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Further Studies on Calf Thymus DNA Polymerase δ Purified to Homogeneity by a New Procedure†

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ABSTRACT: DNA polymerase δ from calf thymus has been purified to apparent homogeneity by a new procedure which utilizes hydrophobic interaction chromatography with phenyl-Sepharose at an early step to separate most of the calcium-dependent protease activity from DNA polymerases δ and α. The purified enzyme migrates as a single protein band on polyacrylamide gel electrophoresis under nondenaturing conditions. The sedimentation coefficient of the enzyme is 7.9 S, and the Stokes radius is 53 Å. A molecular weight of 173K has been calculated for the native enzyme. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the homogeneous enzyme reveals two polypeptides of 125 and 48 kDa. This subunit structure differs from that of DNA polymerase δ prepared by our previous procedure, which was composed of subunits of 60 and 49 kDa [Lee, M. Y. W. T., Tan, C.-K., Downey, K. M., & So, A. G. (1981) *Prog. Nucleic Acid Res. Mol. Biol. 26*, 83–96], suggesting that the 60-kDa polypeptide may have been derived from the 125-kDa polypeptide during enzyme purification, possibly as the result of cleavage of an unusually sensitive peptide bond. DNA polymerse δ is separated from DNA polymerase α by hydrophobic interaction chromatography on phenyl-Sepharose; DNA polymerase δ is eluted at pH 7.2 and DNA polymerase α at pH 8.5. DNA polymerase δ can also be separated from DNA polymerase α by chromatography on hydroxylapatite; DNA polymerase α binds to hydroxylapatite in the presence of 0.5 M KCl, whereas DNA polymerase δ is eluted at 90 mM KCl. DNA polymerase δ is associated with a 3′- to 5′-exonuclease activity, but it is devoid of endonuclease or 5′- to 3′-exonuclease activities. The polymerase activity of DNA polymerase δ is inhibited by aphidicolin, as is the 3′- to 5′-exonuclease activity when the substrate is double-stranded DNA. Aphidicolin does not inhibit exonuclease activity on single-stranded DNA. The coordinated inhibition of both polymerase and exonuclease activities of DNA polymerase δ by aphidicolin is consistent with our observation that both activities reside on the same protein molecule. The present findings are also consistent with the suggestion that the binding of aphidicolin to the enzyme requires the formation of a template-primer–DNA polymerase complex [Huberman, J. A. (1981) *Cell (Cambridge, Mass.) 23*, 647–648].

During the past few years, significant progress has been made on the purification and characterization of high molecular weight species of DNA polymerase from higher eukaryotes (Kornberg, 1980, 1982; Fry, 1982). DNA polymerase α and δ species have been purified to apparent homogeneity from several sources; however, the molecular weights of the native enzymes as well as their subunit composition remain controversial.

A high molecular weight (120K–180K), catalytically active polypeptide has been identified in DNA polymerase α preparations purified from *Drosophila* (Villani et al., 1980; Kaguni et al., 1983) and rat liver (Mechali et al., 1980), whereas no large polypeptide has been found in DNA polymerase α preparation purified from KB cells, rather the peak of polymerase activity on polyacrylamide gel electrophoresis under nondenaturing conditions is found to be associated with a quartet of polypeptides with assigned masses of 70, 65, 59 and 55 kDa[1] (Fisher & Korn, 1977; Filpula et al., 1982). Both high molecular weight and low molecular weight polypeptides have been found to contain polymerase activity in DNA polymerase α species from calf thymus (Holmes et al., 1976; Grummt et al., 1979; Hubscher et al., 1981; Grosse & Krause, 1982) and mouse myeloma (Chen et al., 1979; Karawya & Wilson, 1982).

DNA polymerase δ from rabbit bone marrow has been reported to be a single polypeptide of 122 kDa (Goscin & Byrnes, 1982), whereas we have found that DNA polymerase δ from calf thymus is composed of two subunits of 49 and 60 kDa (Lee et al., 1981). Furthermore, mouse myeloma DNA polymerase α_1, which, like DNA polymerase δ, has an associated 3′- to 5′-exonuclease activity, is composed of subunits of 48 and 52 kDa (Chen et al., 1979).

Proteolysis during enzyme purification has been suggested as a possible explanation for the lack of a large polypeptide in several α polymerases (Banks et al., 1980; Spanos et al., 1981; Grosse & Krauss, 1981). We have been concerned about the possibility that proteolysis during purification of DNA polymerase δ from calf thymus might be responsible for the absence of a high molecular weight subunit in this po-

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[1] Abbreviations: kDa, kilodalton; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

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lymerase. The possible presence of calcium-dependent proteases was our primary concern, since we, like many other investigators, use a hydroxylapatite chromatography purification step. Indeed, we have found that there is a considerable amount of calcium-dependent protease present in calf thymus homogenates.

In this paper we shall describe a purification protocol that removes most of the protease contaminants from both DNA polymerases α and δ at a very early step of enzyme purification. This new method of purification is suitable for large-scale purification of DNA polymerase δ from calf thymus and may be applicable to the purification of other high molecular weight DNA polymerases. By the use of this method, and by including several protease inhibitors of various specificities in the homogenization buffer, we have purified DNA polymerase δ to near homogeneity and demonstrated that it contains a large (125-kDa) polypeptide which was not detected in the preparations of DNA polymerase δ purified by our previous procedure. Additionally we shall report some of the physicochemical and enzymatic properties of the enzymes prepared by this new procedure.

