DNA Polymerase δ: One Polypeptide, Two Activities†

Lee Pletts Goscin and John J. Byrnes*

ABSTRACT: DNA polymerase δ from rabbit bone marrow has an associated 3′–5′-exonuclease. Previous studies demonstrated a Stokes radius of 45.5 Å by gel filtration and a sedimentation coefficient of 6.5 S by zone sedimentation. Thus, a molecular weight of 122000 and a frictional coefficient of 1.39 were calculated [Byrnes, J. J., & Black, V. L. (1978) *Biochemistry 17*, 4226–4231]. Several problems obstructed further purification and definition of DNA polymerase δ. The small amount of protein obtained limited further purification as the nonspecific loss of enzyme in subsequent procedures was excessive. Furthermore, the amount of protein recovered was insufficient for conventional analysis. These difficulties have been overcome, and DNA polymerase δ has been purified to apparent homogeneity. Under conditions of nondenaturing microgel electrophoresis, DNA polymerase δ aggregates to molecular weight species of 300000 and higher. In situ assays for DNA polymerase and exonuclease in these gels generate concordant activity profiles. Upon sodium dodecyl sulfate gel electrophoresis, δ is a single polypeptide of 122000 apparent molecular weight. The DNA polymerase incorporates between 250000 and 300000 nmol of thymidine deoxyribonucleoside monophosphate (dTMP) into poly(dA)/oligo(dT) (mg of protein)$^{-1}$ h^{-1} at 37 °C; the exonuclease simultaneously hydrolyzes 13% of the newly synthesized DNA. Aphidicolin, considered to be a specific inhibitor of DNA polymerase α, inhibits both the DNA polymerase and 3′–5′-exonuclease activities of δ. DNA polymerase α from rabbit bone marrow does not share a common subunit with δ. Therefore, aphidicolin binding is not specific for α, and conclusions based upon the supposition that it is must be reconsidered.

Until 1976, three mammalian DNA dependent DNA polymerases were described: α, β, and γ or mitochondrial. As aphidicolin inhibits the growth of eukaryotic cells and inhibits DNA polymerase α but not β or γ, it has been inferred that α is responsible for eukaryotic nuclear DNA replication [for a review, see Huberman (1981)]. In 1976, Byrnes et al. described DNA polymerase δ from rabbit bone marrow. DNA polymerase δ is similar to DNA polymerase α in many respects: both are of high molecular weight and are sensitive to *N*-ethylmaleimide; neither will synthesize DNA from ribohomopolymers; at low ionic strengths, both enzymes aggregate, and the purification procedures for the bone marrow enzymes are similar. Different substrate preferences occur, but the main difference is that DNA polymerase δ has an associated 3′–5′-exonuclease activity. DNA polymerase δ previously was purified to a specific activity of 30000 units/mg and a molecular weight of 122000 calculated from sedimentation coefficient and Stokes radius determinations (Byrnes & Black, 1978). The small amount of protein limited further purification as the nonspecific loss of enzyme was excessive. Furthermore, the amount of protein obtained was below the amount required for conventional analysis. We have overcome these obstacles and have purified DNA polymerase δ to apparent homogeneity. This has allowed definition of the molecular structure of DNA polymerase δ and its unequivocal distinction from DNA polymerase α. In addition, studies demonstrating that δ also is sensitive to aphidicolin necessitate a reassessment of conclusions based upon the premise that α is the specific target of this inhibitor.

Experimental Procedures

Reagents. ^{3}H-Labeled deoxyribonucleoside triphosphates were purchased from New England Nuclear. Unlabeled deoxyribonucleoside triphosphates, nucleoside monophosphates, oligo(dT)$_{12-18}$, poly(dA), poly(dA-dT), and 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer were obtained from P-L Biochemicals. Dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Calbiochem. Tris(hydroxymethyl)aminomethane (Tris) and poly(ethylene glycol) 6000 (PEG) were from Sigma Chemical Co. Bovine serum albumin (BSA) was purchased from Miles. Ovalbumin, aldolase, and blue dextran 2000 were purchased from Pharmacia. Myosin and lysozyme were gifts from Dr. Bob Rubin. Phosphorylase *b* was obtained from Boehringer Mannheim. Aphidicolin was obtained from Flow Laboratories and dissolved in dimethyl sulfoxide. Coomassie brilliant blue G-250 was from Eastman Kodak Co. Phosphocellulose powder (P11) and GF/C filters were purchased from Whatman. Ultrogel AcA 34 was purchased from LKB Instruments. Poly(ethylenimine)–cellulose (PEI–cellulose) thin-layer chromatography plates were purchased from Brinkmann Instruments, Inc., and were prerun with distilled water and stored at 0 °C before use. The protein dye reagent and all electrophoresis reagents were from Bio-Rad. All other chemicals were reagent grade.

