Purification and Characterization of Two New High Molecular Weight Forms of DNA Polymerase δ†

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ABSTRACT: Two high molecular weight DNA polymerases, which we have designated δ I and δ II, have been purified from calf thymus tissue. Using Bio Rex-70, DEAE-Sephadex A-25, and DNA affinity resin chromatography followed by sucrose gradient sedimentation, we purified DNA polymerase δ I 1400-fold to a specific activity of 10 000 nmol of nucleotide incorporated h^{-1} mg^{-1}, and DNA polymerase δ II was purified 4100-fold to a final specific activity of 30 000 nmol of nucleotide incorporated h^{-1} mg^{-1}. The native molecular weights of DNA polymerase δ I and DNA polymerase δ II are 240 000 and 290 000, respectively. Both enzymes have similarities to other purified δ-polymerases previously reported in their ability to degrade single-stranded DNA in a 3′ to 5′ direction, affinity for an AMP–hexane–agarose matrix, high activity on poly(dA)·oligo(dT) template, and relative resistance to the polymerase α inhibitors N^2-(*p*-*n*-butylphenyl)dATP and N^2-(*p*-*n*-butylphenyl)dGTP. These two forms of DNA polymerase δ also share several common features with α-type DNA polymerases. Both calf DNA polymerase δ I and DNA polymerase δ II are similar to calf DNA polymerase α in molecular weight, are inhibited by the α-polymerase inhibitors *N*-ethylmaleimide and aphidicolin, contain an active DNA-dependent RNA polymerase or primase activity, display a similar extent of processive DNA synthesis, and are stimulated by millimolar concentrations of ATP. We propose that calf DNA polymerase δ I, which also has a template specificity essentially identical with that of calf DNA polymerase α, could be an exonuclease-containing form of a DNA replicative enzyme.

DNA polymerase α has been implicated as the primary replicative enzyme in eukaryotes by two lines of evidence. First, it is known that DNA polymerase α activity increases in regenerating hepatic tissue (Lynch et al., 1975) and other rapidly growing tissue, while remaining low in nonproliferating tissue such as the brain (Baril & Laszlo, 1971). Second, aphidicolin, a potent inhibitor of cell growth, also inhibits purified DNA polymerase α in vitro.

DNA polymerase α from mammalian sources, when purified and incubated with the necessary components for synthesis to occur, has been shown to have a relatively low fidelity of replication compared to prokaryotic DNA polymerases (Loeb & Kunkel, 1982). DNA polymerase α from *Drosophila melanogaster* was shown to have about the same fidelity of replication as DNA polymerase III holoenzyme from *Escherichia coli* of about one mistake made per 10^6 nucleotides replicated (Kaguni et al., 1984). This may still be an unacceptable error rate when genomes on the order of 10^9 nucleotides are replicated. Coincident with this low fidelity is the lack of a 3′–5′-exonuclease "proofreading" activity present in many prokaryotic and viral DNA polymerases (Kornberg, 1980).

Recently, a DNA polymerase sensitive to *N*-ethylmaleimide and another α-polymerase-specific inhibitor, aphidicolin, have been isolated from hyperplastic rabbit reticulocytes (Byrnes, 1984), fetal calf thymus glands (Lee et al., 1984), and mature calf thymus glands (Lee et al., 1980). This enzyme has been designated DNA polymerase δ (Byrnes et al., 1976) and shown to have properties quite different from α-type DNA polymerases. Most striking is the presence of an active 3′–5′-exonuclease intrinsic to the polymerase. This enzyme has also been shown to display differences in template specificity. Synthetic DNA substrates such as alterating dA-dT, alternating dG-dC, or poly(dA) primed with oligo(dT) segments allow the greatest polymerase activity. The template which allows the greatest activity for most α-type DNA polymerases is nuclease-activated calf thymus DNA (Kornberg, 1980).

Here we present a method for the rapid purification of two forms of DNA polymerase δ, which are approximately 1.3 and 1.6 times the molecular weight of the most recently isolated form of DNA polymerase δ from fetal calf thymus (Lee et al., 1984). In addition, we present data on the characterization of these new enzymes.

The enzymes we have purified have been designated as DNA polymerase δ on the basis of template specificity, inhibitor sensitivity, retention by an AMP–agarose resin, and possession of an active 3′–5′-exonuclease activity. Surprisingly, our highly purified enzymes displayed several physical and kinetic characteristics common to α-type DNA polymerases. These are high molecular weight (Kornberg, 1980), possession of an active DNA-dependent RNA polymerase or primase activity intrinsic to the DNA polymerase complex (Conaway & Lehman, 1982), and capacity for stimulation by millimolar concentrations of ATP (Wierowski et al., 1983; Faust et al., 1984).

Materials and Methods

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Polydeoxyadenylate of a chain length between 4000 and 5000 nucleotides [$(dA)_{4000-5000}$], polydeoxyadenylate of a chain length of about 1000 nucleotides

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[$(dA)_{1000}$], oligothymidylate of a chain length of 16 nucleotides [$(dT)_{16}$], polythymidylate of a chain length of about 400 nucleotides [$(dT)_{400}$], alternating dA-dT, polyriboadenylate [poly(rA)], and terminal deoxynucleotidyltransferase (TdT) were all products of The Midland Certified Reagent Co. (Midland, TX). Alternating dA-dT was also obtained from either Miles Scientific (Naperville, IL) or Boehringer Mannheim Biochemicals (Indianapolis, IN). Dithiothreitol (DTT)[1] was obtained from Boehringer Mannheim. DNA polymerase I Klenow fragment and polynucleotide kinase were obtained from New England Biolabs (Wavely, MA). Bacterial alkaline phosphatase was from Bethesda Reseach Labs (Gaithersburg, MD). Bio Rex-70, (200–400 mesh), Affigel-10, Bio-Gel A-5M, Cellex 410, acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, and SDS–polyacrylamide gel electrophoresis high molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, CA). Sodium dodecyl sulfate (SDS) was from either BDH Chemicals, LTD. (Poole, England), or Bethesda Research Labs. DEAE-Sephadex A-25, Sephadex G-50 fine, Sephadex G-100, Sephacryl S-300, and gel filtration chromatography protein standards were from Pharmacia Fine Chemicals (Piscataway, NJ). Ampholines of the pH range 5–7 were from LKB (Paramus, NJ). AMP–hexane–agarose type II and oligo(dT) size standards of 10, 20, and 40–60 nucleotides in length were from Pharmacia-P-L Biochemicals (Milwaukee, WI). Centricon-30 microconcentrators were obtained from Amicon Corp. (Lexington, MA). [^{3}H]Thymidine 5′-triphosphate (specific activity 80 Ci mmol^{-1}), [γ-^{32}P]ATP (specific activity 1000–3000 Ci mmol^{-1}), and Omnifluor were from New England Nuclear Corp. (Boston, MA). [^{3}H]Deoxyadenosine 5′-triphosphate (specific activity 25 Ci mmol^{-1}) and unlabeled deoxynucleoside 5′-triphosphates of HPLC-purified grade were from ICN Pharmaceuticals (Plainview, NY). DEAE-nitrocellulose filters (NA45 of diameter 25 mm and pore size 45 μm) were from Schleicher & Schuell (Keene, NH). α-Polymerase C form was purified as outlined (Holmes et al., 1974, 1975) and was a gift of Dr. Kathy G. Lawton. N^2-(*p-n*-butylphenyl)deoxyguanosine 5′-triphosphate (BuPdGTP) and N^2-(*p-n*-butylphenyl)deoxyadenosine 5′-triphosphate (BuAdATP) were most generous gifts from Dr. George Wright of the Department of Pharmacology, University of Massachusetts Medical School (Shrewsbury, MA).

DNA Substrates. Activated calf thymus DNA was prepared as previously described (Uyemura & Lehman, 1976). $(dA)_{4000-5000}\cdot(dT)_{16}$ substrates were prepared by combining appropriate amounts of $(dA)_{4000-5000}$ and $(dT)_{16}$ such that the desired distance between primers was obtained. The concentration was adjusted to 1.0 mM in $(dA)_{4000-5000}$, in 10 mM Tris-HCl, pH 7.5, and 100 μM EDTA. These mixtures were then heated to 37 °C and slow-cooled for several hours to allow maximum annealing of primer to template. $(dA)_{4000-5000}$ and $(dA)_{1000}$ were both tailed at the 3′OH end with oligo(dT) to form a covalent, fold-back primer-template. The tailed polymer was produced by reacting poly(dA) with excess dTTP in the presence of TdT by the method of Kato et al. (1967). The resultant tailed polymer was purified free of contaminating dTTP and protein by phenol extraction, chloroform/isoamyl alcohol (20:1 v/v) extraction, and then desalting through centrifuge columns of Sephadex G-50 fine resin swollen in 10 mM Tris-HCl, pH 7.5, and 1.0 mM EDTA (Penefsky, 1977). Single-stranded and double-stranded RF bacteriophage fd DNA was prepared by the method of Matson et al. (1980). Specifically primed fd DNA was prepared by annealing a synthetic DNA primer 22 nucleotides in length complementary to nucleotides 1407–1386 on the fd sequence according to Beck et al. (1978). The oligonucleotide primer was made by an Applied Biosystems Model 360A oligonucleotide synthesizer.