Materials and Methods

Unlabeled deoxynucleoside triphosphates (dNTP) and oligo(dA)–cellulose were purchased from P-L Biochemicals. [^{3}H]dTTP (50–80 Ci/mmol) and [^{3}H]dCTP (15–30 Ci/mmol) were obtained from either ICN Pharmaceuticals or New England Nuclear Corp. [γ-^{32}P]ATP was from New England Nuclear. Poly(dA-dT) was purchased from Boehringer Mannheim; other synthetic polydeoxyribonucleotides, oligodeoxyribonucleotides, and polyribonucleotides were from either P-L Biochemicals or Collaborative Research. Fetal calf thymus glands were obtained from Texas Biological Supply Specialists, Inc. DEAE-cellulose (DE-52) and phosphocellulose (P11) were purchased from Whatman, and phenyl-Sepharose CL-4B, Sephacryl S-300, and DEAE-Sepharose CL-6B were obtained from Pharmacia Fine chemicals. Calf thymus DNA was purchased from Worthington Biochemical Corp. Acrylamide, methylenebis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine (Temed), and Coomassie brilliant blue were purchased from Bio-Rad Laboratories. Ampholytes were purchased from LKB. Benzamidine hydrochloride, pepstatin, leupeptin, soybean trypsin inhibitor, and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. Bovine serum albumin (BSA) was from Pentex, and dithiothreitol was from Calbiochem. Aphidicolin was a gift of Dr. A. H. Todd of Imperial Chemical Industries, Macclesfield, England. Molecular size markers and PM2 DNA were obtained from Boehringer Mannheim. Polynucleotide kinase was from Bethesda Research Labs.

Poly(dA-dT)–cellulose was prepared as described by Moss et al. (1981). Hydroxylapatite was prepared by the method of Muench (1971). [^{3}H]d(T)$_{50}$ was synthesized with terminal deoxynucleotidyltransferase by using (dT)$_4$ as primer and [^{3}H]dTTP (200–300 cpm/pmol) as previously described (Que et al., 1978). Poly(dA-dT), labeled at the 3′-termini with [^{3}H]dTMP, was prepared as previously described (Byrnes et al., 1977), and d(T)$_{600}$, labeled at the 5′-termini with ^{32}P was prepared according to Livingston et al. (1975). Activated calf thymus DNA was prepared according to Fansler & Loeb (1974). Succinylated BSA was prepared according to Mellgren et al. (1979).

DNA Polymerase Assays. The standard assay for DNA polymerase δ utilized poly(dA-dT) as template-primer. The reaction mixture contained, in a final volume of 0.125 mL, 0.028 A_{260} unit of poly(dA-dT), 40 mM HEPES buffer, pH 6.5, 10 mM KCl, 0.1 mM $MnCl_2$ or 1 mM $MgCl_2$, 0.04 mM dATP, 100 cpm/pmol [^{3}H]dTTP, 0.2–0.4 unit of DNA polymerase; 0.01 mg of BSA, and 2% glycerol. After incubation at 37 °C for 15 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate (TCA–PP$_i$). The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976).

When poly(dG-dC) was used as template-primer, the reaction mixture was identical with that for poly(dA-dT) synthesis except for the use of poly(dG-dC) instead of poly(dA-dT) and 0.04 mM each of dGTP and [^{3}H]dCTP, 100 cpm/pmol, instead of dATP and [^{3}H]dTTP.

When either poly(dA)/oligo(dT) or poly(rA)/oligo(dT) was used as template-primer, the reaction mixtures were identical with the poly(dA-dT) assay except for the template-primer and the absence of dATP. The size of the oligo(dT) primer was 12–18 nucleotides.

When activated calf thymus DNA was used as template-primer, the reaction mixture contained, in a final volume of 0.125 mL, 0.036 A_{260} unit of activated calf thymus DNA, 60 mM KCl, 0.04 mM each of dATP, dGTP, dCTP, and [^{3}H]-dTTP, 100 cpm/pmol, 40 mM HEPES buffer, pH 7.0, 0.1 mM $MnCl_2$ or 6 mM $MgCl_2$, 0.2–0.4 unit of DNA polymerase, 0.01 mg of BSA, and 2% glycerol (Byrnes et al., 1976).

DNA polymerase α was assayed by the method of Holmes et al. (1974).

One unit of DNA polymerase catalyzes the incorporation of 1 nmol of deoxynucleoside monophosphate/h at 37 °C.

3′- to 5′-Exonuclease Assays. The 3′- to 5′-exonuclease activity of DNA polymerase δ was assayed by measuring the release of [^{3}H]dTMP from either 3′-terminally labeled poly(dA-dT)–[^{3}H]dTMP or [^{3}H](dT)$_{50}$. When poly(dA-dT)–[^{3}H]dTMP was used as substrate, each reaction mixture contained, in a final volume of 0.06 mL, 66 mM HEPES buffer, pH 7.4, 0.2 mM $MnCl_2$, 3.3% glycerol, 0.1–0.2 unit of DNA polymerase δ, 0.004 mg of BSA, and 3.9×10^{-3} A_{260} unit of poly(dA-dT)–[^{3}H]dTMP, 2.2×10^6 cpm/A_{260} unit. When [^{3}H]d(T)$_{50}$ was used as substrate, each reaction mixture contained 0.002 mM [^{3}H]d(T)$_{50}$, 200–300 cpm/pmol, 25 mM HEPES buffer, pH 7.4, 0.005 mg of BSA, 5 mM $MgCl_2$, and 0.3 unit of DNA polymerase δ in a final volume of 0.06 mL. The reaction mixture was incubated at 37 °C, and 0.01-mL aliquots were applied to 2.4-cm circles of Whatman DE-81 paper after 0, 5, 10, 15, and 30 min of incubation. The circles were washed, dried, and counted as described previously (Que et al., 1978).