DNA Polymerase Assay. The reaction contained the following in a final volume of 0.1 mL: 40 mM Mops buffer, pH 6.75, 90 mM KCl, 0.4 mM $MnCl_2$, 0.75 A_{260} unit of poly(dA), 1.88×10^{-2} A_{260} unit of oligo(dT), 19% (v/v) glycerol, 10 μM [^{3}H]dTTP (1000 Ci/mol), 1 μg of BSA, and 1–10 μL of DNA polymerase. For assay of DNA polymerase δ, 0.8 mM guanosine ribonucleoside monophosphate (GMP) is also included to selectively inhibit the exonuclease reaction (Byrnes et al., 1977). The reaction vessels were mixed gently and incubated at 37 °C for 10 min, and the reaction was stopped by the addition of 0.1 mL of 0.1 M sodium pyrophosphate, 1.25 A_{260} units of denatured salmon sperm DNA, and 4 mL of cold 5% trichloroacetic acid (Cl_3CCOOH). The precipitate was collected on a Whatman (GF/C) glass fiber filter and washed with 15 mL of cold 1% Cl_3CCOOH and 5 mL of ethanol. The filter was dried and counted in 10 mL of a toluene Liquifluor (New England Nuclear) solution in a Beckman LS-233 liquid scintillation counter. One unit of DNA polymerase catalyzes the incorporation of 1 nmol of dTMP per h at 37 °C.

† From the Veterans Administration Hospital, Miami, Florida 33125, and the Center for Blood Diseases, Department of Medicine, University of Miami Medical School, Miami, Florida 33125. *Received April 29, 1981.* This research was supported by the Veterans Administration. L.P.G. was a postdoctoral fellow supported by Grant 5 T32 AM07114 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health.

* Correspondence should be addressed to this author at the Department of Medicine, University of Miami Medical School.

0006-2960/82/0421-2513$01.25/0

3′–5′-Exonuclease Assay. The 3′–5′-exonucleolytic activity was assayed by measuring the release of [^{3}H]dTMP from poly(dA-dT)·[^{3}H]dTMP essentially as previously described (Byrnes et al., 1976). Each reaction mixture in a final volume of 0.1 mL contained 0.046 A_{260} unit/mL poly(dA-dT)·[^{3}H]-TMP (6.2 μCi/A_{260} unit), 25 mM Mops, pH 7.0, 1 mM $MnCl_2$, 80 mM KCl, and 5–20 μL of DNA polymerase. The reaction was incubated for 30 min at 37 °C and stopped by the addition of 1 mg of BSA and 1 mL of cold 5% Cl_3CCO-OH. The precipitate was collected by centrifugation at 2600*g*, and a 0.9-mL aliquot of the supernatant was counted in 10 mL of Riafluor (New England Nuclear).

Simultaneous Measurement of DNA Polymerase and 3′–5′-Exonuclease. A DNA polymerase reaction was constituted as described above except that glycerol and GMP were omitted. DNA polymerase δ was added and the mix incubated at 37 °C for 30 min. The reaction was terminated by the addition of ethylenediaminetetraacetic acid (EDTA) to a concentration of 2 mM and rapid chilling on ice. Polynucleotide and nucleoside triphosphate, diphosphate, and monophosphate were separated on PEI–cellulose chromatography and quantitated as previously described (Byrnes et al., 1976).

Preparation of 3′ Terminally Labeled Poly(dA)/Oligo(dT)·[^{3}H]dTMP. Twenty units of DNA polymerase was incubated with 1 A_{260} unit of poly(dA) and 0.04 A_{260} unit of oligo(dT)$_{12-18}$ in 1.38 mL, final volume, of 40 mM Mops, pH 6.75, 10 μg/mL BSA, 0.4 mM $MnCl_2$, 19% (v/v) glycerol, 105 mM KCl, 0.8 mM GMP, and 1.2 μM [^{3}H]dTTP (50.0 Ci/mmol) at 37 °C for 45 min. EDTA was added to a final concentration of 20 mM, and the reaction was heated at 80 °C for 5 min. The unincorporated nucleotides were removed by Sephadex G25 chromatography in 40 mM KCl–10 mM Mops, pH 7.0, at room temperature. The resulting poly(dA)/oligo(dT)·[^{3}H]dTMP had a specific activity of 76 μCi/A_{260} unit.

Other Procedures. Protein concentrations were measured by using the Bradford method (Bradford, 1976), with BSA as the standard through purification step VIII. Protein in slab gels was quantitated by microdensitometry, vide infra. The pH and conductivity of buffer solutions were measured at room temperature with a Radiometer digital pH meter and a Yellowspring conductivity bridge.

