

DNA SYNTHESIS IN CELL-FREE EXTRACTS OF A DNA POLYMERASE-DEFECTIVE MUTANT

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Summary. A DNA-synthesizing activity has been isolated from an *E. coli* mutant defective in DNA polymerase. Like DNA polymerase, the system requires the presence of all four deoxynucleoside triphosphates, magnesium ion and a native DNA template for maximal activity. The activity can be distinguished from *E. coli* DNA polymerase on the basis of its sensitivity to high ionic strength and to p-chloromercuribenzoate. The activity is insensitive to antiserum directed against *E. coli* DNA polymerase. Our results do not exclude the possibility that the activity isolated is composed of, in part, an altered form of DNA polymerase.

INTRODUCTION

The isolation of a DNA polymerase-defective mutant (1) has led to several investigations into the nature of the DNA synthetic capacity of this strain. *In vitro* synthesis using agar-embedded cells and an *in vitro* membrane-associated activity in extracts of Pol A₁⁻ cells have recently been reported (2,3). We will describe the isolation and properties of a soluble DNA synthesizing activity from the Pol A₁⁻ mutant.

MATERIALS AND METHODS

E. coli strains JG 112: W3110 thy⁻, rha⁻, lac⁻, str^r, Pol A₁⁻, its parent W3110 thy⁻ Pol A₁⁺ and JG 142: W3110 met E⁻, thy⁺, Pol A₄ (temperature sensitive polymerase) were obtained from Dr. J. Gross. Purified *E. coli* DNA polymerase, an enzymatically active tryptic fragment of *E. coli* DNA polymerase (4), and anti-DNA polymerase antiserum were obtained from Dr. P. Setlow. ³H-thymidine triphosphate (TTP) was purchased from Schwarz Bio Research and was adjusted to a specific activity of 1.0 × 10⁵ cpm per μmole.

For preparation of cell-free extracts from Pol⁺ or Pol A₁⁻, frozen cells (4-5g) were thawed, washed twice in a solution of tris-acetate pH8.2, 0.02M; 2-mercaptoethanol, 0.005M; Mg(OAc)₂, 0.01M; and E.D.T.A., 0.0005M. The cells were resuspended in two volumes of the above buffer and lysed in a pressure cell (9,000 lbs/inch²). Debris and unbroken cells were removed by centrifugation at 12,000 XG.

The supernatant solution was further clarified by centrifugation at 35,000 XG for 30 min. and the resulting supernatant solution subjected to centrifugation at 100,000 XG for 90 min. (S100).

For purification of enzymatic activity, the S100 was layered on 10-30% glycerol gradients. Low salt gradients were prepared by dissolving glycerol in a solution of tris-HCl pH 7.4, 0.01M; KCl, 0.05M; MgSO₄, 0.005M and 2-mercaptoethanol, 0.002M. High salt gradients were the same except the KCl concentration was 0.5M. 0.5ml of protein solution was layered on each bucket of the SW40 Spinco rotor and centrifugation was at 4°C for 15 hrs. at 40,000 rpm.

For measurement of enzymatic activity, reaction mixtures (0.3ml) contained tris-HCl pH 7.4, 20μmoles; MgCl₂, 2μmoles; 2-mercaptoethanol, 0.3μmoles; dCTP, dGTP, dATP, 10μmoles each; ³H-TTP, 10μmoles; "activated" (5) Calf Thymus DNA, 40μmoles of nucleotide, and enzyme. Incubations were for 5 or 30 min. at 30°C. The reaction was terminated by the addition of cold TCA and the acid insoluble material collected on Whatmann GF/C glass fiber papers. The filters were dried and the radioactivity determined in a liquid scintillation counter. One unit of enzymatic activity is defined as the amount which catalyzes the incorporation of 1 μmole of TTP into an acid insoluble form in 5 min. Incubation mixtures containing DNAase (75μg/ml) served as controls and were generally <10% of the observed activity.