Preparation of Tailed Template Affinity Resins. Tailed template covalently coupled to agarose was produced by reacting 25 optical density units at 260 nm of oligo(dT)-tailed $(dA)_{1000}$ with 5.0 mL of activated Affigel-10 under conditions described by the supplier. DNA which was not linked was washed off the resin by repeated batch washings with an excess of a 2.0 M KCl solution. Tailed template covalently coupled to cellulose was prepared by combining, in an identical polynucleotide to resin ratio as above, the tailed polymer with epoxide-activated cellulose by the method of Moss et al. (1982). Both procedures resulted in the immobilization of between 200 and 800 μM template when the coupling is monitored by the addition of a trace amount of ^{3}H-labeled polymer.

DNA Polymerase Assays. The standard assay (25 μL) contained 20 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), 200 μg mL^{-1} BSA, 10 mM DTT, 15 mM $MgCl_2$, 50 μM [^{3}H]-dTTP (4.0 Ci mmol^{-1}), 40 μM $(dA)_{4000-5000}\cdot(dT)_{16}$ with an interprimer distance of 150 nucleotides, and 1.0–3.0 μL of the indicated polymerase sample. To stimulate polymerase activity as well as inhibit endogenous TdT (Kornberg, 1980), ATP was also added at 2.0 mM. Assays were carried out for a period of 30 min at 30 °C and then terminated by the addition of 10 μL of 0.5 M EDTA, pH 7.5. When activated calf thymus DNA was used as the polymerase substrate, the conditions were the same as in the standard assay with the omission of the $(dA)_{4000-5000}\cdot(dT)_{16}$ template and the addition of 150 μM activated calf thymus DNA and 50 μM each of dATP, dGTP, and dCTP. Lineweaver–Burk analysis of polymerase activity was done with an interprimer distance of 400 nucleotides, and the reaction time was decreased to 10 min. The concentrations of DNA used were 20, 40, 60, 80, 160, and 200 μM (in nucleotides). Separation of labeled, polymeric DNA from precursor monomers was achieved either by sedimentation through centrifuge columns of G-50 fine resin (Penefsky, 1977) or by filtration through DEAE-nitrocellulose filters; then the filters were washed extensively by vacuum with 0.3 M ammonium formate and 10 mM sodium pyrophosphate at pH 7.8 and dried. Aqueous samples from the centrifuge columns were counted in a Triton X-100/toluene/Omnifluor (2 L:1 L:12 g) scintillation mixture. Dried filters were counted in a fluor composed of toluene and Omnifluor in a ratio of 3 L to 12 g. Both types of assays gave similar results. One unit of DNA polymerase incorporates 1 nmol of nucleotide h^{-1} at 30 °C under these conditions.

DNA Primase Assays. DNA primase activity was detected by using one of two substrates with the addition of *Escherichia coli* DNA polymerase I Klenow fragment as a signal amplifier for the detection of DNA-dependent RNA primer synthesis (Conaway & Lehman, 1982). Reactions making use of fd single-stranded DNA template were performed under the same conditions as assays for polymerase except that CTP, UTP, and GTP were added to 1.0 mM, the specific activity of [^{3}H]dTTP was increased to 10.0 Ci mmol^{-1}, dGTP, dCTP,

[1] Abbreviations: BSA, bovine serum albumin; DEAE, diethylaminoethyl; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; M_r, molecular weight; PMSF, phenylmethanesulfonyl fluoride; RF, replicative form; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; HPLC, high-pressure liquid chromatography.

and dATP were added to 50 μM, the DNA substrate was changed to 80 μM fd single-stranded DNA, and 0.5 unit of DNA polymerase I Klenow fragment was added. When $(dT)_{400}$ was used as the DNA substrate, the conditions were the same as those in the standard polymerase assay except that [^{3}H]dATP at a specific activity of 10 Ci mmol^{-1} and a concentration of 20 μM was substituted for [^{3}H]dTTP, $(dT)_{400}$ was added as the DNA substrate to a concentration of 80 μM, and 0.5 unit of DNA polymerase I Klenow fragment was added. All incubations were for 60 min at 30 °C, and incorporated nucleotides were assayed as described for the DNA polymerase assays.

Nuclease/Exonuclease Assays. Two types of deoxyribonuclease substrates were employed. The first was designed to measure nuclease activity but not directionality. $(dT)_{16}$ was extended with 4 Ci mmol^{-1} [^{3}H]dTTP by using TdT under conditions described for the preparation of the tailed templates (see DNA Substrates) to an average chain length of 50 nucleotides (specific activity 200 cpm pmol^{-1}). The second substrate was designed to measure whether the nuclease digests processively 3′ to 5′, 5′ to 3′ or is nondirectional. First, approximately one nucleotide of 40 Ci mmol^{-1} [^{3}H]dTTP was added to $(dT)_{400}$ by brief treatment with TdT. In a separate reaction, $(dT)_{400}$ was treated with bacterial alkaline phosphatase and subsequently with polynucleotide kinase in the presence of 1.0 mM [γ-^{32}P]ATP (specific activity 100 Ci mmol^{-1}) to label the 5′-DNA end (Maniatis et al., 1982). These two templates were deproteinized, and monomeric nucleotide was removed as previously described for the tailed template. The two were then mixed to produce the directional exonuclease substrate (specific activity 1200 ^{3}H cpm/290 ^{32}P cpm per 20 pmol of total nucleotide) of $(dT)_{400}$. The exonuclease assay conditions using either the directional or the nondirectional substrate were the same as for the polymerization except for the deletion of [^{3}H]dTTP, ATP, and $(dA)_{4000-5000}\cdot(dT)_{16}$ polymerase substrate and the addition of the indicated amount of nuclease substrate.

Radioactivity remaining was determined in the same manner as for the DNA polymerase assay. When necessary, dual-channel scintillation counting was performed by using a Beckman Model LS 6800 scintillation counter. The ^{32}P to ^{3}H channel overflow was less than 5%.

Buffers. Buffer I was 50 mM imidazole, pH 6.8, 200 mM NaCl, 10% glycerol (v/v), 20% Me_2SO (v/v), 5.0 mM DTT, 2.0 mM ATP, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin A, and 0.5 mM PMSF. Buffer IA was 50 mM imidazole, pH 6.8 450 mM NaCl, 30% glycerol (v/v), 5.0 mM DTT, 2.0 mM ATP, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin A, and 0.5 mM PMSF. Buffer II was 50 mM imidazole, pH 6.8, 20% glycerol (v/v), 4% Me_2SO (v/v), 60 mM NaCl, 5.0 mM DTT, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin A, and 0.5 mM PMSF. Buffer III was 20 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), 20 mM KCl, 5.0 mM DTT, 4.0 mM ATP, 4.0 mM $MgCl_2$, 100 μM EDTA, 100 μM EGTA, 0.5 μg mL^{-1} leupeptin, and 0.5 μg mL^{-1} pepstatin A. Buffer IV was 50 mM imidazole, pH 6.8, 200 mM KCl, 5.0 mM DTT, 1.0 mM EDTA, 1.0 mM EGTA, 2.0 mM ATP, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin A, and 0.5% Ampholine.

Purification of the Two Forms of DNA Polymerase δ. All procedures were carried out at temperatures between 0 and 4 °C. Detection of protein was by staining with Coomassie brilliant blue (Bradford, 1976). Ionic strength determinations were carried out by using a Model 32 conductivity bridge from Yellow Springs Instruments, Inc. (Yellow Springs, OH). Postmicrosomal supernatant solutions were prepared by the method of Wahl et al. (1984) with the omission of the bacitracin inhibitor. We have found that some lots of bacitracin will strongly inhibit DNA polymerase activity on templates that contain all four deoxynucleoside monophosphate residues but not on homopolymeric templates (data not shown). A 350-mL sample of postmicrosomal supernatant solution was dialyzed against 3.5 L of buffer I, with two changes, for a period of 2–3 h between changes. The solution was then batch-adsorbed onto 200 mL of Bio Rex-70, preequilibrated in the same buffer, and the mixture was poured onto a 4.0 cm × 30 cm column of the same resin, also preequilibrated in buffer I. The resultant column was washed with 1 column volume of buffer I with an additional 100 mM NaCl added and subsequently eluted with a 7 column volume linear gradient from this buffer to buffer IA. Fractions of 14-mL volume were collected by using an LKB Ultra Rack fraction collector and a Buchler peristaltic pump at a flow rate between 0.5 and 0.7 column volume h^{-1}. Every tenth fraction was assayed for polymerase activity in both the standard assay and the assay described for activated calf thymus DNA.

The gradient elution of this Bio Rex-70 column partially resolves peaks of activated calf thymus DNA polymerase activity and $(dA)_{4000-5000}\cdot(dT)_{16}$ DNA polymerase activity. These were designated DNA polymerase δ I and DNA polymerase δ II in order of elution. Fractions containing greater than half-maximal activity were separately pooled. DNA polymerase δ I (300 mL) and DNA polymerase δ II (350 mL) fractions were then dialyzed separately into 10 volumes of buffer II without added NaCl for 3 h and then into 10 volumes of buffer II for an equivalent time. These we termed fraction II. They were subsequently chromatographed over two separate columns of DEAE-Sephadex A-25 (4.0 cm × 40 cm each), preequilibrated in buffer II without added Me_2SO or NaCl. These columns were then washed with 1 column volume of buffer II to remove unadsorbed protein and eluted with a 10 column volume linear gradient from buffer II to buffer II in which the NaCl concentration had been increased to 400 mM and the Me_2SO omitted. A total of 300 fractions of 14 mL each were collected in the same manner as for the Bio Rex-70 column.