Purification of DNA Polymerase δ. DNA polymerase was purified from erythroid hyperplastic rabbit bone marrow through step VIII but with several important modifications of previously described procedures (Byrnes et al., 1976; Byrnes & Black, 1978). Briefly, rabbits were injected with phenylhydrazine to induce erythroid hyperplasia. The marrow obtained from the long bones of 50–54 rabbits yielded approximately 120 g of starting material. The cells were washed and lysed as described except that 0.5% PMSF was added to the lysis buffer. After centrifugation of the cell lysate at 30000*g* for 15 min, the supernatant was brought to 5 mM $MgCl_2$ and then centrifuged at 78000*g* for 2 h. The resulting "microsomal" pellets were homogenized in 50 mL of 50 mM Tris-HCl, pH 7.8, 25% (v/v) glycerol, 0.1 mM EDTA, and 1.0 mM DTT (TGED buffer) containing 1.0 M KCl and kept on ice overnight. The homogenate was centrifuged at 150000*g* for 90 min, and the supernatant was retained. Ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation at 30000*g* for 15 min and resuspended in 25 mL of TGED buffer containing 0.5% PEG. All buffer solutions thereafter contained 0.5% PEG. Phosphocellulose, diethylaminoethyl-Sephadex, hydroxylapatite, a second phosphocellulose, and Ultrogel AcA 34 column chromatography were performed as described. All procedures were done at 4 °C, and between procedures, the material was stored at −70 °C; whenever possible, plastic ware was used in contact with the enzyme. After step VIII, 200–400 units of DNA polymerase δ with a specific activity of about 30000 units/mg was generally obtained.

Further purification was obtained by chromatography on a third phosphocellulose column. Eight milliliters of the most active fractions was diluted 3-fold in 25% (v/v) glycerol, 0.5% PEG, and 1 mM DTT (GPD buffer) and applied to a 0.67 cm^2 × 1.5 cm phosphocellulose column which had been equilibrated with GPD solution. The column was washed with 6 mL of GPD containing 0.1 M ammonium sulfate, and the enzyme was eluted by 1-mL stepwise applications of GPD containing 0.025 M increments of ammonium sulfate. Fractions (0.5 mL) were collected and assayed for DNA polymerase and exonuclease and subjected to gel electrophoretic analysis.

Microelectrophoresis on Gradient Gels. Micro gradient gels were prepared in 30 mm long × 0.65 mm internal diameter capillary tubes containing 10-μL total volume including 6 μL of gel support. The tubes were filled halfway with solution 1 [10% (v/v) glycerol, 0.07% ammonium persulfate, and 350 mM Tris–sulfate, pH 8.4]. A gradient of 1–16% acrylamide was formed by using capillary action to introduce solution 2 [10% (v/v) glycerol, 16% polyacrylamide, 0.6% bis(acrylamide), 350 mM Tris–sulfate, pH 8.4, and 0.25% *N,N,N′,N′*-tetramethylethylenediamine (Temed)] to fill the gel tube from the bottom. Twenty hours before the gels were used, 1 mM DTT was added to the lower buffer and to the solution on top of the gels. Three microliters of sample and 1 μL of 0.5% bromocresol purple in water were loaded on the top of the gels. All enzyme samples and standards were in 25% glycerol to prevent diffusion into the top buffer which contained 10% (v/v) glycerol, 370 mM glycine, and 50 mM Tris, pH 8.2. Thirty volts per gel was applied until the dye showed stacking of the sample on top of the gel, and then 70 V/gel was applied until the tracking dye moved through the gel. The gels were removed with a wire rod and stained with Coomassie brilliant blue. BSA was used as a molecular weight marker, and the monomer, dimer, and trimer were easily visualized. Electrophoresis on gradient gels can be used to determine the molecular weights of proteins. A straight line with a correlation coefficient $r_{\lg,M_r} = -1.000$ is given when the migration distance is plotted against the logarithm of the respective molecular weights (Neuhoff, 1973).

Activity Gels. Gradient gels were formed as stated above except that 0.25 A_{260} unit/mL 3′ terminus labeled poly(dA)/oligo(dT)·[^{3}H]dTMP/mL was added to solutions 1 and 2 and ammonium persulfate was increased to allow for polymerization in 20 min. All buffers were ice cold, and the buffer containers were in ice during the electrophoresis. The poly(dA)/oligo(dT)·[^{3}H]dTMP in the gel approximately doubles the time needed for electrophoresis, but otherwise the proteins migrated in the same way as when template-primer was absent. The template-primer remained evenly distributed in the gel during electrophoresis.

For measurement of DNA polymerase activity in situ, the gels were removed from the capillaries after electrophoresis and placed in 0.9 mL of polymerase reaction mix with 10 mM DTT and 0.6 μM [^{3}H]dTTP (50.0 Ci/mmol) but no template-primer at 37 °C for 1 h. This induced synthesis of poly(dT) by the DNA polymerase. The reaction was stopped, and unincorporated [^{3}H]dTMP was eliminated by washing

the gel in 5 mL of cold 10 mM EDTA twice. The gel was sliced into 1-mm fractions which after remaining 12 h at 37 °C in 10 mL of 0.4% Omnifluor toluene–3% Protosol (New England Nuclear) were counted.

For measurement of nuclease activity in situ, the gels were first sliced into 1-mm fractions and each placed in 200 μL of 10 mM DTT, 40 mM Mops, pH 7.0, 10 μg/mL BSA, 19% (v/v) glycerol, 80 mM KCl, and 1 mM $MnCl_2$. After incubation at 37 °C for 60 min, the reaction was stopped by adding 0.1 mL of 0.1 M pyrophosphate, 1 mg of BSA, and 1 mL of 5% Cl_3CCOOH. After remaining at 0 °C for 10 min, the samples were centrifuged 5 min at 2600*g*. Aliquots (1 mL) of the supernatants were counted in 10 mL of Biofluor (New England Nuclear). [^{3}H]dTMP was released from the gel fractions which contained nuclease.