Other Nuclease Assays. The 5′- to 3′-exonuclease activity was assayed by measuring the release of [^{32}P]dTMP from [^{32}P]d(pT)$_1$–(dT)$_{600}$. Reaction mixtures were identical with those for 3′- to 5′-exonuclease activity with [^{3}H]d(T)$_{50}$ as substrate except that [^{32}P]d(pT)$_1$–(dT)$_{600}$, either alone or annealed to excess d(A)$_{100}$, was the substrate. Endonuclease activity was assayed by measuring the conversion of supercoiled PM2 DNA to relaxed circular or linear forms as determined by agarose gel electrophoresis (Johnson & Grossman, 1977). Reaction mixtures were identical with the 3′- to 5′-exonuclease assay with poly(dA-dT)–[^{3}H]dTMP as substrate except that PM2 DNA was the substrate.

Protease Assay. Protease activity was assayed by the method of Mellgren et al. (1979), which detects the formation of primary amines by measuring the fluorescence of their fluorescamine derivatives. Succinylated BSA was used as substrate, and glycine was used to construct a standard curve. One unit of protease activity is defined as the amount of

activity that releases 1 μmol of glycine-equivalent free amine from succinylated BSA per min at 30 °C.

Buffers. *Buffer A* was 10 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 10 mM KCl, 0.25 M sucrose, 1 mg/mL soybean trypsin inhibitor, 10 mM benzamidine hydrochloride, 0.005 mg/mL pepstatin, 0.05 mg/mL leupeptin, 0.8 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT). *Buffer B* was 40 mM Tris-HCl, pH 7.8, 50 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF. *Buffer C* was 20 mM potassium phosphate buffer (KP_i), pH 7.2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and 114 g/L ammonium sulfate. *Buffer D* was 20 mM KP_i, pH 7.2, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF. *Buffer E* was 40 mM Tris-HCl, pH 7.8, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF. *Buffer F* was 15 mM Tris-HCl, pH 8.5, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF. *Buffer G* was 20 mM KP_i, pH 7.0, 30% glycerol, 1 mM DTT, and 0.2 mM PMSF. *Buffer H* was 20 mM KP_i, pH 7.0, 30% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.2 mM PMSF.

Purification of DNA Polymerase δ. One kilogram of frozen fetal calf thymus was thawed, minced with a pair of scissors, and homogenized in 2 L of buffer A as previously described (Lee et al., 1980). After centrifugation at 25000*g* for 30 min, the supernatant was filtered through glass wool, made 40 mM in Tris-HCl, pH 7.8 (step 1 enzyme), and batch-absorbed to 2 L of DEAE-cellulose (DE-52), preequilibrated with buffer B. After stirring for 1 h at 4 °C, the DEAE-cellulose was packed into a column (9 × 35 cm) and washed with 3 column volumes of buffer B. DNA polymerase activity was eluted stepwise with 0.4 M KCl in buffer B. The pooled enzyme (550 mL; step 2 enzyme) was made 20% saturated in ammonium sulfate and loaded onto a phenyl-Sepharose column (5 × 10.5 cm), preequilibrated in buffer C. After the column was washed with 2–3 bed volumes of the same buffer, DNA polymerase δ activity was eluted stepwise with buffer D. Active fractions were pooled (293 mL; step 3 enzyme) and dialyzed against buffer E. DNA polymerase α activity was eluted from the phenyl-Sepharose column with buffer F. Active fractions were pooled, precipitated with ammonium sulfate (47.5% saturation), and stored at −70 °C for future studies.

The dialyzed step 3 enzyme was loaded onto a phosphocellulose column (5 × 7 cm), preequilibrated in buffer E containing 100 mM KCl, and washed with 2 column volumes of the same buffer. Enzyme activity was eluted with a 1000-mL linear KCl gradient (100–650 mM KCl in buffer E). Active fractions (235–320 mM KCl) were pooled (156 mL; step 4 enzyme) and dialyzed against buffer E. The dialyzed step 4 enzyme was loaded onto a DEAE-Sepharose column (3.2 × 7.8 cm), preequilibrated in buffer E containing 50 mM KCl. After the column was washed with 2–3 bed volumes of the same buffer, DNA polymerase δ activity was eluted with a 500-mL linear gradient (50–400 mM KCl in buffer E). Active fractions were pooled (40 mL; step 5 enzyme) and dialyzed against buffer G. The dialyzed step 5 enzyme was loaded onto a hydroxylapatite column (1 × 5 cm) preequilibrated in buffer G and washed with 3 column volumes of buffer G. DNA polymerase δ activity was eluted with a 120-mL linear gradient (0–550 mM KCl in buffer G). One-milliliter fractions were collected into tubes containing EDTA and EGTA such that the final concentration of each was 0.5 and 0.1 mM, respectively. The active fractions were pooled (30 mL; step 6 enzyme) and dialyzed against buffer H. Dialyzed step 6 enzyme was loaded onto a poly(dA-dT)–cellulose column (1 × 3 cm) preequilibrated in buffer H at 30 mL/h. After the column was washed with 3 column volumes of buffer H, DNA polymerase δ was eluted stepwise with 0.1 M KP_i, pH 7.0, in buffer H. Active fractions were pooled (4.5 mL; step 7 enzyme), dialyzed against buffer H, and loaded onto an oligo(dA)–cellulose column (1 × 7 cm) preequilibrated in buffer H. After the column was washed with 2–3 column volumes of buffer H, the enzyme was eluted stepwise with 0.2 M KCl in buffer H. Active fractions were pooled, bacitracin was added to 0.1 mg/mL, and the enzyme was stored at −70 °C.

Polyacrylamide Gel Electrophoresis under Nondenaturing Conditions. Electrophoresis was carried out with 5% nondenaturing cylindrical gels (5 × 100 mm) according to Fisher & Korn (1977). The enzyme was dialyzed against buffer containing 25 mM KP_i, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM mercaptoacetic acid, 20% ethylene glycol, and 30% sucrose prior to electrophoresis at 3 mA/tube for 3 h at 0 °C. Following electrophoresis the gels were either sliced into 42 slices with a manual Hoefer gel slicer, extracted, and assayed for both polymerase and exonuclease activities as previously described (Lee et al., 1981) or stained with Coomassie brilliant blue, destained with 7% acetic acid, and scanned with a laser densitometer equipped with an electronic integrator.