Fractions which contained at least half-maximal activity, as judged by the standard assay, were again pooled for each of the δ I and δ II polymerases, placed in dialysis membranes, and concentrated against solid poly(ethylene glycol) (M_r 16 000–20 000) to a protein concentration of at least 200 μg mL^{-1}. These fractions were then dialyzed against 50 volumes of buffer III overnight and either used immediately in the next step or frozen under liquid nitrogen in 1.0-mL aliquots and stored at −70 °C for later use. These were termed fraction III DNA polymerases δ I and II.

For the final purification steps, between 1.0 and 3.0 mL of either δ I or δ II fraction III DNA polymerase was applied to a 3.0-mL packed bed volume of the tailed template affinity matrix which had been preequilibrated in buffer III without any KCl. The flow was stopped and the polymerase sample allowed to adsorb onto the matrix for 45 min. Then unbound protein was washed off by the application of 4 column volumes of buffer III containing 100 mM KCl. Elution was achieved by the addition of 300 mM KCl to buffer III and passage of several column volumes of this buffer through the affinity resin. A total of 14 fractions of 1.5 mL were collected by using a Gilson micro-fractionator at a column flow rate of 2 column volumes h^{-1}. Each fraction was assayed for polymerase activity

by using 3.0 μL of sample. Fractions containing polymerase activity (4.5 mL) were pooled and concentrated to a volume of 250 μL with a Centricon-30 microconcentrator. Samples were diluted to 4% glycerol by the addition of 1.25 mL of buffer IV, and then concentration was continued to a final volume of between 75 and 150 μL. Concentrators were then inverted and samples centrifuged off the membrane into a collection tube. To remove all polymerase from the Centricon membrane, 50 μL of buffer IV, containing 4% sucrose, was added to the reverse side of the membrane during the step in which the sample is removed from the membrane. This is fraction IV DNA polymerase δ I and DNA polymerase δ II.

Sucrose gradients (5–20% w/v) were generated in buffer IV in tubes measuring 16 mm × 60 mm by using a Beckman density gradient former. Between 100 and 200 μL of fraction IV DNA polymerase δ I or II was gently layered onto these sucrose gradients and subsequently spun in a Beckman SW 60 rotor at 40000 rpm for 18 h at 2 °C. Tubes were punctured from the bottom by using a Beckman fraction recovery system and five-drop fractions collected by a Gilson micro-fractionator (31 fractions/gradient). Molecular weight standards of thyroglobulin (200 μg), catalase (100 μg), and BSA (60 μg) in a total volume of 100 μL were layered on a parallel gradient to act as markers for the determination of sedimentation coefficients. Their positions were determined by staining the fractionated tubes for protein content. Fractionated samples were assayed for polymerase activity in the standard assay, the fractions containing at least half peak level activity were pooled. These fractions, designated fraction V DNA polymerase δ I or fraction V DNA polymerase δ II, have been used to perform all subsequently reported experiments unless otherwise noted.

Gel Filtration for Estimation of the Stokes Radius. Gel filtration was performed with a column of Sephacryl S-300 (0.8 cm × 47 cm) which had been preequilibrated in 50 mM imidazole, pH 6.8, 20% glycerol, 200 mM KCl, 5.0 mM DTT, 4.0 mM ATP, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin A, and 0.1% Ampholine. KCl was added to discourage aggregation, and Ampholine was added to retain activity. Five-drop fractions were collected at a flow rate of 4.0 mL h^{-1} by using an LKB peristaltic pump and Multirack fraction collector. The void volume and included volume of the column were determined from the elution positions of blue dextran and orange G. respectively. Prior to the application of polymerase samples, protein standards were passed over the column and their positions determined by protein staining aliquots of the fractions collected. The protein standards used were BSA, aldolase, catalase, ferritin, and thyroglobulin. When either of the two forms of DNA polymerase δ were passed over the column, 500 μL of fraction III polymerase was applied, the peak of eluted polymerase activity was determined, and fractions were pooled to determine the volume of elution.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carred out according to the method of Laemmli (1970). Protein separation was carried out in 7.5% polyacrylamide gels with a 4.0% polyacrylamide stack. All polyacrylamide gels were preelectrophoresed prior to use and subsequently fixed, stained, and destained according to the method of Marshall (1984). We found this technique to be superior to several others we have tried for the detection of the small quantities of protein present in our polymerase samples.

Analytical AMP–Agarose Chromatography. Since deoxynucleoside monophosphates are the products of DNA exonucleases, immobilized AMP potentially can serve as an affinity matrix for binding DNA polymerases that contain such activity (Lee & Whyte, 1984). We used a slightly modified version of this method to detect binding of exonuclease-containing DNA polymerase δ I and II polymerases and non-exonuclease-containing α-polymerase C form. A volume of 100 μL of fraction III DNA polymerase δ I, fraction III DNA polymerase δ II, or α-polymerase C form (Holmes et al., 1974, 1975) was dialyzed into buffer III without added ATP. This sample was loaded onto a 950-μL column of AMP–hexane–agarose type II which had been preequilibrated in buffer III minus ATP and KCl at a flow rate of 2 column volumes h^{-1}. The flow was stopped as with the tailed template affinity chromatography purification step and the given polymerase sample allowed to adsorb onto the matrix for a period of 30 min. The flow of buffer was then resumed and the column washed with 3.0 mL of the preequilibration buffer to remove unadsorbed protein. Fractions of 20 drops (450 μL) were collected by using a Gilson micro-fractionator. Bound polymerase was removed from the resin by elution with 3.1 mL of buffer III minus ATP and with the KCl concentration increased to 0.35 M. Three microliters of each fraction was assayed for polymerase activity under conditions outlined previously.

Primer Elongation Product Size Analysis: Measurement of Processive DNA Synthesis. To determine the size of products produced per polymerase–DNA binding event by each of the two purified forms of DNA polymerase δ, the method of Fay et al. (1980) was used. Polymerization was allowed to proceed in a reaction (50 μL) under the same conditions outlined for the standard assay, except that a $(dA)_{4000-5000}$·$(dT)_{16}$ template with a 400-nucleotide interprimer distance was used at a concentration of 200 μM and the specific activity of the [^{3}H]dTTP label was increased to 10 Ci $mmol^{-1}$. ATP (2.5 mM) either was added or was not added. After 4 min, the reaction was stopped by the addition of 10 μL of 0.5 M EDTA and placed on ice. An aliquot of the reaction was removed for the determination of the number of picomoles of [^{3}H]TMP incorporated per picomole of 3′-hydroxyl termini present. The amount of polymerase was adjusted such that the enzyme statistically never bound and synthesized at the same 3′-terminus more than once. This was accomplished by keeping the amount of incorporation below 1 mol of nucleotide incorporated per mole of 3′ termini present. As a result, the sizes of the primers labeled by nucleotide addition represent the original primer length plus the average extent of processive DNA synthesis.

The remainder of the reaction mixture was desalted by passing it over a column of Sephadex G-100 (0.6 cm × 27 cm) preequilibrated in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1.0 mM EDTA. The tube which contained the peak of excluded radioactivity, as determined by counting a 10-μL aliquot of each tube in the Triton X-100 based scintillation fluor, was denatured by the addition of NaOH to 0.10 M and heating to 37 °C. One hundred microliters of this DNA was passed through a column of Bio-Gel A-5M (0.6 cm × 27 cm) which was preequilibrated in 0.1 M NaOH and 5.0 mM EDTA at room temperature. Ten-drop fractions were collected and assayed for radioactivity by the addition of 1.8 mL of the Triton X-100 containing aqueous fluor previously described. To calibrate this column, polymers of oligo(dT) of known sizes were used. For standardization, samples of 100 μL in 0.1 M NaOH at 5.0 optical density units at 260 nm mL^{-1} were passed through the Bio-Gel A-5M column. Again, 10-drop fractions were collected, and the position of elution of these standards

Table I: Purification of DNA Polymerases δ I and II from Calf Thymus[a]

step	δ-polymerase form	total act. (units)	sp act. (units mg^{-1})	recovery[b] (%)
(I) postmicrosomal supernatant solution		60200	7.3	100
(II) Bio Rex-70	I	4600	24	7.5
	II	13500	86	22.5
(III) DEAE-Sephadex A-25	I	1230	151	2.0
	II	3750	331	6.25
step	γ-polymerase form	units recovered/ units applied[c]	sp act. (units mg^{-1})	recovery[b] (%)
(IV) tailed template chromatography	I	32/120	2000	0.53
	II	15/21	4000	4.46
(V) sucrose gradient ultracentrifugation	I	13.5/30	10000	0.24
	II	10.0/13.4	30000	3.33

[a] Procedures were carried out as described under Materials and Methods. One unit of DNA polymerase catalyzes the incorporation of 1 nmol of nucleotide h^{-1} at 30 °C in the standard assay. [b] Recovery values are given on the basis of complete processing of the activity in step I. [c] Only part of fraction III was used for subsequent steps.