Sodium Dodecyl Sulfate (NaDodSO$_4$) Slab Gel Electrophoresis. The polyacrylamide (16 × 12 × 0.075 cm) slab gel system of Laemmli (1970) and O'Farrel (1975) was used. Final concentrations in the separation gel were 0.21% bis(acrylamide) and 8% polyacrylamide, while the stacking gel containing 0.12% bis(acrylamide) and 4.5% acrylamide. Sample buffer was 10% glycerol, 0.0001% bromophenol blue, 0.0625 M Tris, pH 6.8, 2% NaDodSO$_4$, and 5% 2-mercaptoethanol. Sample buffer (30 μL) was added to 40 μL of sample and heated for 30 s at 100 °C. Gels were run at 15 mA through the stacking gel and 20 mA through the lower gel. The gels were fixed in 25% Cl_3CCOOH for 45 min.

Gels were stained by using the Oakley et al. (1980) modification of the silver stain of Switzer et al. (1979). Because of the sensitivity of the method, great care was taken to avoid nonspecific staining in the silver staining gel. In particular, it was necessary to recrystallize the NaDodSO$_4$ and use ultrapure water. In spite of these precautions, a faint speckled band often developed about the middle of the gels. For this reason, control gel lanes containing only sample buffer were included in each run. Gels were photographed with a 35-mm camera and traced by using a Zeineh soft laser scanning densitometer with integrator. The amount of protein in a band was quantitated by comparison to known concentrations of myosin electrophoresed in the same gel. The silver stain was linear and directly proportional over a range of 10–100 ng of protein. The absolute quantity of protein may be somewhat more or less due to amino acid composition differences between DNA polymerase δ and myosin. However, myosin was selected for a reference as it migrates near DNA polymerase δ in the gel. (Using BSA as a standard gives a 1.7-fold increase in specific activity.) Molecular weights were determined by the method of Weber & Osborn (1969) by using five standards: myosin (M_r 200000), phosphorylase *b* (M_r 92500), BSA (M_r 68000), ovalbumin (M_r 43000), and chymotrypsinogen (M_r 25000).

Results

Purification of DNA Polymerase δ (Step IX). The addition of PMSF and PEG to the purification protocol improved the yield of enzyme activity at each step such that after step VIII 200–400 units of DNA polymerase δ was generally obtained. The specific activity of the most pure fraction at this point was about 30000 units/mg of protein. However, analysis in native gels revealed five to six protein bands, and generally 10 polypeptides appeared in NaDodSO$_4$ gels.

Various procedures, including several affinity columns, were explored for further purification. However, the most effective has been an additional, third, phosphocellulose column. The efficiency of this step was refined by NaDodSO$_4$ gel analysis of the elute fractions. We were able to correlate the appearance of one polypeptide in the ionic gradient eluate at 0.13 M ammonium sulfate with DNA polymerase δ. Most of the contaminating polypeptides began to elute earlier, but they trailed into the δ eluate. Subsequently, similar columns were first washed extensively with six column volumes of buffer containing 0.10 M ammonium sulfate followed by 1-mL aliquots containing stepwise increases in ammonium sulfate. This elution procedure resulted in highly pure DNA polymerase δ (vide infra). Recovery of the applied DNA polymerase activity has been excellent, in the range of 75–90%. Insufficient protein remained to be detected in the Bradford microassay.

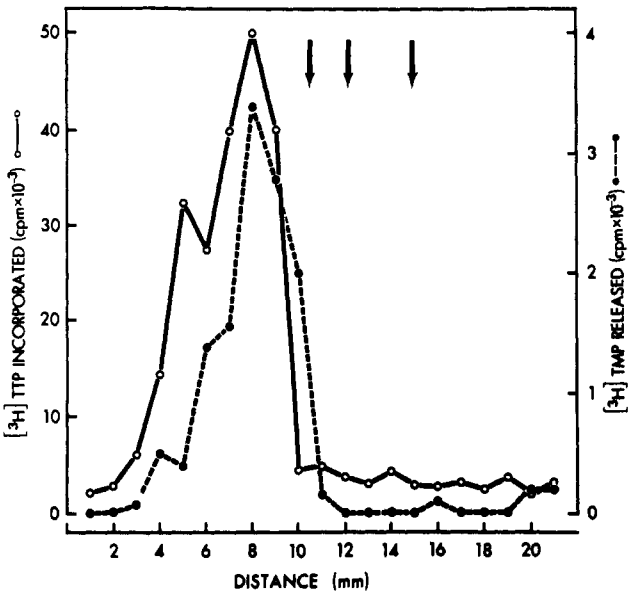

FIGURE 1: Activity microgels of DNA polymerase δ. Electrophoresis is from left to right. All gels contained immobilized poly(dA)/oligo(dT)·[^{3}H]TMP. The three arrows show the migration of BSA monomer, dimer, and trimer which were used as molecular weight markers in one gel. DNA polymerase was indicated by synthesis of poly(dT) on poly(dA)/oligo(dT)·[^{3}H]TMP in a second gel. Nuclease was measured by the release of [^{3}H]TMP in a third gel. The specific activity of the TMP was 12300 cpm/pmol.