RESULTS

Cell-free extract (S100) was layered on low-salt glycerol gradients and subjected to centrifugation as described in Materials and Methods. Enzymatic activity was present as a discrete peak sedimenting slightly faster than the bulk protein (Fig. 1A). Fractions corresponding to the middle two thirds of the activity peak were pooled and the protein concentrated by precipitation with ammonium sulfate (85% saturation). The pellet was dissolved in the high salt gradient buffer (one tenth the original volume) and dialyzed against the same buffer for 1 hr. at 4°C. The dialyzed solution was then layered on a high salt gradient and subjected to centrifugation as described above. Enzymatic activity

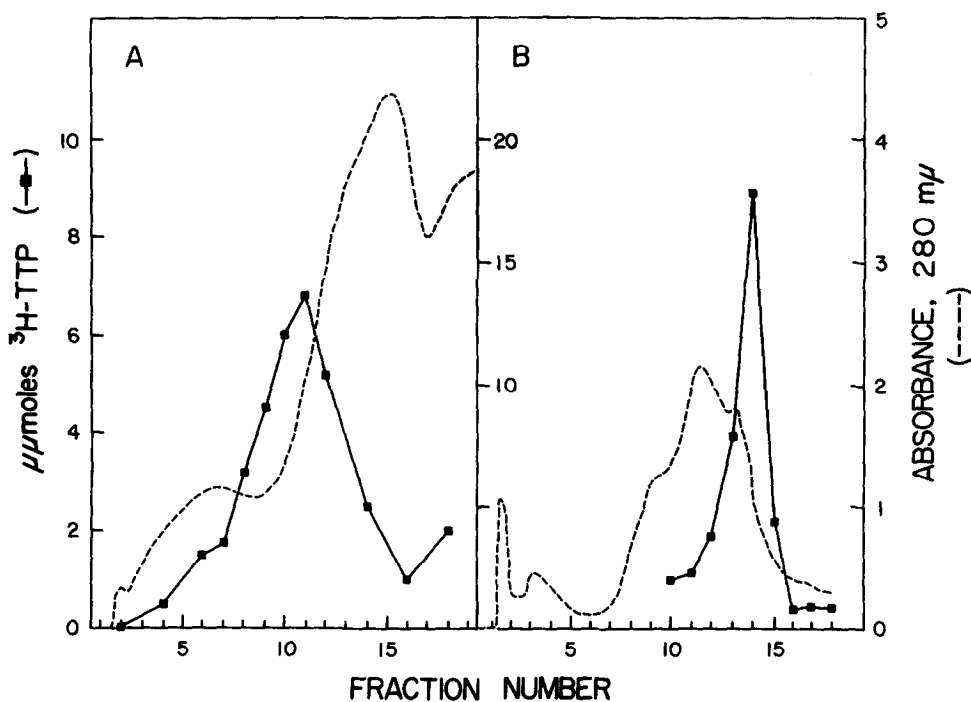


Figure 1. Sedimentation of protein in low salt (A) and high salt (B) glycerol gradients. Conditions for sedimentations are described in the text. Sedimentation is from right to left. The enzymatic activity of each fraction was determined under standard assay conditions. Incubations were for 30 min.

was present as a discrete peak sedimenting slower than the bulk protein (Fig. 1B). Fractions containing enzymatic activity were pooled and the protein concentrated ten-fold by precipitation with ammonium sulfate as described above. The enzyme preparation was dialyzed for 2 hrs. against a solution of 0.01M tris-HCl pH7.4, 0.002M dithiothreitol. This preparation was used for all experiments to be described.

The specific activity (units per mg protein) of the S100 was generally 20-30.[†] Thirty to forty percent of the total activity was recovered in the final preparation and had a specific activity of 600. The preparation could be stored at 0°C for one month without significant loss of activity. Incorporation of TTP

[†]Specific activity measured immediately after obtaining the S100 was often several fold higher than 20 but decayed to this value after storage for several hours at 0°C.