SDS–Polyacrylamide Gel Electrophoresis. Slab gels were prepared and run as described by Laemmli (1970) with a 4% stacking gel and a 10% separating gel. Prior to electrophoresis samples were precipitated overnight with 4 volumes of acetone and centrifuged for 2 min in an Eppendorf centrifuge, and the precipitate was dissolved in 65 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The samples were heated for 2 min at 100 °C. Standards of BSA, *Escherichia coli* RNA polymerase, and soybean trypsin inhibitor were run simultaneously. After electrophoresis the gels were either Coomassie stained or silver stained (Oakley et al., 1980).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was carried out according to a modification of the method of O'Farrell (1975). DNA polymerase δ was electrophoresed on a 5% cylindrical gel under nondenaturing conditions in the presence of Triton X-100 at 2 mA for 3 h. The cylindrical gel was then soaked in 5 mL of SDS-sample buffer for 1 h, placed on an SDS slab gel (4% stacking gel and 10% running gel), and electrophoresed at 20 mA for 4 h. The gel was silver stained according to Oakley et al. (1980).

Density Gradient Centrifugation. The sedimentation coefficient of DNA polymerase δ was determined as described by Martin & Ames (1961) in 4.8-mL glycerol gradients (10–30%) in 50 mM HEPES buffer, pH 7.4, 5 mM dithiothreitol, 0.1 mM EDTA, and 0.5 M KCl. Standards were BSA (4.4 S), aldolase (7.35 S), and catalase (11.3 S). After centrifugation at 44 000 rpm for 17 h in a SW-65 rotor at 4 °C, 0.17-mL fractions were collected from the bottoms of the tubes, and aliquots of each fraction were assayed for DNA polymerase and 3′- to 5′-exonuclease activities as described above.

Other Methods. Protein concentrations were determined by the method of Bradford (1976) with BSA as standard. Stokes radius and isoelectric point determinations were carried out as previously described (Lee et al., 1980).

Results

Purification of DNA Polymerase δ. Table I summarizes the purification of DNA polymerase δ from 1 kg of fetal calf

Table I: Purification of DNA Polymerase δ from Calf Thymus[a]

step	total protein (mg)	total activity (units)	specific activity (units/mg)	recovery (%)
(1) 25000*g* supernatant	13 000	269 000	21	100
(2) DEAE-cellulose	4 500	132 500	30	49
(3) phenyl-Sepharose	930	48 670	52	18
(4) phosphocellulose	28.6	21 900	766	8.1
(5) DEAE-Sepharose	5.9	12 900	2 186	4.8
(6) hydroxylapatite	0.35	5 160	14 743	1.9
(7) poly(dA-dT)–cellulose	0.104	2 510	24 100	0.9
(8) oligo(dA)–cellulose	0.033	1 580	47 900	0.6

[a] The purification procedure was carried out as described under Materials and Methods. DNA polymerase activity was followed by using poly(dA-dT) as template-primer because of the preference of DNA polymerase δ for alternating copolymer template-primers (see Table III). In the earlier stages of purification, a portion of the total poly(dA-dT) activity is due to other DNA polymerases, particularly DNA polymerase α, which is not separated from DNA polymerase δ until after chromatography on phenyl-Sepharose.

thymus. The values reported are averages of several preparations carried out over several months. We have scaled up the preparation to 3 kg of tissue by accumulating 1-kg preparations after step 2 and storing the enzyme at −70 °C in the presence of 20% saturated ammonium sulfate.

Because we were concerned about the likelihood that proteolysis may occur during purification of DNA polymerase δ, we homogenized the tissue in the presence of a "cocktail" of protease inhibitors of different specificities: PMSF, benzamidine hydrochloride, soybean trypsin inhibitor, pepstatin, and leupeptin, in addition to EDTA and EGTA. In subsequent steps PMSF, EDTA, and EGTA were included in all buffers. Futhermore, we followed neutral protease activity during the purification procedure. We specifically assayed for calcium-dependent neutral proteases, since we utilize a hydroxylapatite chromatography step which might activate calcium-dependent proteases. We were able to demonstrate a considerable amount of calcium-dependent protease activity in crude homogenates of calf thymus. Most of this activity was separated from DNA polymerase activity upon hydrophobic interaction chromatography.

We systematically examined the adsorption of DNA polymerase δ to a series of alkyl-Sepharoses in 20% saturated ammonium sulfate and found that DNA polymerase δ does not bind to butyl- or pentyl-Sepharose and binds too tightly to octyl-, decyl-, and dodecyl-Sepharose. Phenyl-Sepharose, however, was found to be suitable. Both DNA polymerases α and δ were adsorbed to phenyl-Sepharose in 20% saturated ammonium sulfate at pH 7.0, whereas the calcium-dependent protease activity was not absorbed. DNA polymerase δ activity was eluted with buffer at pH 7.2, and DNA polymerase α was eluted at pH 8.5 (data not shown). This has proven to be a key step, since we removed most of the neutral proteases and separated DNA polymerase δ from DNA polymerase α at an early step. The low levels of protease activity that remained after phenyl-Sepharose chromatography were reduced to nondetectable levels after phosphocellulose chromatography; thus, hydorxylapatite chromatography was delayed until after this step.

The small amount of DNA polymerase α activity that remained following DEAE-Sepharose chromatography was completely removed after chromatography on hydroxylapatite; DNA polymerase δ eluted from hydroxylapatite at 90 mM KCl whereas DNA polymerase α was not eluted by KCl up to a concentration of 0.5 M.