Electrophoresis under Nondenaturing Conditions. Step IX enzyme was examined in situ on gradient microgels containing template-primer. Aliquots of enzyme were electrophoresed in three separate gels. One was examined for DNA polymerase, the second for exonuclease, and the third for protein. The enzymatic activities remained very active after electrophoresis. (However, no attempt was made to quantitate the activity recovered, as substrate saturation in the gels could not be assured.) The DNA polymerase and the exonuclease assay gave a similar pattern (Figure 1). They were broad-based with peaks of activity corresponding to an M_r of 300000. A faint band of protein in the third gel corresponded to the activities; otherwise, no protein was visible in the gel (not shown). Apparently DNA polymerase δ aggregates under the low ionic strength conditions of electrophoresis. Previous studies demonstrated that δ aggregates at low ionic strength (Byrnes et al., 1976). Attempts to sharpen the protein band by the inclusion of nonionic detergents in the electrophoresis buffer at concentrations not inhibitory to the enzyme activity were unsuccessful. Similar results were obtained by using microgels not containing template-primer; however, the extraction of enzyme from the gels was much more time and effort consuming and provided a less effective demonstration of enzymatic activity.

Electrophoresis under Denaturing Conditions. Electrophoresis of refined step IX eluate carried out on 8% poly-

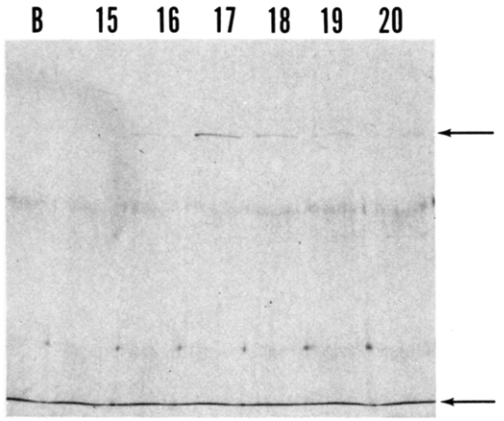

FIGURE 2: $NaDodSO_4$ slab gel electrophoresis of step IX eluate. The lower arrow indicates the migration of the tracking dye while the upper arrow indicates DNA polymerase δ. Forty microliters of fractions 15–20 was electrophoresed in their respective lanes, and 40 μL of GPD was run in lane B as a control for the silver stain procedure.

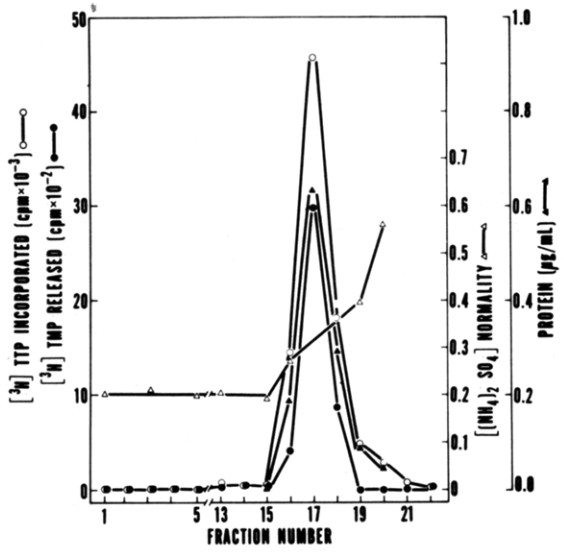

FIGURE 3: Step IX, third phosphocellulose column. Step VIII DNA polymerase δ was chromatographed on a phosphocellulose column and eluted as described. Fractions were assayed for polymerase and exonuclease, and the conductivity was measured. Each fraction was also electrophoresed on $NaDodSO_4$ slab gels (Figure 2), and the amount of protein in each fraction was quantitated.

acrylamide slab gels containing $NaDodSO_4$ and mercaptoethanol revealed one polypeptide band across the peak of DNA polymerase δ (Figure 2). The protein was quantitated by microdensitometry and is plotted with the DNA polymerase and exonuclease profiles in Figure 3. The protein elution pattern was identical with that of the DNA polymerase and exonuclease activities. DNA polymerase δ appears nearly homogeneous (Figure 5). Similar preparations of DNA polymerase δ have been obtained 7 times, and this polypeptide band has strictly correlated with the DNA polymerase δ activities each time. The specific activity of DNA polymerase δ has ranged between 250000 and 300000 units/mg of protein. The R_f of DNA polymerase δ in the $NaDodSO_4$ gels corresponds to a polypeptide of molecular weight 122000.

