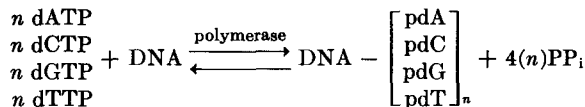


[6] T4 DNA Polymerase¹

By I. R. LEHMAN

**Assay Method**

Reagents. The reaction mixture (total volume 0.3 ml) contains:

Tris·HCl buffer, 0.067 M, pH 8.6

MgCl₂, 6.7 mM

2-Mercaptoethanol, 10 mM (or dithiothreitol, 1 mM)

(NH₄)₂SO₄, 16.7 mM

KF, 7.5 mM

EDTA, 6.7 μM

dCTP, dATP, dGTP, and dTTP, 0.33 mM each; dTTP labeled with ³H or α-³²P at a specific radioactivity of 2 to 10 × 10⁶ cpm/μmole

Denatured salmon sperm DNA, 0.2 mM (nucleotide). The DNA is denatured by treatment with 0.1 M NaOH for 10 minutes at room temperature and then neutralized with HCl.

Enzyme fraction, diluted, if necessary in a solution composed of 0.05 M Tris·HCl, pH 7.5, 0.1 M (NH₄)₂SO₄, 1 mM dithiothreitol, and bovine plasma albumin, 1 mg/ml.

Procedure. The assay measures the conversion of ³²P-labeled deoxy-nucleoside triphosphates to an acid-insoluble product. After a 30-minute incubation at 37°, the reaction is stopped by the addition of 0.1 ml of a carrier DNA solution containing 0.1 M sodium pyrophosphate, 0.1 M EDTA, and 100 μg of calf thymus DNA per milliliter. Five-tenths milliliter of cold 7% perchloric acid and 3 ml of cold 1 N HCl are added; after 5 minutes at 0°, the acid-insoluble material is collected on a GF/C Whatman glass filter (2.4 cm diameter). The filter is washed 5 times with 3-ml aliquots of cold 1 N HCl, twice with 95% ethanol, and once with ethyl ether. After drying, the radioactivity of the filter is measured by liquid scintillation counting. Sodium fluoride, which serves to inhibit the T4-induced deoxycytidine triphosphatase activity is omitted in assays of fractions beyond the phosphocellulose step in the purification procedure. A unit of enzyme activity is defined as the amount catalyzing the incorporation of 10 nmoles

¹ M. Goulian, Z. J. Lucas, and A. Kornberg, *J. Biol. Chem.* **243**, 627 (1968).

of total nucleotide into an acid-insoluble product during the period of incubation. The assay is linear in the range of 0.04–0.40 unit of enzyme.

Isolation Procedure

Except as noted, all steps are carried out at 0–4°. Centrifugations are done at 15,000 *g* for 15 minutes. The purification procedure is summarized in the table.

Preparation of Extract. *Escherichia coli* B cells are grown from 1% inocula in 100-liter quantities, at 37°, with aeration. The medium contains the following, per liter: K₂HPO₄, 13.2 g; KH₂PO₄, 3.26 g; Difco Casamino acids, 10 g; DL-tryptophan, 0.1 g; cysteine-HCl, 0.13 g; (NH₄)₂SO₄, 2.6 g; MgSO₄, 0.25 g; glucose, 10 g; Fe(NH₄)₂(SO₄)₂, 0.4 mg. When the culture reaches an optical density (590 nm) of 1.0, it is infected with 3 × 10¹² T4 *am* N82 phage per liter (multiplicity of 4).² After 75 minutes of further incubation, the culture is chilled and harvested by centrifugation, and the cell paste is stored at –20° until the next step.

Pooled cell paste from two 100-liter cultures, totaling 552 g, is mixed with 370 ml of glycyglycine buffer, 50 mM, pH 7.0, in a large Waring Blendor (5-liter capacity) equipped with a cooling jacket and connected to a Variac. Slow stirring is begun and after 5 minutes, 1660 g of acid-washed glass beads (Superbrite, average diameter 200 μ, Minnesota Mining and Manufacturing Company) are gradually added to the suspension. When the mixture appears homogeneous, stirring is increased to approxi-

PURIFICATION OF T4-INDUCED DNA POLYMERASE

Fraction	Units (×10 ⁻⁵)	Protein (mg/ml)	Specific activity (units/mg)	Overall yield (%)
I. Extract	30	16.2	78	100
II. Streptomycin	38	14.8	200	127
III. Autolysis	26	3.1	370	87
IV. Ammonium sulfate	20	29.5	530	67
V. Phosphocellulose	11	0.33	15,300	37
VI. DEAE-cellulose	7.3	0.67	33,000	24
VII. Hydroxyapatite	1.2 ^a	0.86	31,000	8

^a Only 10.6 mg of the 22 mg of fraction VI were purified on hydroxyapatite; the yield for this step is corrected to apply to the total amount of fraction VI, but includes the 16% loss in activity of the latter after storage in liquid nitrogen.