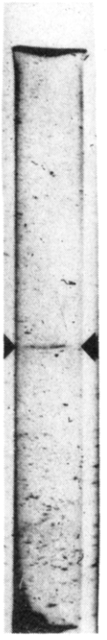

FIGURE 1: Polyacrylamide gel electrophoresis of DNA polymerase δ under nondenaturing conditions. Experimental details were as described in the text.

This procedure resulted in a 2280-fold purification of DNA polymerase δ with a yield of 0.6%. The specific activity of the step 8 enzyme was 47 900 units/mg. Our previous eight-step purification procedure resulted in an enzyme with a specific activity of 28 000 units/mg.

Further purification of DNA polymerase δ was carried out by "preparative" gel electrophoresis (Fisher & Korn, 1977). Aliquots of step 8 enzyme were electrophoresed on cylindrical gels under nondenaturing conditions. Following electrophoresis the gels were sliced, extracted, and assayed for enzyme activity. The relative mobilities of both the polymerase and 3′- to 5′-exonuclease activities were the same (R_m = 0.57) and somewhat higher than that of DNA polymerase δ prepared by our previous procedure (R_m = 0.53). The specific activity of the enzyme after preparative gel electrophoresis was estimated by analytical gel electrophoresis (Fisher & Korn, 1977). An aliquot of the enzyme (120 units) was dialyzed and rerun on a single 5 × 100 mm 5% nondenaturing gel (Figure 1). After staining, the amount of protein in the single protein band (R_m = 0.57) was estimated from a gel scan to be approximately 500 ng, on the basis of protein standard (1 μg of BSA) run in a parallel gel. By this quantitative method, the specific activity of the homogeneous enzyme was estimated to be 240 000 units/mg, assuming that protein concentration is proportional to Coomassie staining.

Characterization of the Physical Properties of DNA Polymerase δ. The isoelectric point of DNA polymerase δ (step 8 enzyme) was determined and found to be identical with the enzyme prepared by our previous procedure (p*I* = 5.5). A sedimentation coefficient of 7.9 S was obtained by sedimentation in a 10–30% glycerol gradient in the presence of 0.5 M KCl with catalase (11.3 S), aldolase (7.35 S), and BSA (4.4 S) as standards, and a Stokes radius of 53 Å was determined by gel filtration on Sephacryl S-300 with ferritin (61 Å), catalase (52 Å), aldolase (46 Å), BSA (35 Å), ovalbumin (27.3 Å), and cytochrome *c* (16.4 Å) as standards. Assuming a partial specific volume of 0.725, the molecular weight of DNA polymerase δ was calculated to be 173K.

The subunit structure of DNA polymerase δ was determined by SDS–polyacrylamide gel electrophoresis with BSA, *E. coli*

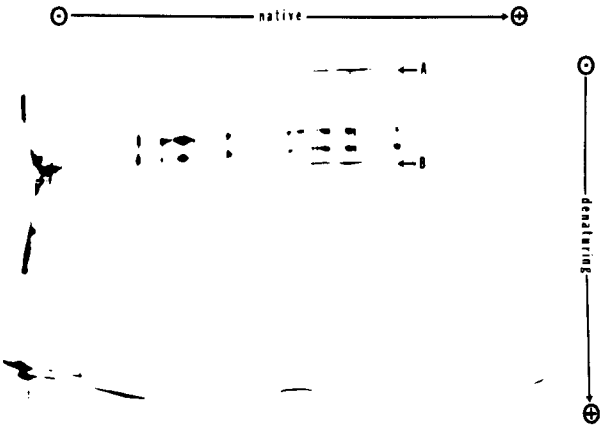

FIGURE 2: Two-dimensional gel electrophoresis of DNA polymerase δ. Experimental details are given under Materials and Methods. The subunits of DNA polymerase δ are indicated by (A) (125 kDa) and (B) (48 kDa). The other stains are artifacts that appear across the gel in the absence of any protein, probably due to the extreme sensitivity of silver nitrate stains.

Table II: Physical Properties of DNA Polymerase δ

Stokes radius (Å)	53
$s_{20,w}$ (S)	7.9
calcd M_r	173 000
f/f_0	1.44
pI	5.5
subunit M_r	125 000; 48 000

RNA polymerase, and soybean trypsin inhibitor as standards. The molecular weights of the subunits were estimated to be 125K and 48K. This is in contrast to the enzyme prepared by our previous procedure, which was composed of subunits of 60 and 48 kDa (Lee et al., 1981). Two-dimensional gel electrophoresis was also carried out in order to analyze the subunit structure of DNA polymerase δ according to a modification of the procedure of O'Farrell (1975). Polyacrylamide gel electrophoresis under nondenaturing conditions in a cylindrical gel (first dimension) was followed by SDS slab gel electrophoresis (second dimension). The results (Figure 2) indicate that homogeneous DNA polymerase δ is composed of two subunits of 125 and 48 kDa. These results suggest that proteolytic degradation of DNA polymerase δ may have been a significant problem in our previous purification procedure. The sum of the molecular weights of the two subunits corresponds to the molecular weight calculated for the native enzyme (173K), suggesting that the native enzyme is composed of one of each subunit. The physical properties of DNA polymerase δ purified by the new procedure are summarized in Table II.

Enzymatic Characterization of DNA Polymerase δ. The template-primer specificity of DNA polymerase δ is shown in Table III. The alternating copolymers poly(dA-dT) and poly(dG-dC) were nearly 3 times as active as activated calf thymus DNA and 10 times as active as poly(dA)/oligo(dT) at template:primer ratios of 1:1, 10:1, and 20:1. Poly(rA)/oligo(dT) had no template activity with DNA polymerase δ.