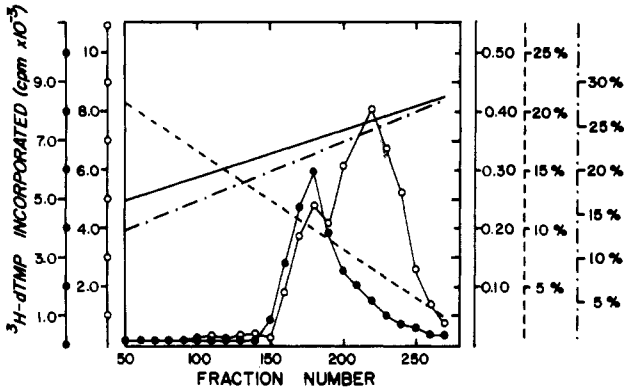

FIGURE 1: Bio Rex-70 chromatography of calf thymus postmicrosomal supernatant solution. Chromatography was as described under Materials and Method. Polymerase activity was assayed by using either $(dA)_{4000-5000}\cdot(dT)_{16}$ (○) or activated calf thymus DNA (●) as the primer-template under previously described conditions. To standardize the amount of incorporation to a molar level, the cpm values obtained from the activated calf thymus DNA assays were multiplied by 4. Shown also are the calculated concentrations of NaCl (—) in molarity, Me_2SO (- - -), and glycerol (–·–).

was determined by the location of the fraction which contained the peak optical absorbance at 260 nm.

Results

Purification Procedure. Table I summarizes the purification of the two forms of DNA polymerase δ present in calf thymus postmicrosomal supernatant solutions. Figure 1 depicts the initial fractionation of this dialyzed solution through a column of Bio Rex-70 resin. Two polymerase fractions were eluted at NaCl concentrations below 0.40 M. One of these forms was active to about the same degree on either the activated calf thymus DNA template or the homopolymeric DNA template $(dA)_{4000-5000}$ with segments of $(dT)_{16}$ annealed to form primers for synthesis. We have designated this form DNA polymerase δ I. The other form of DNA polymerase δ elutes after the peak I form and so has been designated DNA polymerase δ II. This material utilizes homopolymeric DNA for synthesis, while it is virtually inactive on activated calf thymus DNA.

These two partially separated fractions were separately pooled and further fractionated through separate DEAE-

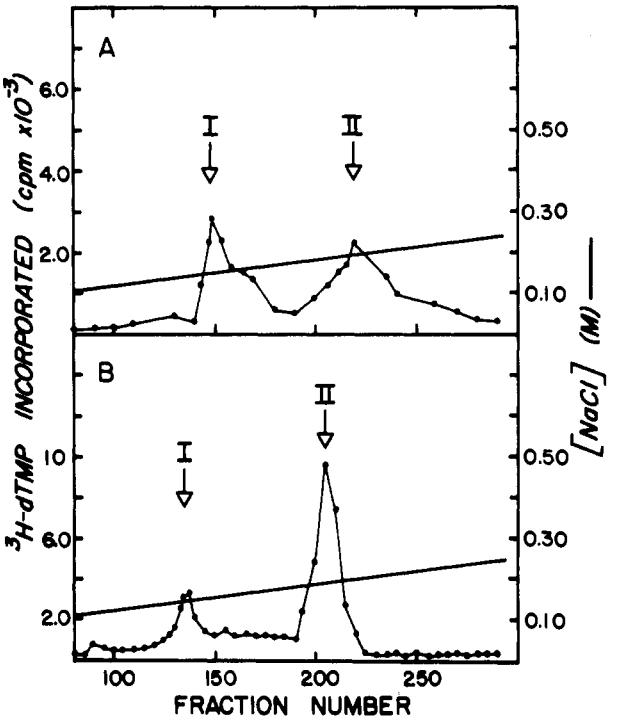

FIGURE 2: Chromatography of DNA polymerse δ I and DNA polymerase δ II through a column of DEAE-Sephadex A-25 resin. Panel A represents the DNA polymerase profile obtained from chromatography of fraction II DNA polymerase δ II. Procedures were as described under Materials and Methods. NaCl concentrations are indicated by a solid line (—). DNA polymerase δ I (labeled I in both panels) eluted at a NaCl concentration of 100 mM, and DNA polymerase δ II (labeled II in both panels) eluted at a NaCl concentration of 200 mM.

Sephadex A-25 columns. This resulted in complete resolution of the two different polymerases, as shown in Figure 2. The first peak of polymerase activity to elute from the DEAE-Sephadex column is DNA polymerse δ I, again identified by its activity on the calf thymus DNA primer-template. The later eluting peak is polymerase δ II, identified by its singular activity on poly(dA)·oligo(dT) templates and negligible polymerizing activity on activated calf thymus DNA. These polymerases were separately adsorbed onto an affinity column of immobilized $(dA)_{1000}$ which was tailed at the 3′ end with oligo(dT). This polymer can form a stable primer-template structure capable of binding DNA polymerases. After unadsorbed protein was washed free of the column matrix by a 100 mM KCl added salt wash, the resultant adsorbed polymerase was eluted by a buffer containing 300 mM KCl, concentrated, and applied to separate 5–20% sucrose gradients. The protein was sedimented through the gradients, and they were fractionated and assayed for several enzyme activities. Results of assays for polymerase, DNA-dependent RNA polymerase (primase), and DNA exonuclease activities are shown in Figure 3 for DNA polymerase δ I and in Figure 4 for DNA polymerase δ II. In the cases of both polymerases, all three of these activities cosedimented. When ATP was omitted from the primase assays, rates of incorporation fell 10–20-fold (data not shown). In addition, when exonuclease activity was assessed throughout the purification procedure, a peak of exonuclease was always found to copurify with each of the DNA polymerases (data not shown). This entire purification scheme takes between 5 and 6 days to complete.

After purification to fraction V, DNA polymerases δ I and δ II were conservatively assessed to have specific activities of 10 000 and 30 000 units mg^{-1}, respectively. This represents a 1400-fold purification of DNA polymerase δ I and a

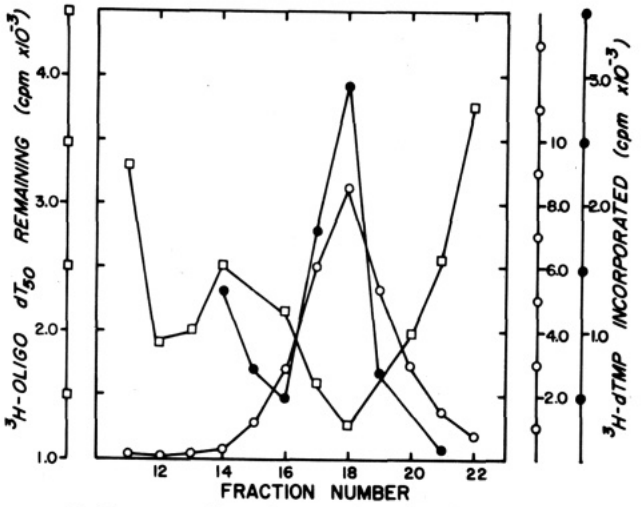

FIGURE 3: Sucrose gradient ultracentrifugation of DNA polymerase δ I. DNA polymerase δ I fraction IV was sedimented through a 5–20% (w/v) sucrose gradient as described under Materials and Methods. Assays performed on the resultant fractions are shown for polymerase (1.0 μL) in the standard assay (○), DNA primase activity (3.0 μL) utilizing fd single-stranded DNA (●), and nuclease activity (5.0 μL) using the [^{3}H](dT)$_{50}$ substrate (□). The bottom of the gradient is to the left.

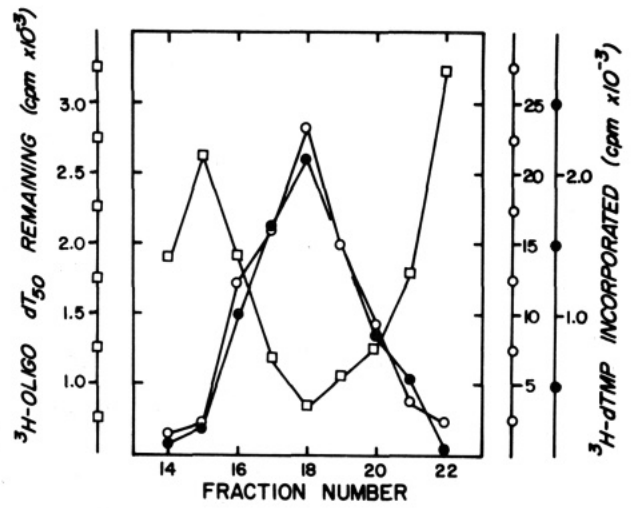

FIGURE 4: Sucrose gradient ultracentrifugation of DNA polymerase δ II. DNA polymerase δ II was sedimented through a 5–20% sucrose gradient as described under Materials and Methods. Assays were performed on the resultant fractionated gradients: polymerase (2.0 μL) as in the standard assay (○); DNA primase activity (3.0 μL) using the (dT)$_{400}$ DNA template (●); nuclease activity (5.0 μL) using the [^{3}H](dT)$_{50}$ substrate (□). The bottom of the gradient is to the left.

4100-fold purification of DNA polymerase δ II. This is also a low estimate of fold purification as the crude supernatant solution is contaminated with other DNA polymerases than these purified. Because of the low protein concentration in the fraction V polymerases, protein concentration was determined by laser densitometric scans of the polymerase samples and standard proteins of known concentrations, after SDS–polyacrylamide gel electrophoresis and staining with ammonical silver. The major protein bands which cosedimented with the enzymatic activity of the polymerase–primase–nuclease complex were of M_r 245 000, 164 000, 120 000, 110 000, 60 000, and 45 000 for DNA polymerase δ I and of M_r 245 000, 135 000, 110 000, 60 000, and 45 000 for DNA polymerase δ II as shown in Figure 5. Four polypeptides could be common to both polymerase forms.