² The amber mutant is used, with the nonpermissive host *E. coli* B because the specific activity of crude extracts is approximately 5–10 times the level attained with wild-type phage T4 or T2.

mately one-third of maximal speed. After 20 minutes an additional 1500 ml of the same buffer are added and the homogenization is continued for 10 minutes at reduced speed to prevent excessive foaming. During the period of homogenization, the temperature is not permitted to rise above 12°. The beads are then allowed to settle out, and the broken cell suspension is decanted and saved. An additional 1500 ml of buffer are added to the glass beads, and the residual broken cells are extracted by a 10-minute homogenization at slow speed. The beads are again allowed to settle out, and the supernatant fluid is decanted and combined with the first supernatant fluid to give a final volume of 2300 ml (fraction I, see table).

Streptomycin Precipitation. To fraction I are added with stirring, 5100 ml of 2 mM reduced glutathione, 2 mM EDTA, followed by 2200 ml of 5% streptomycin sulfate during a 30-minute interval. After additional stirring for 20 minutes, the precipitate is collected by centrifugation and suspended in 1290 ml of potassium phosphate buffer, 50 mM, pH 7.0, containing 2 mM reduced glutathione (fraction II, table).

Autolysis. Fraction II is made 3 mM in MgCl₂ and incubated at 37° until 95% of the ultraviolet absorbing material at 260 nm has become acid-soluble (approximately 90 minutes).³ The autolyzate is then rapidly chilled to 0°, and the supernatant fraction (fraction III, table) is separated by centrifugation.

Ammonium Sulfate Fractionation. To each liter of fraction III are added 218 g of ammonium sulfate with stirring during a 30-minute interval. After an additional 30-minute stirring period, the precipitate is removed by centrifugation. Additional ammonium sulfate, 78 g per liter of supernatant fluid, is added with the same procedure. The precipitate is collected and dissolved in 120 ml of potassium phosphate buffer, 50 mM, pH 7.4, containing 2 mM EDTA and 2 mM reduced glutathione. This solution is passed through a column of Sephadex G-25 (20 cm² × 50 cm) equilibrated with potassium phosphate buffer, 50 mM, pH 6.5, and 0.01 M 2-mercaptoethanol, and followed by the same buffer. Active fractions are pooled (fraction IV, table).

Phosphocellulose Chromatography. Fraction IV is applied to a column of phosphocellulose (10 cm² × 20 cm) that has been equilibrated with potassium phosphate buffer, 50 mM, pH 6.5, and 10 mM 2-mercaptoethanol. This is followed by a linear gradient of 3 liters of potassium phosphate buffer, pH 6.5, with limits of 50 mM and 0.4 M, containing 10 mM 2-mercaptoethanol. The flow rate is 2.5 ml per minute, and 40-ml fractions

³ At 30-minute intervals, 1-ml aliquots are removed and centrifuged and the optical density of the supernatant fluid after suitable dilution in Tris-HCl buffer, 50 mM, pH 7.4 is determined at 260 nm; a portion of the supernatant fluid is precipitated with an equal volume of cold 1 N perchloric acid, and the optical density of the acid-soluble fraction is determined.

are collected. The peak of enzyme activity emerges after passage of approximately 2 liters of the gradient solution. Fractions with specific activity of greater than 12,000 are pooled (fraction V, table).

DEAE-Cellulose Chromatography. The volume of fraction V is reduced from 330 ml to approximately 20 ml by dialysis against solid sucrose; fresh changes of dry sucrose are applied as necessary to achieve the volume reduction. The sample is then dialyzed against potassium phosphate buffer, 20 mM, pH 7.4, containing 10 mM 2-mercaptoethanol and applied to a column of DEAE-cellulose (1.6 cm² × 25 cm) that has been equilibrated against the same buffer. A linear gradient of 800 ml of potassium phosphate buffer, pH 6.3, is applied, with limits of 20 mM and 0.15 M, containing 10 mM 2-mercaptoethanol. The flow rate is 0.6 ml per minute; 5-ml fractions are collected. The peak of protein and polymerase activity appear after passage of approximately 200 ml of gradient solution. The active fractions are pooled, concentrated by vacuum ultrafiltration,⁴ and dialyzed against 0.1 M Tris-HCl buffer, pH 7.5, and 1 mM reduced glutathione (fraction VI, table). Fraction VI, stored in liquid nitrogen, retained 84% of the original activity after 10 months.