The pH optimum as well as the monovalent and divalent cation optima for both the polymerase and the 3′- to 5′-exonuclease activities of DNA polymerase δ were found to vary with the structure of the template-primer. For example, in Figure 3A is shown the effect of increasing KCl concentrations on the polymerase activity with either poly(dA-dT), poly(dG-dC), or activted calf thymus DNA as template-primer. The calf thymus DNA directed reaction was relatively insensitive to KCl concentration, whereas the poly(dA-dT)-directed reaction was slightly stimulated at low KCl concentrations and markedly inhibited at high KCl concentrations; e.g., at 100 mM KCl, the synthesis of poly(dA-dT) was inhibited over 80%. With poly(dG-dC) as template-primer, the optimal KCl concentration was 20–60 mM, and synthesis was about 70% inhibited at 100 mM KCl.

Table III: Template-Primer Specificity of DNA Polymerase δ [a]

template-primer	metal ion	dNMP incorporated (pmol/30 min)
poly(dA-dT)	0.1 mM Mn^{2+}	210
poly(dA-dT)	1.0 mM Mg^{2+}	180
poly(dG-dC)	0.2 mM Mn^{2+}	202
poly(dG-dC)	2.0 mM Mg^{2+}	129
activated calf thymus DNA	0.1 mM Mn^{2+}	78
activated calf thymus DNA	6.0 mM Mg^{2+}	56
poly(dA)/oligo(dT) (1:1)	0.1 mM Mn^{2+}	26
poly(dA)/oligo(dT) (10:1)	0.1 mM Mn^{2+}	19
poly(dA)/oligo(dT) (20:1)	0.1 mM Mn^{2+}	17
poly(rA)/oligo(dT) (1:1)	0.1 mM Mn^{2+}	<2
poly(rA)/oligo(dT) (10:1)	0.1 mM Mn^{2+}	<2

[a] Assays were carried out as described under Materials and Methods with the indicated template-primers and divalent cations.

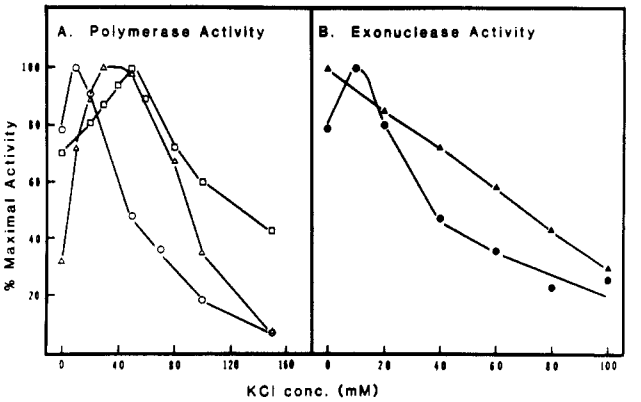

FIGURE 3: Effect of KCl concentration on polymerase and 3′- to 5′-exonuclease activities with different template-primers. Assay conditions were as described in the text. Polymerase activity with poly(dA-dT) (○), poly(dG-dC) (△), or activated calf thymus DNA (□) as template-primer. The 3′- to 5′-exonuclease activity with poly(dA-dT)–[^{3}H]dTMP (●) or [^{3}H]d(T)$_{50}$ (▲) as substrate.

The effects of KCl concentration on the 3′- to 5′-exonuclease activity were also dependent upon the structure of the DNA substrate (Figure 3B). With single-stranded poly(dT) as substrate, the exonuclease activity was optimal in the absence of KCl and was 50% inhibited at about 90 mM. With poly(dA-dT)–[^{3}H]dTMP as substrate, exonuclease activity was slightly stimulated at 10–20 mM KCl and inhibited at higher concentrations, being 50% inhibited at 40 mM. The exonuclease activity was extremely sensitive to inhibition by phosphate buffer; the activity was 50% inhibited at 25 mM and greater than 90% inhibited at 80 mM when assayed with [^{3}H]d(T)$_{50}$ (data not shown).

Either Mg^{2+} or Mn^{2+} can serve as divalent cation activator for both polymerase and 3′- to 5′-exonuclease activities. With poly(dA-dT) as template-primer, the Mn^{2+} optimum was found to be 0.1 mM, and the Mg^{2+} optimum was approximately 10-fold higher. The divalent cation optima for the exonuclease activity with poly(dA-dT)–[^{3}H]dTMP as substrate were 0.1–0.4 mM Mn^{2+} and 2–6 mM Mg^{2+} (data not shown).

The pH optima for polymerase and exonuclease activities varied somewhat with divalent cation. For example, the optimal pH for polymerase activity with poly(dA-dT) as template-primer was 6.5 when Mn^{2+} was divalent cation and 6.0

Table IV: Apparent K_m Values for Deoxynucleoside Triphosphates[a]

template-primer	divalent cation	variable substrate	apparent K_m (μM)
poly(dA-dT)	Mn^{2+}	dATP	0.4
poly(dA-dT)	Mn^{2+}	dTTP	1.1
poly(dG-dC)	Mn^{2+}	dGTP	0.6
poly(dG-dC)	Mn^{2+}	dCTP	8.5
poly(dA-dT)	Mg^{2+}	dATP	0.5
poly(dA-dT)	Mg^{2+}	dTTP	2.7

[a] Assay conditions were as described under Materials and Methods, with either poly(dA-dT) or poly(dG-dC) as template-primer and either Mg^{2+} or Mn^{2+} as divalent cation, as indicated. The concentration of the variable substrate was varied over a 200-fold range, and the concentration of the nonvariable substrate was constant and saturating. Apparent K_m values were determined by means of both Lineweaver-Burk and Hanes-Woolf plots by the method of least squares.

in the presence of Mg^{2+}. The pH optimum for exonuclease activity with poly(dA-dT)–[^{3}H]dTMP as substrate was 6.5 in the presence of Mn^{2+} and 7.0–7.5 in the presence of Mg^{2+}.