Molecular Weight Determination of the Two Forms of

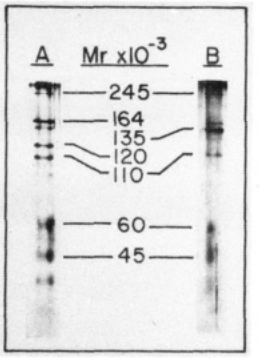

FIGURE 5: SDS–polyacrylamide gel electrophoresis of fraction V DNA polymerases δ I and II. SDS–polyacrylamide gel electrophoresis, gel staining, and subsequent destaining were carried out on an aliquot (70 μL) of either fraction V DNA polymerase δ I (lane A) or fraction V DNA polymerase δ II (lane B) as described under Materials and Methods. Protein bands which comigrated upon sucrose gradient ultracentrifugation with either polymerase are indicated.

DNA Polymerase δ. According to the correlation of Siegel & Monty (1966), the Stokes radius obtained from gel filtration and the $s_{20,w}$ value from sedimentation data were used to calculate the molecular weight of each form of DNA polymerase δ. Both polymerase forms sediment to 10.1 S in the final sucrose gradient ultracentrifugation purification step. Upon gel filtration under the conditions outlined under Materials and Methods, we find a significant difference in the Stokes radii of these two polymerases. DNA polymerase δ I has a Stokes radius of 58.2 Å, and DNA polymerase δ II has a Stokes radius of 69.6 Å. When fraction V DNA polymerase δ I or II are gel-filtered under identical conditions using a Sephacryl S-300 column of dimensions 0.6 cm × 27 cm, the same difference in Stokes radius is observed (data not shown). On both sedimentation and gel filtration analysis, the presence of Ampholine is required to retain activity. Under these conditions, the molecular weights of DNA polymerases δ I and II are 240 000 and 290 000, respectively.

Demonstration That DNA Polymerases δ I and II Behave as a Complex of Polymerase Associated with Exonuclease. The method of Lee & Whyte (1984) was used to show that partially purified DNA polymerases δ I and II each exist as a stable polymerase–nuclease complex. The products of DNA exonuclease are 5′-deoxynucleoside monophosphates. AMP has the ability to bind to the nuclease active site on several exonuclease-containing DNA polymerases (Byrnes et al., 1977) and upon binding is found to inactivate these exonucleases. Because of this property, immobilized AMP has been found to serve as an affinity ligand for binding DNA polymerase δ but not for DNA polymerase α which lacks an exonuclease. Figure 6 shows the result obtained when either DNA polymerase δ I or DNA polymerase δ II is passed through a column of AMP–hexane–agarose type II resin under conditions outlined under Materials and Methods. DNA polymerase α C form (Holmes et al., 1974, 1975) was used as the non-exonuclease-containing control enzyme. About 58% and 85% of DNA polymerases δ I and II, respectively, were retained by the AMP–agarose matrix, while 10% of the applied α-polymerase was found to bind. This result implies that the DNA polymerase δ I and II species have an exonuclease activity which is tightly bound to the polymerase.

DNA Polymerases δ I and II Are Both Associated with a 3′-OH-Specific Exonuclease. Figure 7 presents the results obtained when either DNA polymerase δ I or II was reacted with a substrate designed to measure the directionality of the associated nuclease activities. This substrate is a segment of poly(dT), 400 nucleotides in length, labeled at the 5′ end with

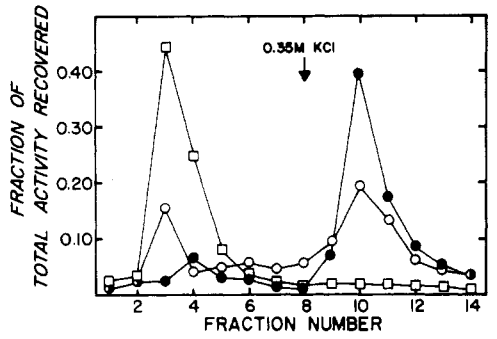

FIGURE 6: Analytical AMP–agarose chromatography of the two forms of DNA polymerase δ. DNA polymerase δ I (○), DNA polymerase δ II (●), and DNA polymerase α C form (□) were passed through a column of AMP–agarose type II as described under Materials and Methods. The total yields of the chromatography polymerase samples were 86%, 94%, and 91% for DNA polymerase δ I, DNA polymerase δ II, and DNA polymerase α C form, respectively, as compared to the applied activity. The majority of the α-polymerase activity recovered (86%) flowed through the column to elute between fractions 2 and 7, inclusive. After the application of the 0.35 M added KCl step elution buffer, 58% of the DNA polymerase δ I and 85% of the DNA polymerase δ II activity recovered were found to lie between fractions 9 and 14, inclusive. Less than 10% of the total recovered α-polymerase activity was found to elute in these latter fractions.

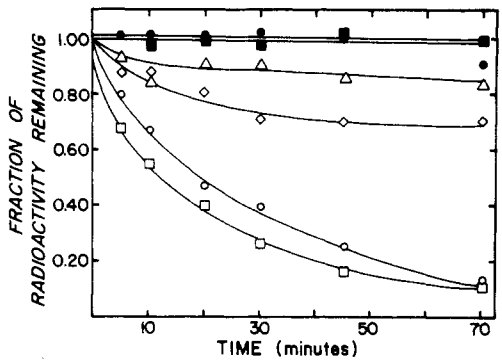

FIGURE 7: Determination of the directionality of the nucleases associated with DNA polymerases δ I and II. Conditions used were as described under Materials and Methods except that the volume of the reaction was increased to 70 μL; 140 pmol (in nucleotides) of the $^3H/^{32}P$-labeled $(dT)_{400}$ was added as substrate, and 10 μL of the indicated polymerase sample was added. The total number of polymerase units added was 0.18 for DNA polymerase δ I and 0.21 for DNA polymerase δ II. Aliquots (10 μL) were removed at the indicated times, added to 10 μL of 0.5 M EDTA, and then placed on ice to stop the reaction. Radioactivity remaining was determined as previously described. When AMP was added, it was added at a concentration of 5.0 mM. Plotted are the fractions of initial ^3H remaining (○) or ^{32}P remaining (●) after addition of DNA polymerase δ I, ^3H remaining (□) or ^{32}P remaining (■) after addition of DNA polymerase δ II, ^3H remaining after addition of DNA polymerase δ I and AMP (△), and ^3H remaining after addition of DNA polymerase δ II and AMP (◇). The fraction of initial ^{32}P cpm remaining for the two experiments which contained AMP was unchanged through the time course of these experiments. Therefore, the ^{32}P profiles in the presence of AMP were omitted to enhance clarity. When no enzyme was added as a control, there was less than 3% loss of either ^{32}P or ^3H during the course of the assay. When the curves were fitted to a simple exponential decay and the initial rates of ^3H loss calculated from the first derivative of those equations, the addition of AMP resulted in an 85% inhibition of the DNA polymerase δ I exonuclease and an 75% inhibition of the DNA polymerase δ II exonuclease. The rate of loss of ^{32}P was determined by separate linear regressions of the data points for the two experiments not containing added AMP. The ratio of the initial rate of ^3H loss to the rate of ^{32}P loss is 100 for DNA polymerase δ I and 500 for DNA polymerase δ II.

^{32}P and at the 3′ end with [^3H]dTMP. Release of only ^{32}P from this substrate implies the action of a 5′–3′-exonuclease; release of only ^3H implies the action of a 3′–5′-exonuclease activity. Coincident release of both ^{32}P and ^3H denotes the presence of an endonuclease, since the entire template would be degraded nondirectionally. The rate of release of ^3H-labeled nucleotides from the 3′-terminus was found to be more than 40-fold higher than the rate of release of [^{32}P]phosphate molecules or nucleotides from the 5′-terminus when either DNA polymerase δ I or DNA polymerase δ II was allowed to react with the substrate. In fact, if the initial rates are calculated for these exonuclease reactions, assuming that digestion follows a simple exponential decay, the rate of release of [^3H]/[^{32}P] release was 100 for DNA polymerase δ I and 500 for DNA polymerase δ II.

Table II: DNA Polymerase Inhibitors

inhibitor[a]	fraction of control activity: DNA polymerase δ I	DNA polymerase δ II
none	1.00	1.00
aphidicolin (25 μg mL^{-1})	0.07	0.14
10 mM *N*-ethylmaleimide	0.10	0.08
anti-α-polymerase Ab (1×)[b]	1.00	0.91
anti-α-polymerase Ab (10×)[c]	1.00	0.65
BuPdGTP (100 μM)	0.20	0.82
BuAdATP (100 μM)	0.20	0.80

[a] Inhibitors were used as previously described (Wahl et al., 1984) in the standard assay described under Materials and Methods. BuPdGTP and BuAdATP were added at the indicated concentrations to the polymerase assay. 1.0×10^{-2} unit of DNA polymerase δ I or 1.3×10^{-2} unit of DNA polymerase δ II was used per assay. [b] This concentration inhibits calf α-polymerase 50%. [c] This concentration inhibits calf α-polymerase greater than 95%.