*Hydroxyapatite Chromatography.*⁵ Fraction VI (15.8 ml), from liquid nitrogen storage, is diluted with an equal volume of 20 mM 2-mercaptoethanol and adjusted to pH 6.5 with 50 mM KH₂PO₄ containing 10 mM 2-mercaptoethanol. The diluted fraction is applied to a column of hydroxyapatite (4.5 cm² × 15 cm) that has been washed with 2500 ml of 50 mM potassium phosphate buffer, pH 6.5, and 10 mM 2-mercaptoethanol. The column is washed with 100 ml of the same buffer, and then a linear gradient of 1500 ml of potassium phosphate, pH 6.5, with limits of 50 mM and 0.3 M, containing 10 mM 2-mercaptoethanol, is applied. The flow rate is 0.8 ml per minute. Protein and enzyme activity appear in a single peak midway in the gradient. Tubes with greater than 300 units/ml are pooled, concentrated by dialysis against solid sucrose, and dialyzed exhaustively against 50% glycerol, potassium phosphate, 0.2 M, pH 6.5, containing 10 mM 2-mercaptoethanol. This material (fraction VII) has a protein concentration of 0.86 mg/ml and when stored at -20° retained full activity for 10 months.

Properties of the Enzyme

Homogeneity. The hydroxyapatite fraction of the T4 DNA polymerase is a homogeneous protein as judged by three criteria: (1) There is a con-

⁴ C. C. Richardson and A. Kornberg, *J. Biol. Chem.* **239**, 242 (1964).

⁵ Although the hydroxyapatite step results in a large loss of activity with no increase in specific activity, it is included because it removes traces of residual endonuclease activity still detectable in fraction VI.

stant specific activity across the protein peak in the hydroxyapatite chromatogram. (2) A single protein band is obtained on SDS-polyacrylamide gel electrophoresis (20 μg of protein). (3) There is a linear relationship between log of concentration and distance squared in Yphantis equilibrium sedimentation.⁶

Physical Properties. The T4 DNA polymerase consists of a single polypeptide chain with a molecular weight of 114,000. In contrast to *E. coli* DNA polymerase I, which has only 3 half-cystine residues, the T4-induced polymerase has 15.

pH Optimum. Maximum activity is observed over the pH range 8–9 (Tris or glycine buffers). At pH's 7.5 and 9.7, approximately 50% of maximal activity is observed.

Divalent Metal Requirement. There is no detectable polymerase activity in the absence of a divalent cation. Maximal activity is observed at 6 mM Mg^{2+} . Mn^{2+} at an optimal concentration of 0.1 mM gives a rate about one-fourth of that obtained with 6 mM Mg^{2+} .

Requirement for Sulfhydryl Reagents. DNA polymerase activity is the same in the presence and in the absence of 10 mM 2-mercaptoethanol during the initial 15-minute interval of incubation. At later times (30 minutes) the rate of polymerization in the absence of mercaptoethanol drops to 50% of that observed in its presence. Addition of *p*-hydroxymercuribenzoate at 4×10^{-4} M reduces polymerase activity to less than 5% of control values obtained in the absence of the mercurial.

Effect of Salt Concentration. Optimal activity is observed in the presence of a total salt concentration of approximately 0.10 M. At 0.30 M, activity is inhibited by 97%.

Nuclease Activity. The purified T4 polymerase is free of endonuclease as judged by its inability to cleave phosphodiester bonds in single-stranded (M13) or double-stranded (SV40) closed circular DNA's. The enzyme does, however, possess an associated exonuclease activity that has a strong preference (100-fold) for single-stranded over double-stranded DNA. *E. coli* DNA, glucosylated T4 DNA, and synthetic polydeoxynucleotides are all attacked. Hydrolysis proceeds exclusively in the 3' \rightarrow 5' direction to produce deoxynucleoside 5'-monophosphates and a dinucleotide derived from the 5' terminus of the polynucleotide substrate.⁷ In contrast to the polymerization reaction, which is stimulated by the product of T4 gene 32^{7a} (see below), the exonuclease activity is completely inhibited.