Simultaneous Quantitation of DNA Polymerase and 3′–5′-Exonuclease. The ratio of the DNA polymerase to exonuclease is an important determinant of proofreading efficiency of the 3′–5′-exonuclease. The quantitation of the nuclease in relation to the polymerase is best determined on the same template-primer under similar conditions and substrate saturation. The DNA polymerase reaction was analyzed on

Table I: Simultaneous Measurement of DNA Polymerase and 3′–5′-Exonuclease[a]

	[³H]TMP as DNA (pmol)	[³H]TMP as 5′-monophosphate (pmol)
step IX enzyme + template	73.1	10.3
	80.8	14.3
step IX enzyme − template	0	0
	0	0

[a] A DNA polymerase reaction was constituted as described under Experimental Procedures and after incubation was chromatographed on PEI–cellulose thin-layer plates to separate the reaction components. Zero-time incubation and no enzyme added controls were also run and produced no detectable DNA or monophosphate. The specific activity of the [³H]TMP was 750 cpm/pmol.

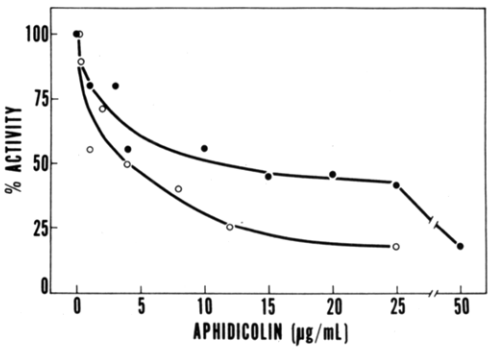

FIGURE 4: Inhibition of DNA polymerase δ (○) and the 3′–5′-exonuclease (●) by aphidicolin. The enzyme was assayed in the standard mixture with various concentrations of inhibitor.

PEI–cellulose to do this. When poly(dA)/oligo(dT) was used to direct dTMP incorporation, step IX DNA polymerase δ simultaneously hydrolyzed approximately 13% of the newly synthesized DNA to nucleoside 5′-monophosphate (Table I). As previously demonstrated, the generation of nucleoside 5′-monophosphate was template dependent. This requirement reflects the sequential actions of the DNA polymerase, template dependence and the 3′–5′-exonuclease release of 5′-monophosphate.

Aphidicolin Inhibition of DNA Polymerase and Exonuclease. Aphidicolin was added to the DNA polymerase and exonuclease reactions over a concentration range which is inhibitory to purified calf thymus DNA polymerase α (Grosse & Krauss, 1981) and to bone marrow DNA polymerase α. Both the DNA polymerase and the exonuclease were inhibited by aphidicolin in parallel fashion; the DNA polymerase was 50% inhibited at 4 μg/mL, and the 3′–5′-exonuclease was 50% inhibited at 10 μg/mL (Figure 4).

Molecular Structural Comparisons to DNA Polymerase α. There are a number of similarities between the α and δ polymerases, including *N*-ethylmaleimide and aphidicolin sensitivity. Aphidicolin binds to DNA polymerase α noncovalently, and this interaction has been considered specific for α. For these reasons, it is important to consider whether α and δ may share a common subunit. Therefore, DNA polymerase α has been purified from rabbit bone marrow and the DNA polymerase core isolated (this will be the subject of a separate report). DNA polymerase α core is a polypeptide, of 135000 molecular weight. It has a similar polymerase specific activity as δ and is similarly sensitive to aphidicolin but has no detectable exonuclease. The molecular structures of DNA polymerase α and δ are compared in Figure 5. Clearly, α is distinct from δ. Aphidicolin may bind to similar regions of the α- and δ-polypeptides. The question of

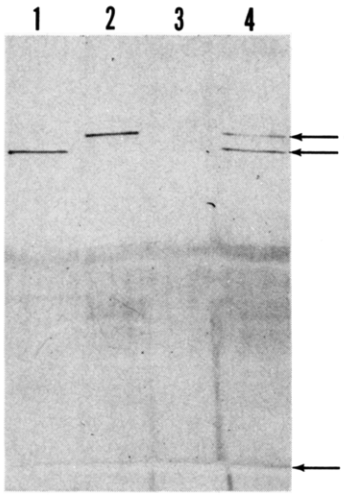

FIGURE 5: $NaDodSO_4$ slab gel electrophoresis of step IX DNA polymerases δ and α. The lowest arrow indicates the tracking dye. The four lanes contain the following: (1) 4.7 units of δ; (2) 4.3 units of α; (3) buffer as a control for the silver stain; and (4) 3.5 units of δ mixed with 3.2 units of α.

primary structure similarity is being pursued.