TABLE I

ENZYME SOURCE	POL A ₁ ⁻		*POL ⁺		DNA POLYMERASE	
	μμ MOLES INCORPORATED	PERCENT ACTIVITY	μμ MOLES INCORPORATED	PERCENT ACTIVITY	μμ MOLES INCORPORATED	PERCENT ACTIVITY
Complete System	22.3	100	20.2	100	20	100
" - MgCl ₂ + E.D.T.A. (0.3mM)	4	18	<1	<5	<1	<5
" - dGTP, - dATP, - dCTP	<1	<5	<1	<5	<1	<5
" + DNAase (75μg/ml)	<1	<5	<1	<5	<1	<5
" - Activated Calf Thymus DNA	<1	<5	<1	<5	<1	<5
" - Act. DNA + Denatured T7 DNA	1.1	5	--	<10	--	<1
" - Act. DNA + Native T7 DNA	23	107	--	68	--	48
" - 2-mercaptoethanol + p.c.m.b. (0.3mM)	<1	<5	12.4	62	11	55
" + KCL (0.2M)	1.0	5	--	172	23	115
" + Anti-DNA Polymerase Antiserum (5μl)	27	121	<1	<5	<1	<5

Table 1. Requirements and properties of enzymatic activity from Pol A₁⁻ cells, Pol⁺ cells, and purified *E. coli* DNA polymerase. The complete system is as described in Materials and Methods and was the same for all three preparations. Incubations were for 30 min. at 30°C. Values in parentheses represent final concentrations in the reaction mixtures. *The activity from Pol⁺ cells was purified using the same gradient procedures as described for Pol A₁⁻. Pol⁺ activity sedimented 20% faster than the activity from Pol A₁⁻ in the low salt gradient and essentially the same as Pol A₁⁻ activity in the high salt gradient.

was linear for 30 min. over a ten-fold range in enzyme concentration (2-20 units). Incorporation continued to increase for 2-3 hrs. and then began to decline. In contrast to what was observed with the final preparation, all incorporation ceased after 1 min. of incubation when S100 was used as a source of enzyme, and in addition, the system showed a three-fold dependence on the presence of ATP. This dependency was not evident in the final preparation.

The enzymatic activity shows a complete dependence on the presence of all 4 deoxynucleoside triphosphates, magnesium ion, native DNA as template and a thiol reagent. The presence of KCl (0.2M final concentration) or p-chloromercuribenzoate (p.c.m.b.) (0.3mM) in the reaction mixture leads to complete loss of activity. The presence of anti-DNA polymerase antiserum in the reaction mixture is without effect. The properties of this enzyme preparation, a similar preparation from Pol⁺ cells, and *E. coli* DNA polymerase are summarized in Table 1. As shown, all three preparations are similar with respect to the deoxynucleoside triphosphate, magnesium ion, and DNA requirements. In contrast to the Pol A₁⁻ activity, the activity from Pol⁺ cells and DNA polymerase are stimulated by the presence of KCl and are inhibited completely by the presence of anti-DNA polymerase antiserum. The activity from Pol A₁⁻ cells is at least ten-fold more sensitive to p.c.m.b. than the activity from Pol⁺ cells or DNA polymerase.⁺⁺

Titration of the activity from Pol A₁⁻ cells with anti-DNA polymerase antiserum has shown that it is approximately 100-fold less sensitive than is DNA

⁺⁺The fifty percent reduction in activity observed with the Pol⁺ and DNA polymerase preparations is also observed at a p.c.m.b. concentration of 3×10^{-5} M. Higher concentrations of p.c.m.b. $>3 \times 10^{-4}$ M do not reduce the level of activity below 50%. The observed inhibition is believed to be due to the inhibition by p.c.m.b. of exonuclease III present in these preparations.