No 5′- to 3′-exonuclease activity could be detected with purified DNA polymerase δ when assayed by the release of acid-soluble [^{32}P]dTMP from [^{32}P](dT)$_1$–(dT)$_{600}$, either in the presence or in the absence of d(A)$_{100}$. Similarly, no endonuclease activity was detected when assayed by measuring the conversion of supercoiled PM2 DNA to relaxed circular or linear forms by agarose gel electrophoresis.

The apparent K_m values for deoxynucleoside triphosphates with alternating copolymer template-primers are shown in Table IV. The K_m values for dATP and dTTP were determined with poly(dA-dT) as template-primer, and the K_m values for dGTP and dCTP were determined with poly(dG-dC). With Mn^{2+} as divalent cation, the K_m values for dATP, dGTP, and dTTP were of the order of 1 μM while that for dCTP was approximately 10-fold higher. These values are of the same order of magnitude as those reported for homogeneous DNA polymerase α from KB cells (Fisher et al., 1979). The K_m values for dATP and dTTP were only slightly higher when Mn^{2+} was replaced by Mg^{2+} as divalent cation. This is in contrast to KB cell DNA polymerase α, where the K_m for dTTP was found to increase 10-fold when Mg^{2+} was substituted for Mn^{2+}.

Effect of Aphidicolin on Polymerase and 3′- to 5′-Exonuclease Activities of DNA Polymerase δ. The effect of increasing concentrations of aphidicolin on both polymerase and 3′- to 5′-exonuclease activities of DNA polymerase δ is shown in Figure 4. With poly(dA-dT) as template-primer, the polymerase activity was inhibited 50% at 2 μg/mL aphidicolin when the dTTP concentration was 10 μM. At higher dTTP concentrations the polymerase activity was more resistant to inhibition by aphidicolin; e.g., at 20 μM dTTP, 50% inhibition was seen at 4 μg/mL. The effect of aphidicolin on the 3′- to 5′-exonuclease activity was dependent on the secondary structure of the substrate. With double-stranded poly(dA-dT)–[^{3}H]dTMP as substrate the 3′- to 5′-exonuclease activity was extremely sensitive to inhibition by aphidicolin, whereas with single-stranded [^{3}H]d(T)$_{50}$ as substrate, no inhibition was seen even at 10 μg/mL aphidicolin.

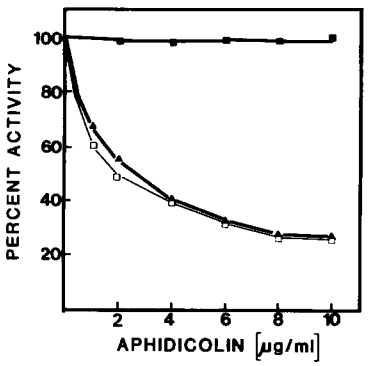

FIGURE 4: Inhibition of DNA polymerase and 3′- to 5′-exonuclease activities by aphidicolin. Assay conditions were as described under Materials and Methods except for the addition of aphidicolin as indicated. Polymerase activity (▲), exonuclease activity assayed with [^{3}H]d(T)$_{50}$ (■), and exonuclease activity assayed with poly(dA-dT)–[^{3}H]dTMP (□).

Discussion

The purification of high molecular weight DNA polymerases from calf thymus has not been a simple and straightforward process. Multiple species of DNA polymerase have been identified in this tissue by column chromatography and density gradient centrifugation (Holmes et al., 1974). Determination of the molecular structures of these DNA polymerases, as well as those from other eukaryotic sources, has been hampered by the presence of proteolytic enzymes in tissue extracts (Brakel & Blumenthal, 1977). Uncontrolled proteolysis during enzyme purification might explain the heterogeneity of these DNA polymerases (Holmes & Johnston, 1975; Brakel & Blumenthal, 1977), and this possibility has been of great concern to many investigators (Banks et al., 1979; Hubscher et al., 1981; Gross & Krause, 1981). Recently, Kaguni et al. (1982) have demonstrated that proteolytic degradation of the large subunit of DNA polymerase α was occurring during all the steps of purification of the enzyme from *Drosophila melanogaster*, in spite of the presence of the protease inhibitors PMSF and sodium bisulfite.

We have developed a new procedure for purification of DNA polymerase δ which may be of use in the purification of other eukaryotic DNA polymerases. By this procedure we have removed most of the calcium-dependent protease activity at an early stage of purification and have purified DNA polymerase δ to apparent homogeneity. A molecular weight of 173K has been calculated for the native enzyme from measurements of Stokes radius and sedimentation coefficient. SDS–polyacrylamide gel electrophoresis of the homogeneous enzyme reveals two polypeptides of M_r 125K and 48K. The sum of the molecular weights of the two subunits corresponds to the molecular weight calculated for the native enzyme, suggesting that the native enzyme is composed of one of each subunit.

The subunit composition of calf thymus DNA polymerase δ differs from that of DNA polymerase δ purified from rabbit bone marrow, which has recently been reported to consist of a single polypeptide chain of 122 kDa (Goscin & Byrnes, 1982). The reasons for this discrepancy are not obvious at present; however, it is worth noting that rabbit bone marrow DNA polymerase δ prefers poly(dA)/oligo(dT) as template-primer, whereas DNA polymerase δ from calf thymus utilizes poly(dA-dT) as template-primer 10 times more efficiently than poly(dA)/oligo(dT).

Comparison of the subunit structure of DNA polymerase δ prepared by our previous method, i.e., subunits of 49 and 60 kDa, with that of the enzyme prepared by the present method, in which the 60-kDa subunit is replaced by a 125-kDa subunit, suggests that the 60-kDa polypeptide may have been derived from the 125-kDa polypeptide, possibly as the result of cleavage of an unusually sensitive peptide bond, during enzyme purification. DNA polymerase δ prepared by either procedure has the same isoelectric point (pI = 5.5). Fur-

thermore, the enzymes prepared by the two procedures have essentially identical properties in terms of template-primer specificity, pH optimum, and optimal cation concentrations.