Exonuclease activity of either of these polymerases on the dual-labeled $(dT)_{400}$ substrate is significantly inhibited by the addition of AMP, as also found for δ-polymerase by Byrnes et al. (1977). At a concentration of 5.0 mM AMP, the initial rate of δ I exonuclease was inhibited by 85%, compared to the initial rate without added AMP. The DNA polymerase δ II associated exonuclease was inhibited by a similar factor of 75%. Both of these findings further indicate a relationship between the enzyme species we have purified and the previously described DNA polymerase δ (Byrnes et al., 1977).

Effects of DNA Polymerase Inhibitors on the Two Forms of δ-Polymerase. Table II depicts the responses of DNA polymerases δ I and II to several inhibitors of mammalian replicative DNA polymerases. Both forms of δ-polymerase are sensitive to the α-polymerase inhibitors aphidicolin and *N*-ethylmaleimide. The monoclonal antibody SJK-287-38 developed against human KB cell α polymerase by Tanaka et al. (1983) can inhibit calf α-polymerase more than 95% (Wahl et al., 1984). However, it is not able to inhibit DNA polymerase δ I and shows only 35% inhibition of DNA polymerase δ II at a concentration that inhibits α polymerase more than 95%. Another type of α-polymerase-specific inhibitor, BuPdGTP (Wright & Dudyez, 1984), and the more recently synthesized dATP analogue BuAdATP both show inhibitory effects on DNA polymerases δ I and II. At a concentration of 100 μM for either compound, DNA polymerase δ I is inhibited 80%, while at this concentration DNA polymerase δ II is inhibited only 20%. We find that α-polymerase purified by the method of Wahl et al. (1984) is significantly more sensitive than either of the δ forms and is inhibited 95% at the same concentration of these compounds (data not shown).

Differential Template Activity. Table III shows the results obtained when various templates are used as substrates for DNA polymerases δ I and II. A notable feature of this table is the relative inability of the DNA polymerase δ II to perform

Table III: Differential Template Activity[a]

template	IPD[b]	fraction of standard assay activity: DNA polymerase δ I	DNA polymerase δ II
poly(dA)·$(dT)_{16}$	8	1.19	1.23
	20	1.96	1.30
	50	1.21	1.61
	100	1.14	1.31
	150	1.00	1.00
	290	0.60	0.66
	400	0.61	1.09
	4000	0.07	0.31
poly(rA)·$(dT)_{16}$[c]	20	0.01	0.01
	150	0.00	0.01
poly(dT)·$(dA)_{16}$[d]			
−ATP	20	0.01	0.01
−ATP	150	0.01	0.01
−ATP	no primers	0.01	0.01
+ATP	20	0.05	0.01
+ATP	150	0.05	0.01
+ATP	no primers	0.04	0.01
poly(dA-dT)[e]		0.262	0.01
tailed template[f]		0.36	0.36
activated calf thymus DNA[g]		1.02	0.03
denatured calf thymus DNA[h]		0.21	0.05
fd single-stranded DNA (+ATP)[i]		0.04	0.03
fd single-stranded DNA (+4 NTPs)[j]		0.09	0.02
singly primed fd DNA[k]		0.06	0.04
fd RF DNA[l]		0.02	0.02

[a] Assays were carried out as described under Materials and Methods with the deletion of the standard template and the addition of the indicated template to a concentration of 80 μM (in nucleotides). All assays were done in duplicate, averages were taken, and the resultant number of moles of total nucleotide incorporated was listed as a fraction of the standard assay. 2.2×10^{-2} unit of DNA polymerase δ I and 1.2×10^{-2} unit of DNA polymerase δ II were added per assay. For comparison purposes, the standard template of $(dA)_{4000-5000}$·$(dT)_{16}$ incorporation is listed as 1.00. [b] IPD is the average interprimer distance on the template given in nucleotides. [c] This template was made in a manner identical with the $(dA)_{4000-5000}$·$(dT)_{16}$ template, except poly(rA) was used as the template polymer. [d] $(dT)_{400}$·$(dA)_{16}$ primer-templates with the indicated interprimer distance were produced in the same manner as for the $(dA)_{4000-5000}$·$(dT)_{16}$ primer-templates. The ^3H label used in *a* was changed to 10 Ci mmol^{-1} [^{3}H]dATP at a concentration of 50 μM. ATP was either deleted or added at 2.5 mM as indicated. [e] The result listed is that obtained with poly(dA-dT) obtained from the Midland Certified Reagent Co. 50 μM dATP was added in addition to the components of the standard assay. When polymer was obtained from Miles Pharmaceuticals, relative synthesis was 0.04 for δ I and 0.03 for δ II. The relative synthesis for DNA polymerase δ I and II was 0.02 and 0.01, respectively, using poly(dA-dT) obtained from Boehringer Mannheim. [f] $(dT)_{4000-5000}$ covalently tailed with oligo(dT) as described under Materials and Methods was the DNA substrate. [g] Conditions were the same as for the activated calf thymus DNA assay described under Materials and Methods except the concentration of primer-template was reduced to 80 μM. [h] Denatured calf thymus DNA was obtained by heating a 5.0 mM stock solution of calf thymus DNA in 10 mM Tris-HCl, pH 7.5, and 100 μM EDTA to 95 °C for 10 min and then chilling on ice. Conditions used were the same as in *g* except for deletion of the activated calf thymus DNA template. [i] Conditions used were the same as in *g* except for the omission of the calf thymus DNA template and the addition of fd single-stranded DNA. [j] Conditions used were the same as in *i* except for the addition of CTP, GTP, and UTP to 1.0 mM each. [k] Conditions were the same as in *i* except that the DNA substrate was changed to the specifically primed fd DNA substrate described under Materials and Methods. [l] Conditions used were the same as in *g* except that the DNA substrate was changed to double-stranded fd RF DNA.

significant synthesis on any substrate other than $(dA)_{4000-5000}$·$(dT)_{16}$. Synthesis on this primer-template by the δ II polymerase is also less sensitive to the decrease of $(dT)_{16}$ primer density than is synthesis by the δ I polymerase. When the mean interprimer distance is 4000 nucleotides, synthesis by the δ II polymerase is 31% of that in the standard assay. Under these same conditions, synthesis by the δ I enzyme dropped to 7% of its value in the standard assay.

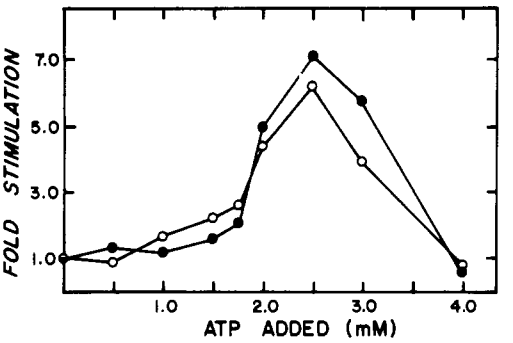

FIGURE 8: ATP titration of DNA polymerase δ I and DNA polymerase δ II. ATP stimulation was monitored under the conditions outlined for the standard assay except that ATP was adjusted to the level shown and the interprimer distance changed to 400 nucleotides. After 10 min of incubation, incorporation was assayed as described. The response of DNA polymerase δ I (○) and DNA polymerase δ II (●) is as indicated. Each assay contained 2.3×10^{-2} unit of DNA polymerase δ I and 1.4×10^{-2} unit of DNA polymerase δ II.

DNA polymerase δ I is able to efficiently synthesize on a number of templates. In fact, the rate of nucleotide incorporation in assays using activated calf thymus DNA as the enzyme substrate is slightly greater than that in the standard assay which utilizes the homopolymeric DNA template. Neither polymerase was able to utilize the mixed primer-templates of poly(rA)·$(dT)_{16}$, thus characterizing these enzymes as different from the 10.1S α-polymerase purified by Masaki et al. (1978), and from γ-polymerase (Kornberg, 1980). Alternating dA-dT polymers from several suppliers were assayed as substrates for synthesis activity. Only polymer from Midland Certified Reagent Co. proved to be an effective substrate, and this only by the DNA polymerase δ I. Even when the alternating dA-dT was activated by treatment with pancreatic DNase, as in the protocol to produce activated calf thymus DNA, no increase in activity was noted with the DNA polymerase δ II (data not shown).

Stimulation of the Two Forms of DNA Polymerase δ by ATP. Both forms of DNA polymerase δ are stimulated by the addition of millimolar concentrations of ATP as shown in Figure 8. The two forms are identical with respect to their degree of stimulation (7-fold) as well as the optimum concentration of ATP necessary for this stimulation (2.5 mM). This ATP-dependent stimulation was found to affect two kinetic parameters. The K_m for the DNA substrate was reduced and the processivity of the enzymes increased. Lineweaver–Burk analysis of synthesis on the homopolymeric DNA template, as described under Materials and Methods, demonstrated the effect of the addition of ATP on the K_m of both forms of DNA polymerase δ. The K_m of DNA polymerase δ I is depressed from 220 to 18 μM for its DNA substrate by the addition of ATP at 2.5 mM. DNA polymerase δ II enzyme shows a similar shift in K_m from 400 to 25 μM with the addition of 2.5 mM ATP. The addition of 2.5 mM ATP had no effect on the maximum reaction velocity (V_{max}) for either DNA polymerase δ I or DNA polymerase δ II, as determined from *y*-intercept values.