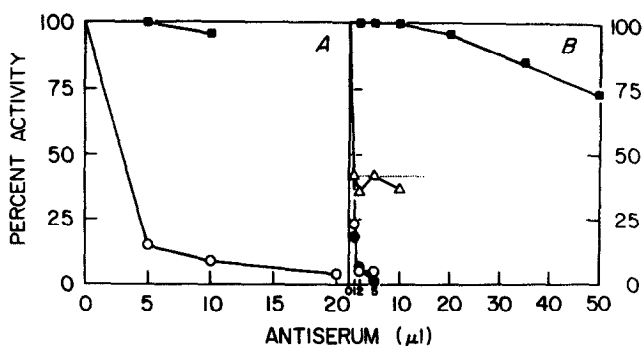


Figure 2. Titration of enzymatic activities with anti-DNA polymerase antiserum. Part A represents titration of activity in the S100 extracts of Pol A₁⁻ (49 units) —■— and Pol⁺ (290 units) —○—. Part B represents titration of partially purified activity from Pol A₁⁻ (3.7 units) —■— purified *E. coli* DNA polymerase (3.3 units) —○—, tryptic fragment of *E. coli* DNA polymerase (4.0 units) —●— and a mixture of Pol A₁⁻ activity (3.7 units) and tryptic fragment (4.0 units) —△—. The dotted line represents the theoretical amount of antiserum-resistant activity present in the mixture. Enzyme and antiserum were incubated with the components of the standard assay mixture at 30°C for 5 min. in the absence of deoxynucleoside triphosphates and DNA. DNA and deoxynucleoside triphosphates were then added and incubations were for 5 min. (Part A) and 30 min. (Part B) at 30°C. Reactions done in the absence of antiserum were also incubated at 30°C prior to the addition of the other components.

polymerase or the active tryptic fragment of DNA polymerase. The presence of Pol A₁⁻ extract does not protect DNA polymerase against inhibition by antiserum (Fig. 2B).

The presence of the components of the S100 do not affect the antiserum reaction; the differential antiserum sensitivity can also be observed in the S100 extracts of Pol⁺ and Pol A₁⁻ cells (Fig 2A). The addition of "activated" Calf Thymus DNA (33 μm moles of nucleotide) to the antiserum reaction is without effect. In addition, treatment of the Pol A₁⁻ activity with sodium desoxycholate (DOC) (0.4% final concentration) does not render it sensitive to antiserum.

We have also partially purified a DNA synthesizing activity from extracts of *E. coli* strain JG 142. The Pol A₄ mutation renders the cell sensitive to methylmethanesulfonate (MMS) at 25°C and it is relatively insensitive to M.M.S. at 37°C. Extracts of this strain have the same specific activity as Pol A₁⁻ extracts and show no antiserum-sensitive polymerization activity. The activity observed in the S100 and in the partially purified preparation is not cold-

sensitive. Its properties are identical to the activity from Pol A₁⁻ cells; it is insensitive to antiserum and is sensitive to 0.2M KCl and to p.c.m.b. (0.3mM).

DISCUSSION

We have partially purified a DNA-synthesizing activity from two *E. coli* mutants defective in DNA polymerase. The activity can be distinguished from DNA polymerase in that it is sensitive to high ionic strength and to p.c.m.b. and is insensitive to anti-DNA polymerase antiserum. Since the activity is present in extracts of an apparent non-amber mutant of DNA polymerase (Pol A₄), the activity in Pol A₁⁻ cells is not a result of chain termination within the polymerase gene.

These results indicate that the residual DNA polymerase-like activity reported by DeLucia and Cairns (1) to be present in extracts of Pol A₁⁻ cells is not due to "DNA polymerase" as described by Kornberg (5) or to a fragment of DNA polymerase. The relationship of this activity to that reported by Knippers and Strätling (3) is not clear since in our hands, the activity in Pol A₁⁻ cells is not membrane bound.

The activity that we have described can be a result of: 1) a mutant DNA polymerase, 2) a mutant DNA polymerase complexed with another protein or membrane component, or 3) a new DNA-synthesizing enzyme. We believe that 1) is unlikely since one would not expect that two independent mutants of DNA polymerase would have identical properties and yet both be unrelated to DNA polymerase antigenically. Hypotheses 2) and 3) are difficult to resolve. We have detected anti-serum-resistant activity in extracts of wild-type cells. However, one might expect this result if DNA polymerase naturally exists, in part, as a membrane-associated complex. If such a complex exists, then both polymerase mutants could have been selected as having inactive "free" enzyme but active "complex" enzyme. The properties of such a complex would have to be such that the antigenic determinant is masked and that it is not dissociable in DOC, 4M ammonium sulfate or 0.5M KCl but is inactive in the presence of 0.2M KCl. The inactivation by p.c.m.b. would have to be explained in terms of dissociation of the complex

and subsequent denaturation of the "mutant" polymerase. We have no evidence to suggest that such a complex exists but the possibility of its existence cannot be eliminated. Although we favor the existence of a new DNA synthesizing enzyme, purification of this activity from wild-type cells is essential in order to decide absolutely between hypotheses 2) and 3).

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