We have recently carried out comparative studies on the relative sensitivity of DNA polymerases α and δ from calf thymus to several inhibitors of DNA replication (Lee et al., 1981). We found that DNA polymerase δ, similar to DNA polymerase α, is sensitive to inhibition by aphidicolin, NEM, and ara-ATP, whereas it is resistant to dideoxythymidine triphosphate. Thus, DNA polymerase δ has many properties in common with DNA polymerase α. However, in contrast to DNA polymerase α, DNA polymerase δ is associated with a 3′- to 5′-exonuclease activity whereas DNA polymerase α is usually found to be devoid of this activity. The exception is DNA polymerase α_1 from mouse myeloma (Chen et al., 1979), which does have an associated 3′- to 5′-exonuclease activity.

Other characteristics which distinguish DNA polymerases α and δ include the following: (1) template-primer specificity, where DNA polymerase δ prefers alternating copolymers whereas DNA polymerase α is most active with activated DNA; (2) elution behavior on hydroxylapatite chromatography, where DNA polymerase α binds to hydroxylapatite in the presence of 0.5 M KCl (Fisher & Korn, 1977; Mechali et al., 1980) whereas DNA polymerase δ is eluted at 90 mM KCl; (3) elution behavior on hydrophobic interaction chromatography, where DNA polymerase δ is eluted from phenyl-Sepharose at pH 7.2 whereas DNA polymerase α is eluted at pH 8.5.

Recently a catalytically active "core polymerase", which consists of a single polypeptide of approximately 120 kDa, has been demonstrated in crude extracts as well as in highly purified DNA polymerase preparations from calf thymus and mouse myeloma (Hubscher et al., 1981; Albert et al., 1982; Karawya & Wilson, 1982). It has been suggested that this 120-kDa polypeptide is a component of mouse myeloma DNA polymerase α, since it is resistant to inhibition by dideoxy-TTP and sensitive to aphidicolin (Karawya & Wilson, 1982). Whether the 125-kDa subunit of calf thymus DNA polymerase δ is related to the 120-kDa core polymerase found in calf thymus and mouse myeloma must await further studies.

The template-primer specificity of DNA polymerase δ distinguishes it from other mammalian DNA polymerases. In contrast to DNA polymerase α, it utilizes alternating copolymer template-primers in preference to activated DNA, in the presence of either Mg^{2+} or Mn^{2+}. Similar to DNA polymerase α, but in contrast to DNA polymerases β (Wang et al., 1974; Chang, 1974) and γ (Knopf et al., 1976), DNA polymerase δ has little or no ability to utilize a primed polyriboadenylate template. DNA polymerase δ is also very inefficient in utilizing a primed polydeoxyriboadenylate template; however, the ability of the enzyme to utilize poly(dA)/oligo(dT) is markedly stimulated by a 37 000-dalton protein from calf thymus (unpublished results).

Both the polymerase and 3′- to 5′-exonuclease activities of DNA polymerase δ are able to utilize either Mg^{2+} or Mn^{2+} as divalent cation activator, and the optimal Mg^{2+} concentration is approximately 10 times the Mn^{2+} optimum. However, the optimal divalent cation concentration, the pH optimum, and the optimal monovalent cation concentration for either the polymerase or the 3′- to 5′-exonuclease activity differ with the nature of the template-primer.

The replication inhibitor aphidicolin has been extensively studied in vivo and in vitro and has been utilized to implicate DNA polymerase α as the enzyme responsible for chromosomal DNA replication (DePamphilis & Wasserman, 1980; Huberman, 1981). The exact mechanism of inhibition of either DNA polymerase α or DNA polymerase δ is not known. Kinetic studies have shown that, with activated calf thymus DNA as template-primer, aphidicolin is a competitive inhibitor of DNA polymerase α activity when dCTP is the variable substrate and a noncompetitive inhibitor when dATP, dTTP, or dGTP is the variable substrate (Oguro et al., 1979; Pedrali-Noy & Spadari, 1980). However, when poly(dA)/oligo(dT) is the template-primer, aphidicolin is a competitive inhibitor with respect to dTTP (Holmes, 1981; Krokan et al., 1981).

We have found that the 3′- to 5′-exonuclease activity of DNA polymerase δ, similar to the polymerase activity, is sensitive to inhibition by aphidicolin when the template-primer is double-stranded DNA, whereas the hydrolysis of single-stranded poly(dT) is resistant to inhibition. The coordinated inhibition of both polymerase and exonuclease activities of DNA polymerase δ is consistent with our observation that both activities reside on the same protein molecule. Furthermore, the present findings are consistent with the suggestion that the binding of aphidicolin to the polymerase molecule requires the formation of a template-primer–DNA polymerase complex (Huberman, 1981). The mechanism of inhibition of DNA polymerase appears to be more complex than simple competitive inhibition with respect to pyrimidine nucleoside triphosphates. Indeed, with yeast DNA polymerase I, a mixed type of inhibition is seen with dCTP as variable substrate, suggesting that the binding of aphidicolin interferes with the binding of dCTP, possibly by binding at an overlapping site (Plevani et al., 1980). The mechanism of inhibition of DNA polymerase δ by aphidicolin is currently under investigation in our laboratory.

Acknowledgments

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Registry No. dATP, 1927-31-7; dTTP, 365-08-2; dGTP, 2564-35-4; dCTP, 2056-98-6; Mn, 7439-96-5; Mg, 7439-95-4; poly(dA-dT), 26966-61-0; poly(dG-dC), 36786-90-0; poly(dA)/oligo(dT), 24939-09-1; poly(rA)/oligo(dT), 27156-07-6; aphidicolin, 38966-21-1; DNA polymerase, 9012-90-2.

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