The exonuclease activity of the T4 polymerase has essentially the

⁶ D. A. Yphantis, *Biochemistry* **3**, 297 (1964).

⁷ W. M. Huang and I. R. Lehman, *J. Biol. Chem.* **247**, 3139 (1972).

^{7a} B. M. Alberts and L. Frey, *Nature (London)* **227**, 1313 (1970).

same pH optimum and divalent metal ion requirement as the polymerase activity. The two do, however, differ in their requirement for sulfhydryl reagents. Thus, the rate of hydrolysis of denatured DNA is actually 50% higher in the absence of added 10 mM 2-mercaptoethanol (or 1 mM dithiothreitol) than in its presence; *p*-hydroxymercuribenzoate at 50 mM does, however, cause complete inhibition of the exonuclease activity. Unlike *E. coli* DNA polymerase I, T4 DNA polymerase is completely devoid of 5 → 3' exonuclease activity.⁸

Contaminating Enzyme Activities. The purified T4 DNA polymerase contains a low level of ATP-dependent DNA ligase activity.⁹ Using ³H-poly-(dA-dT) as substrate, the specific activity of the ligase is about 43 units/mg, an amount that is equivalent to about 0.1% contamination.

Primer-Template Requirement. T4 DNA polymerase requires a single-stranded template with a free 3'-hydroxyl terminus, which is replicated to an extent that approaches but never exceeds the DNA input. The product is a helical structure in which the newly synthesized strand is covalently linked from its 5' terminus to the 3' terminus of the template. Double-stranded DNA made partially single-stranded by digestion with *E. coli* exonuclease III⁴ is also capable of supporting synthesis by T4 DNA polymerase. The extent of synthesis is equivalent to the extent of prior digestion by exonuclease III. A model has been proposed to explain the template specificity of T4 polymerase in which the 3' terminus of the single-stranded template first loops back upon itself and then serves as a priming end for replication of the remainder of the template. The "looped back" configuration would then resemble a DNA molecule partially digested with exonuclease III.¹

The T4 DNA polymerase, like *E. coli* DNA polymerase I is unable to catalyze the initiation of new strands. Unlike the *E. coli* enzyme, the T4 polymerase is unable to initiate DNA synthesis at a single-strand interruption ("a nick") in double stranded DNA.

T4 DNA polymerase activity is markedly and specifically stimulated by the T4 gene 32 protein.¹⁰

Requirement for Deoxynucleoside Triphosphates. Like all other purified DNA polymerases, the T4 polymerase requires all four deoxynucleoside triphosphates, dATP, dGTP, dCTP, and dTTP, or their analogs for the synthesis of DNA. The concentration of each deoxynucleoside triphosphate ordinarily used for assays is 0.033 mM; however, enzyme activity is augmented 3-fold by increasing this to 0.33 mM. Omission of one, two, or three triphosphates reduces activity to 0.63, 0.23, and 0.10%, respectively, of that

⁸ N. R. Cozzarelli, R. B. Kelly, and A. Kornberg, *J. Mol. Biol.* **45**, 513 (1969).

⁹ B. Weiss and C. C. Richardson, *Proc. Nat. Acad. Sci. U.S.* **57**, 1021 (1967).

¹⁰ J. A. Huberman, A. Kornberg, and B. M. Alberts, *J. Mol. Biol.* **62**, 39 (1971).

obtained with all four present. Unlike *E. coli* DNA polymerase I, replacement of Mg^{2+} by Mn^{2+} does not permit substitution of a ribonucleoside triphosphate for a deoxynucleoside triphosphate.

Interaction of Polymerase and 3' → 5' Exonuclease Activities

In the presence of the complementary deoxynucleoside triphosphates, a template and a primer, T4 DNA polymerase may act either as polymerase or exonuclease depending on the mode of association of the template and primer. Polymerization occurs when all three of the following components are present simultaneously: (a) the appropriate deoxynucleoside triphosphate (or triphosphates) complementary to the template, (b) a polynucleotide template, and (c) a 3' hydroxyl-terminated primer at least one nucleotide residue shorter than the template. In the absence of any one of these components the enzyme functions as exonuclease. As an example, consider a structure in which the 3' hydroxyl terminus of one of the strands of a homopolymer pair protrudes beyond the 5' terminus of the opposing strand. Hydrolysis of the protruding nucleotides will continue until the 3' end occupies a position at least one residue interior to the 5' terminus. At this point, all three of the necessary components are present, the enzyme becomes a polymerase and can restore the missing nucleotide. The ends then fall into register and the enzyme once again acts as an exonuclease removing a residue from the 3' end. The cycle can be repeated again with the enzyme alternating as a polymerase or exonuclease in a process which consumes triphosphates without net DNA synthesis. Once the triphosphates are exhausted, the enzyme functions exclusively as a nuclease and complete degradation follows.