Figure 9 depicts the shift in the size of products synthesized processively by these two forms of the DNA polymerase. This analysis is performed in template excess such that the size of the extended primers represents the extent of processive synthesis during a single interaction of polymerase and DNA

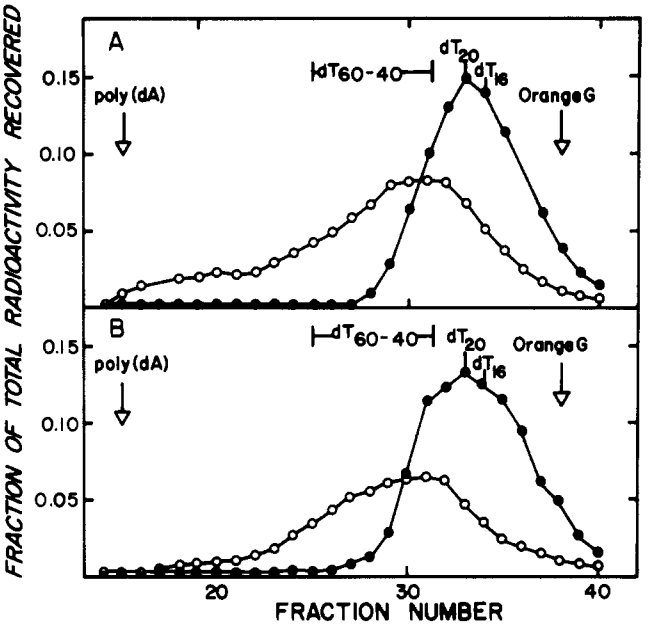

FIGURE 9: Processivity determination of DNA polymerse δ I and DNA polymerase δ II via Bio-Gel A-5M gel filtration. Reactions and subsequent manipulations were carried out as described under Materials and Methods. Panel A shows the resultant product profile obtained for DNA polymerase δ I without ATP [(●) 1.1×10^{-2} mol of nucleotide incorporated/mol of 3′OH termini present] or with the addition of ATP at 2.5 mM [(○) 1.8×10^{-2} mol of nucleotide incorporated/mol of 3′OH present]. Panel B depicts the result obtained when DNA polymerase δ II was allowed to synthesize processively when ATP was either not added [(●) 1.1×10^{-2} mol of nucleotide incorporated/mol of 3′OH termini present] or added at 2.5 mM [(○) 2.9×10^{-2} mol of nucleotide incorporated/mol of 3′OH termini present].

template (Fay et al., 1980). Both DNA polymerases δ I and II produce similar sized products in the absence of ATP, representing processive addition of about four nucleotides to the primer terminus. In the presence of ATP, 30–40 nucleotides are added per polymerase–template encounter.

Discussion

We have been able to purify two novel high molecular weight polymerases from calf thymus. These enzymes most resemble previously described DNA polymerase δ (Byrnes, 1984; Lee et al., 1984) since they have an associated 3′–5′-exonuclease activity. They share other properties described below. These enzymes are higher molecular weight than the δ-polymerases previously reported. Our results derive from the use of different, potentially less disruptive purification methods and the inclusion of multiple protease inhibitors. The techniques we have used were initially chosen because they retained the integrity of the polymerase III holoenzyme complex through its purification from *Escherichia coli* (McHenry & Kornberg, 1977).

Of note is that both polymerase species obtained (designated DNA polymerase δ I and DNA polymerase δ II) have, as a constitutive part of the enzyme complex, an active DNA-dependent RNA polymerase or primase activity (Conaway & Lehman, 1982). Both polymerases have other properties in common with mammalian α DNA polymerases in that they are both inhibited by aphidicolin and *N*-ethylmaleimide. Both polymerases are sensitive to the drugs N^2-(*p*-*n*-butylphenyl)-dGTP and N^2-(*p*-*n*-butylphenyl)dATP, but inhibition requires higher levels than those required to inhibit α-polymerase (Khan et al., 1984). This is a similar result to that found with rabbit α- and δ-polymerases (Byrnes, 1985). Antibody developed against and capable of inactivating human KB cell α-polymerase does not significantly inhibit either δ I or δ II polymerase at levels where α-polymerase is inhibited by 50%.

The two peaks of δ-polymerase activity eluted from the column of Bio Rex-70 resin represent approximately 50% of the total polymerase activity originally present in calf thymus postmicrosomal supernatant solution as determined in the standard assay. The fact that homopolymeric DNA substrates are rarely used in most laboratories for the primary assays during polymerase purification may account for the lack of previous detection of δ II polymerase, which is virtually inactive on other commonly used templates. This may also partially account for the infrequency with which high molecular weight exonuclease-containing mammalian DNA polymerases have been reported. However, there has been some investigation of these polymerases in several systems: calf thymus (Lee et al., 1980), fetal calf thymus (Lee et al., 1984), rabbit (Byrnes, 1984), and mouse (Chen et al., 1979).

The two types of DNA polymerase δ that we have purified differ from each other in several characteristics. DNA polymerase δ II is significantly higher in molecular weight (M_r 290 000) than DNA polymerase δ I (M_r 240 000). The lower molecular weight enzyme displays much greater activity on natural DNA substrates as compared to the δ II enzyme, when activity is standardized to the homopolymer assay. Activated calf thymus DNA, denatured calf thymus DNA, alternating dA-dT, and fd DNA all proved to be effective substrates of DNA polymerase δ I.

DNA polymerase δ II shows comparatively more activity on low primer density homopolymeric DNA templates. This implies an ability to more efficiently find and productively bind 3′-DNA termini, as compared to δ I polymerase.

Both DNA polymerase δ I and DNA polymerase δ II have the ability to synthesize RNA de novo. The primase activity associated with the δ-polymerase I is active on either fd single-stranded DNA or $(dT)_{400}$. The DNA polymerase δ II primase is active almost exclusively on $(dT)_{400}$ and ineffective at priming fd single-stranded DNA, causing only a 2-fold increase in nucleotide synthesis on addition of ATP rather than the 10-20-fold observed with the $(dT)_{400}$ template (data not shown).

Both DNA polymerases δ I and II are stimulated 7-fold by the addition of 2.5 mM ATP. ATP stimulation assays have been performed as previously described (Wierowski et al., 1983) using $(dA)_{4000-5000}$·$(dT)_{16}$, a template on which the stimulation cannot be attributed to utilization of ATP for priming. The effects of ATP on both enzyme forms are identical in that the K_m values for the DNA substrate are decreased and the processivities of the enzymes are increased. Both enzymes add about 4 nucleotides after binding to a primer terminus in the absence of ATP and approximately 30–40 nucleotides per binding event in the presence of 2.5 mM ATP. A similar result was obtained (Wierowski et al., 1983; Lawton et al., 1984) when DNA polymerase α A form was used (Holmes et al., 1974, 1975).

The molecular weight of calf DNA polymerase δ I is almost identical with that of calf DNA polymerase α, 240 000 in the presence of Ampholine and more than 430 000 in its absence (data not shown). Both the α and δ I polymerases are able to efficiently utilize poly(dA) templates which have been primed with oligo(dT). They are stimulated by ATP by apparently the same mechanism, since the same kinetic parameters are affected (Wahl et al., 1984). These results suggest that DNA polymerase δ I may consist of an α-type DNA polymerase with an associated exonuclease. This exonuclease

could be lost during previously reported purifications of α-polymerase (Holmes et al., 1974, 1975; Grosse & Krauss, 1981) either by subunit removal or by proteolysis.

The associated nuclease may alter the structure of the antigenic site, interfering with the binding of the monoclonal anti-α-polymerase antibody that we have used in our attempts to inactivate DNA polymerase δ I. Since the antibody has been produced from a polymerase lacking an exonuclease activity (Tanaka et al., 1983), it is likely that the nuclease–polymerase complex is not efficiently recognized. This explanation is consistent with the inability of immunoaffinity procedures to purify DNA polymerase δ I (Wahl et al., 1984). When many more anti-α-polymerase antibodies are available, it may be possible to identify portions of the α and δ enzymes that have common antigenic sites.

DNA polymerase δ II has very little activity on DNA templates composed of the four deoxynucleoside monophosphate residues and slight sensitivity to the anti-α-polymerase antibody. Yet it has several features common to the δ I and α enzyme forms: high molecular weight, primase activity, the ability to be stimulated by ATP, similar processivity, and similar inhibitor sensitivity. DNA polymerase δ II may share structures, or one or more subunits, with DNA polymerases α and δ I but could still be unparalleled.

As yet, neither the δ I nor the δ II form has been purified in sufficient quantities for peptide analysis. In fact, specific subunit assignments cannot be made because SDS–polyacrylamide gel electrophoretic patterns of both enzymes show several major bands between molecular weights of 250000 and 40000. Some may be contaminants. The M_r 245000 polypeptide that we find common to the δ-polymerases and the immunoaffinity-purified α-polymerase (Wahl et al., 1984) is also similar in size to the proposed active-site subunit of DNA polymerase α from monkey kidney cells (Karawya et al., 1984). The question of the structural relationship of the subunits cannot be immediately resolved.