Discussion

In previous studies, Byrnes & Black (1978) calculated that DNA polymerase δ has a molecular weight of 122 000. The nuclease activity of DNA polymerase δ was shown to be a 3′–5′-exonuclease and to participate in the template-dependent generation of a monophosphate series of reactions (Byrnes et al., 1976). To this we have added the inhibition of both activities by aphidicolin. This inhibitor has been shown to bind noncovalently to DNA polymerase, but the mechanism of inhibition is not completely understood. Aphidicolin has been considered a highly specific inhibitor of DNA polymerase α and similar viral DNA polymerases. It does not inhibit eukaryotic DNA polymerases β or γ, *Escherichia coli* DNA polymerases I, II, or III, T_4 DNA polymerase, terminal transferase, reverse transcriptase (Pedrali-Noy & Spadari, 1979, or thymidine kinase (Ohashi et al., 1978). This is the first description of inhibition of an enzymatic activity other than DNA polymerase by aphidicolin. Herpes simplex viral DNA polymerase and DNA polymerase II of yeast are sensitive to aphidicolin and have associated 3′–5′-exonucleases, but inhibition studies upon their exonuclease activities have not been reported.

The ultimate proof that one or more enzymatic activities are associated with a protein is the demonstration of a direct quantitative relationship and exclusion of other proteins. Generally this involves the purification of the protein to homogeneity; this has been our goal. Since our last report (Byrnes & Black, 1978), further purification and definition of the molecular structure of DNA polymerase δ were limited by the small quantity of enzyme protein available. There were two aspects to the limitations. First, the small quantity, about 1 μg, led to very high nonspecific enzyme loss upon subsequent procedures. This probably was due to adsorption of the protein to various surfaces in spite of the usual precautions. Other investigators have added back protein, such as BSA or gelatin, to alleviate this problem (Chen et al., 1979; Fischer & Korn, 1977; Lee et al., 1980). We felt this was not satisfactory, especially in light of future plans for proteolytic "fingerprint" studies of the enzyme. We found that the addition of 0.5% PEG to the buffer solutions greatly enhanced the recovery of enzyme by reducing the nonspecific loss and, thus, allowed the necessary further purification.

The second limitation was that standard analytic methodology for the demonstration of protein purity and subunit molecular structure requires much more than the approximately 100 ng of pure protein that is ultimately obtained. Two methodologies allowed examination of such small quantities of protein.

Microgel electrophoresis in capillary tubes gives similar resolution as standard gels but offers a 1000-fold economy of sample; 1 ng of BSA can be seen in a 6-μL gel. Another advantage of the microgels is that they allowed us to develop an immediate in situ assay of the DNA polymerase and exonuclease activities. The relatively short running time, 20 min, the high surface:volume ratio which facilitated gel cooling, and the addition of glycerol, Cleland's reagent, and PEG to the running buffers led to apparent good enzyme stability. The immobilization of template-primer in the gel and the short distance for other reaction substrates to diffuse into the gel allowed for rapid in situ enzyme activity assay rather than first extracting the enzyme from the gel. Assay of DNA polymerase in one gel and exonuclease in a simultaneously run gel demonstrated a similar electrophoretic migration pattern. Both were spread over the M_r 150 000–1 000 000 range, as was the faint protein stain; both activity profiles peaked at M_r 300 000 and were virtually identical with the allowance that they were determined in separate gels. Aggregation at low ionic strength apparently is responsible for this pattern. This was further evidence of the integral relationship of the two activities. However, the broad pattern limited the native gels as a criteria of protein purity.

The second method, the recently introduced silver stain, which is 100-fold more sensitive than the Coomassie brilliant blue procedure, facilitated the definition of DNA polymerase δ and the degree of purity. As little as a 1-ng protein band can be detected in slab gel analysis. This method allowed us to clearly visualize the polypeptide components in the final steps of purification and to refine the elution procedure to eliminate unrelated proteins. Thus, we have demonstrated DNA polymerase δ is one polypeptide of molecular weight 122 000. The exact correspondence of the value of DNA polymerase δ upon $NaDodSO_4$ gel analysis and that calculated from the Stokes radius and sedimentation coefficient is reassuring.

Since we reported the association of a mammalian DNA polymerase with a 3′–5′-exonuclease, similar associated activities have been reported by Chen et al. (1979) from a mouse myeloma cell and by Lee et al. (1980) from calf thymus. The enzyme described by Chen et al., referred to as α_1, has a native M_r of 190 000 and is reported to be composed of 48 000 and 52 000 M_r subunits. Calf thymus DNA polymerase δ has been reported to have a calculated molecular weight of 152 000 but to be composed of 49 000 and 60 000 molecular weight subunits. Thus, DNA polymerase δ from bone marrow is the first described mammalian polymerase with exonuclease that is a single high molecular weight polypeptide. All prokaryotic DNA polymerases with associated 3′–5′-exonucleases have both activities on the same polypeptide. Likewise, the molecular weight, 122 000, and the specific activity of the δ polymerase and exonuclease are comparable to those of the well-studied prokaryotic enzymes (Kornberg, 1980).

On the basis of aphidicolin studies, it is commonly assumed that DNA polymerase α is responsible for nuclear DNA replication (Huberman, 1981). However, as shown here, DNA polymerase δ is inhibited in a similar aphidicolin concentration range as α. Thus, δ cannot be precluded from chromosomal DNA replication on the basis of aphidicolin sensitivity, as can

DNA polymerases β and γ. Consequently, the relation of DNA polymerase δ from bone marrow to the α-type DNA polymerase is an important issue. It is conceivable that δ is derived from α by the loss of a 13000 molecular weight polypeptide. Proteolytic peptide analyses to explore a possible primary structure relationship between δ and α are necessary to explore such a possible derivative relationship. However, it is now clear that δ is distinct from α in the integral presence of a 3′–5′-exonuclease and in molecular structure. The roles of DNA polymerases α and δ in cellular DNA replication remain an unanswered question.