Mutants of T4 DNA Polymerase

T4 gene 43 is the structural gene for the T4 DNA polymerase.¹¹ *Amber* mutants which map in gene 43 fail to induce the synthesis of T4 polymerase but do produce peptides that show immunological cross reactivity with antibody prepared against purified T4 DNA polymerase.¹² One *amber* mutant, *amb22* induces the synthesis of a polypeptide of molecular weight approximately 90,000 that lacks polymerase activity but does have the 3' → 5' exonuclease activity characteristic of the wild type enzyme.¹³ Several of the temperature-sensitive gene 43 mutants induce the synthesis of abnormally thermolabile DNA polymerases. Since *amber* and temperature-sensitive mutants of gene 43 are unable to induce the synthesis of phage DNA under restrictive conditions, the T4 DNA polymerase must be required for T4 DNA replication *in vivo*. Several, but not all tempera-

¹¹ A. DeWaard, A. V. Paul, and I. R. Lehman, *Proc. Nat. Acad. Sci. U.S.* **54**, 1241 (1965).

¹² W. M. Huang and I. R. Lehman, *J. Biol. Chem.* **247**, 3139 (1973).

¹³ N. G. Nossal and M. S. Hersfield, *J. Biol. Chem.* **246**, 5414 (1971).

ture-sensitive gene 43 mutants cause a large (in one instance up to 2000-fold) increase in reversion frequency of rII mutants, involving both transitions and transversions; that is, they act as mutators.¹⁴ Other temperature-sensitive gene 43 mutants produce a decrease in reversion frequency of rII mutants, especially AT → GC transitions, hence have the properties of antimutators.¹⁵ *In vitro* measurement of exonuclease-polymerase ratios of T4 DNA polymerases induced by mutator and antimutator phages indicate that the mutators have low exonuclease:polymerase ratios relative to the wild-type enzyme and the antimutators have correspondingly high exonuclease:polymerase ratios.¹⁶

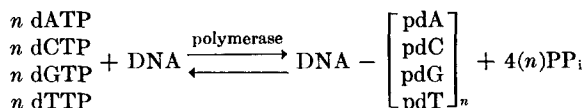
¹⁴ J. F. Speyer, *Biochem. Biophys. Res. Commun.* **21**, 6 (1965).

¹⁵ J. W. Drake and E. F. Allen, *Cold Spring Harbor Symp. Quant. Biol.* **33**, 339 (1968).

¹⁶ N. Muzyczka, R. L. Poland, and M. J. Bessman, *J. Biol. Chem.* **247**, 7116 (1973).

[7] Sea Urchin Nuclear DNA Polymerase¹

By BRADFORD S. FANSLER and LAWRENCE A. LOEB



Sea urchin embryos are particularly well suited as a source of DNA polymerase. In the initial cell divisions after fertilization the rate of DNA synthesis is about 60 times greater than in most other animal cells. This high DNA synthetic activity in the embryos is accompanied by a correspondingly high level of DNA polymerase activity.

The biology of this enzyme during early development of the embryo has been studied.²⁻⁵ It is synthesized during oogenesis and present in the unfertilized egg cytoplasm. With each cell division after fertilization, more and more is sequestered in the nuclei until, by the blastula stage, the majority of the embryo's polymerase activity can be isolated with the nuclei. It is for this reason that we chose to start the enzyme purification with the nuclear fraction.

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² L. A. Loeb, B. Fansler, R. Williams, and D. Mazia, *Exp. Cell Res.* **57**, 298 (1969).

³ B. Fansler and L. A. Loeb, *Exp. Cell Res.* **57**, 305 (1969).

⁴ L. A. Loeb and B. Fansler, *Biochim. Biophys. Acta* **217**, 50 (1970).

⁵ B. Fansler and L. A. Loeb, *Exp. Cell Res.* **75**, 433 (1972).