Our work to this point indicates that exonuclease-containing polymerases are present in sufficient quantities to have an important role in chromosomal replication. It is of particular note that the DNA polymerase δ I form represents approximately 30–40% of the polymerase activity present in clarified calf thymus supernatant solutions capable of synthesis on activated calf thymus DNA templates. This estimation is based on the quantity of activity on calf thymus DNA templates which elutes with DNA polymerase δ I from the Bio Rex-70 column, as compared to the activity on activated calf thymus DNA present in the unfractionated supernatant solution. The presence of an α-polymerase-associated exonuclease could account for the high fidelity of mammalian DNA replication, on the order of 10^{-9}–10^{-11} misincorporated nucleotides per nucleotide replicated in vivo (Loeb & Kunkel, 1982). This is inconsistent with the high error frequencies in vitro, on the order of one mistake made per 10^4 nucleotides incorporated, found in tests of highly purified, exonuclease-free α-polymerase from mammalian cells (Abbotts & Loeb, 1984). Whether all replicative DNA polymerases in vivo have an associated exonuclease, some of which is always lost during purification, will be a difficult question to answer. Our current efforts are directed toward the separation and isolation of the exonuclease from each of the δ-polymerases. Subsequently, we will attempt reconstitution with each nuclease-free δ-polymerase and with the exonuclease-lacking immunoaffinity-purified α-polymerase.

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Registry No. ATP, 56-65-5; DNA polymerase, 9012-90-2; 3′–5′-exonuclease, 79393-91-2; primase, 64885-96-7; poly(dA)·$(dT)_{16}$, 24939-09-1.

REFERENCES

Abbotts, J., & Loeb, L. A. (1984) *J. Biol. Chem. 259*, 6712–6714.

Baril, E., & Laszlo, J. (1974) *Adv. Enzyme Regul. 9*, 183–204.

Beck, E., Sommer, R., Auerswald, E. A., Kurz, C., Zink, B., Osterburg, G., & Schaller, H. (1978) *Nucleic Acids Res. 5*, 4495–5003.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Byrnes, J. J. (1984) *Mol. Cell. Biochem. 62*, 13–24.

Byrnes, J. J. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1599A.

Byrnes, J. J., Downey, K. M., Black, V. L., & So. A. G. (1976) *Biochemistry 15*, 2817–2823.

Byrnes, J. J., Downey, K. M., Que, B. G., Lee, M. Y. W., Black, V. L., & So, A. G. (1977) *Biochemistry 16*, 3740–3746.

Chen, Y., Bohn, E. W., Planck, J. R., & Wilson, J. H. (1979) *J. Biol. Chem. 254*, 11678–11687.

Conaway, R. C., & Lehman, I. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2523–2527.

Faust, E. A., Gloor, G., MacIntyre, M.-F., & Nagy, R. (1984) *Biochim. Biophys. Acta 781*, 216–224.

Fay, P. J., Johanson, K. O., McHenry, C. S., & Bambara, R. A. (1981) *J. Biol. Chem. 256*, 976–983.

Grosse, F., & Krauss, G. (1981) *Biochemistry 20*, 5470–5475.

Holmes, A. M., Hesslewood, I. P., & Johnston, I. R. (1974) *Eur. J. Biochem. 43*, 487–499.

Holmes, A. M., Hesslewood, I. P., & Johnston, I. R. (1975) *Nature (London) 255*, 420–422.

Kaguni, L. S., DiFrancesco, R. A., & Lehman, I. R. (1984) *J. Biol. Chem. 259*, 9314–9319.

Karawya, E., Swack, J., Albert, W., Fedorko, J., Minna, J. D., & Wilson, S. H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7777–7781.

Kato, K., Goncalves, J. M., Houts, G. E., & Bollum, F. J. (1967) *J. Biol. Chem. 242*, 2780–2789.

Khan, N. N., Wright, G. E., Dudycz, L. W., & Brown, N. C. (1984) *Nucleic Acids Res. 12*, 3695–3706.

Kornberg, A. (1980) in *DNA Replication*, pp 201–229, W. H. Freeman, San Francisco, CA.

Laemmli, U. K. (1970) *Nature (London) 226*, 680–685.

Lawton, K. G., Wierowski, J. V., Schechter, S., Hilf, R., & Bambara, R. A. (1984) *Biochemistry 23*, 4294–4300.

Lee, M. Y. W. T., & Whyte, W. A. (1984) *Anal. Biochem. 138*, 291–297.

Lee, M. Y. W. T., Tan, C., So, A. G., & Downey, K. M. (1980) *Biochemistry 19*, 2096–2101.

Lee, M. Y. W. T., Tan, C., Downey, K. M., & So, A. G. (1984) *Biochemistry 23*, 1906–1913.

Loeb, L. A., & Kunkel, T. A. (1982) *Annu. Rev. Biochem. 51*, 429–457.

Lynch, W. E., Surrey, S., & Lieberman, J. (1975) *J. Biol. Chem. 250*, 8179–8183.

Maniatis, T., Fritch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, pp 97–148, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marshall, T. (1984) *Anal. Biochem. 136*, 340-346.
Masaki, S., Koiwai, O., & Yoshida, S. (1982) *J. Biol. Chem. 257*, 7172-7177.
Matson, S. W., Fay, P. J., & Bambara, R. A. (1980) *Biochemistry 19*, 2089-2096.
McHenry, C. S., & Kornberg, A. (1977) *J. Biol. Chem. 252*, 6478-6484.
Moss, L. G., Moore, J. P., & Chan, L. (1981) *J. Biol. Chem. 256*, 12655-12658.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346-362.
Tanaka, S., Hu, S., Wang, T. S., & Korn, D. (1982) *J. Biol. Chem. 257*, 8386-8390.
Uyemura, D., & Lehman, I. R. (1976) *J. Biol. Chem. 251*, 4078-4084.
Wahl, A. F., Kowalski, S. P., Harwell, L. W., Lord, E. M., & Bambara, R. A. (1984) *Biochemistry 23*, 1895-1899.
Wierowski, J. V., Lawton, K. G., Hockensmith, J. W., & Bambara, R. A. (1983) *J. Biol. Chem. 258*, 6250-6254.
Wright, G. E., & Dudycz, L. W. (1984) *J. Med. Chem. 27*, 175-181.

Transcription by T7 RNA Polymerase Is Not Zinc-Dependent and Is Abolished on Amidomethylation of Cysteine-347†

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ABSTRACT: T7 RNA polymerase has been purified to homogeneity from an overproducing clone of *Escherichia coli* containing pAR1219. Preparations have a zinc content as low as 0.01 mol/mol of enzyme and a high specific activity, 300 000–500 000 units/mg. There are no intrinsic zinc sites. Furthermore, extrinsic Zn^{2+} does not function as an activator. Supplementation of the assay mix with up to 5 mM ethylenediaminetetraacetic acid has little effect on activity while added Zn^{2+} is strongly inhibitory at concentrations above 10 μM. This monomeric RNA polymerase is not a zinc metalloenzyme, unlike its multimeric bacterial counterparts. Titration of the urea-denatured protein with 5,5′-dithiobis(2-nitrobenzoic acid) reveals that all 12 Cys residues are present in the free sulfhydryl form, 5 of which are readily accessible to reagent in the native enzyme. More preferential labeling of the sulfhydryls can be achieved with low concentrations of [^{14}C]iodoacetamide, where inactivation of the enzyme proceeds with incorporation of approximately 1.2 mol of [^{14}C]iodoacetamide/mol of polymerase. Amidomethylation primarily occurs at Cys-347, with lesser reaction at Cys-723 and Cys-839. Cys-347 and Cys-723 are in segments of the primary sequence containing numerous basic residues. These same segments have previously been implicated in promoter binding, suggesting that both residues are located within or near the active site region.

While considerable progress has been made toward understanding the molecular basis of transcription (von Hippel et al., 1984), the identities of the RNA polymerase functional groups involved in promoter recognition and catalysis remain unknown. Zinc has been postulated to play a role in catalysis, since all RNA polymerases analyzed to date have been reported to contain at least 1 mol of zinc/mol of enzyme (Mildvan & Loeb, 1979; Coleman, 1983). Amongst the simplest of RNA polymerases is the monomeric protein encoded by gene 1 of the T7 bacteriophage (M_r 98 856) and isolated from T7-infected *Escherichia coli* (Chamberlin et al., 1970; Niles et al., 1974; Moffatt et al., 1984). Significant amounts of zinc were reported to be associated with extensively purified preparations of this enzyme (Coleman, 1974). Recently, the study of T7 RNA polymerase has taken a large advance with the successful cloning and expression of gene 1 by J. J. Dunn, F. W. Studier, and colleagues (Davanloo et al., 1984). It became clear during our own isolation of the overproduced protein that Zn was not present in sufficient concentration to satisfy even a 1:1 stoichiometry. This finding prompted a reinvestigation of the relationship between Zn and the enzyme activity.

T7 RNA polymerase is known to be inhibited by sulfhydryl-selective reagents (Chamberlin & Ring, 1973; Oakley et al., 1975). A previous study from our laboratory has reported that reaction with a large excess of [^{14}C]iodoacetamide resulted in the incorporation of 1 mol of reagent/mol of enzyme, causing complete inactivation of the protein (Oakley et al., 1975). The selectively amidomethylated polymerase was found to interact with DNA fragments containing a T7 promoter, but in an aberrant manner and without initiation of RNA synthesis (Oakley et al., 1975, 1979; Strothkamp et al., 1980). These observations are consistent with, but not sufficient proof of, the presence of a sulfhydryl group within the active site of the enzyme. As a first step toward clarifying this issue, we have identified the site(s) of amidomethylation by peptide analysis after reaction with [^{14}C]iodoacetamide. The results of earlier studies of sulfhydryl content and reactivity are updated in the light of new, more reliable data obtained for the overproduced protein.

Materials and Methods

T7 RNA polymerase was prepared from *E. coli* strain BL 21 containing plasmid pAR1219 (Davanloo et al., 1984),

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