Acknowledgments

We thank Maria E. Serrano and Teresa Ferrer for their technical assistance. Also we appreciate the advice and generous assistance of Drs. Frans Huijing and Bob Rubin.

References

Bradford, M. (1976) *Anal. Biochem. 72*, 248–252.
Byrnes, J. J., & Black, V. L. (1978) *Biochemistry 17*, 4226–4231.
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. C., Bohn, E. W., Planck, S. R., & Wilson, S. H. (1979) *J. Biol. Chem. 254*, 11678–11687.
Fischer, P. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 6528–6535.
Grosse, F., & Krauss, G. (1981) *Biochemistry 20*, 5470–5475.
Huberman, J. A. (1981) *Cell (Cambridge, Mass.) 23*, 647–648.
Kornberg, A. (1980) in *DNA Replication*, W. H. Freeman, San Francisco.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lee, M. Y. W. T., Tan, C. K., So, A. G., & Downey, K. M. (1980) *Biochemistry 19*, 2096–2101.
Neuhoff, V. (1973) in *Micromethods in Molecular Biology*, p 66, Springer-Verlag, New York.
Oakley, B. R., Kirsch, D. R., & Morris, N. R. (1980) *Anal. Biochem. 105*, 361–363.
O'Farrel, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Ohashi, M., Tagucchi, T., & Ikegami, S. (1978) *Biochem. Biophys. Res. Commun. 82*, 1084–1090.
Pedrali-Noy, G., & Spadari, S. (1979) *Biochem. Biophys. Res. Commun. 88*, 1194–1202.
Switzer, R. C., Merril, C. R., & Shifrin, S. (1979) *Anal. Biochem. 98*, 231–237.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Purification of Three Iron–Sulfur Proteins from the Iron–Protein Fragment of Mitochondrial NADH–Ubiquinone Oxidoreductase†

C. Ian Ragan,* Yves M. Galante, and Youssef Hatefi

ABSTRACT: A fragment containing non-heme iron and acid-labile sulfide but little flavin can be solubilized from the mitochondrial NADH–ubiquinone oxidoreductase complex with chaotropic agents. This iron–protein fragment [Hatefi, Y., & Stempel, K. E. (1969) *J. Biol. Chem. 244*, 2350] has been resolved with detergents and ammonium sulfate fractionation into iron and acid-labile sulfide containing fractions, here called ISP-I and ISP-(II + III). ISP-I consists predominantly of a single polypeptide of molecular weight 75000. ISP-(II + III) consists predominantly of three polypeptides in equimolar concentrations with molecular weights of 49000, 30000, and 13000. Treatment of the latter with sodium trichloroacetate followed by ammonium sulfate fractionation results in separation of the 49000 molecular weight polypeptide from the two smaller subunits. Both of these subfractions (ISP-II and ISP-III, respectively) contain non-heme iron. The three iron–sulfur proteins have been characterized by their absorption spectra and iron and acid-labile sulfide contents. On the basis of the distribution of iron among the fractions obtained from chaotropic resolution of the NADH–ubiquinone oxidoreductase complex, a minimum of six or seven iron–sulfur centers are present in this enzyme.

There is still considerable uncertainty as to the number and cluster structure of the iron–sulfur centers of mitochondrial NADH–ubiquinone oxidoreductase (e.g., Ohnishi, 1979). While such uncertainty exists, it is extremely difficult to propose plausible pathways of electron transfer within the enzyme and to suggest mechanisms by which this process is coupled to proton translocation across the inner mitochondrial membrane. EPR[1] spectroscopy has been invaluable in providing information on the iron–sulfur clusters of the enzyme, but the technique suffers from several drawbacks. First, the line shape and intensity of the EPR signals are extremely sensitive to apparently trivial modification of the enzyme during purification. Second, the midpoint potentials of certain centers are also prone to extensive alterations, making their detection difficult in certain instances (e.g., Ohnishi et al., 1981). Third, the spin–spin interaction between neighboring clusters can render them "EPR silent".

Despite these problems, it is widely agreed that there are at least four centers in the enzyme (designated N-1, N-2, N-3, and N-4 by Ohnishi) as originally put forward by Orme-

† From the Department of Biochemistry, University of Southampton, Southampton, S09 3TU, United Kingdom (C.I.R.), and the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037 (Y.M.G. and Y.H.). *Received December 1, 1981.* This research was supported by grants from the Wellcome Trust, the Minna-James-Heineman-Stiftung, and the Science Research Council to C.I.R. and U.S. Public Health Service Grants AM 08126 (to Y.H.) and GM 27812-01 (to Y.M.G.).

[1] Abbreviations: EPR, electron paramagnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; ISP, iron–sulfur protein; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

0006-2960/82/0421-2518$01.25/0