.00	3	67
	0.15	0.08	32	62

Pol I was purified as described (11); Pol II and Pol III were purified by the method of Kornberg and Gefter (5). Units of Pol I (12), Pol II (13), and Pol III (5) have been defined. Units of Pol III* are defined in *Methods*. Fractions IV and VII were used.

On the other hand, the gap-filling defects in mutant cells that lack adequate levels of Pol I suggests that this function is efficiently performed by Pol I and not by Pol III*–Copol III*.

NOTE ADDED IN PROOF

A protein fraction that stimulates *E. coli* DNA polymerases I, II, and III in extension of a template-primer has just been reported [Hurwitz, J., Wickner, S. & Wright, M. (1973) *Biochem. Biophys. Res Commun.* **51**, 257–267]. This fraction may contain Copol III* as well as additional proteins that stimulate this assay of DNA polymerases I and II.

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