Human Placental DNA Polymerase δ: Identification of a 170-Kilodalton Polypeptide by Activity Staining and Immunoblotting†

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Received July 18, 1986; Revised Manuscript Received September 24, 1986

ABSTRACT: DNA polymerase δ was isolated from human placenta and identified as such on the basis of its association with a 3′- to 5′-exonuclease activity. The association of the polymerase and exonuclease activities was maintained throughout purification and attempted separations by physical or electrophoretic methods. Moreover, ratios of the two activities remained constant during the purification steps, and both activities were inhibited by aphidicolin, oxidized glutathione, and *N*-ethylmaleimide. The purified enzyme had an estimated molecular weight of 172000, on the basis of a Stokes radius of 53.6 Å and a sedimentation coefficient of 7.8 S. On sodium dodecyl sulfate (SDS) gel electrophoresis, polymerase δ preparations contained a band of ca. 170 kilodaltons (kDa) as well as several smaller polypeptides. The 170-kDa polypeptide was identified as the largest polypeptide component in the preparation possessing DNA polymerase activity by an activity staining procedure following gel electrophoresis in the presence of SDS. Western blotting of DNA polymerase δ with polyclonal antisera also revealed a single 170-kDa immunoreactive polypeptide. Monoclonal antibodies to KB cell polymerase α inhibited placental polymerase α but did not inhibit DNA polymerase δ, while the murine polyclonal antisera to polymerase δ inhibited δ but not α. These findings establish the existence of DNA polymerase δ in a human tissue and support the view that both its polymerase and its exonuclease activities may be associated with a single protein.

Mammalian DNA polymerases have been extensively studied and can be classified as being of three types, α, β, and γ [see DePamphillis & Wassarman (1980) and Fry (1982) for reviews]. DNA polymerase α has been implicated in cellular DNA replication, and it has been distinguished from polymerases β and γ by its larger size, template-primer specificity, and sensitivity to *N*-ethylmaleimide and aphidicolin. DNA polymerase α enzymes have been shown to differ from the procaryotic enzymes such as *Escherichia coli* Pol I and Pol III (Kornberg, 1982, 1984) in that they do not possess an intrinsic 3′- to 5′-exonuclease activity. This difference is significant because of the proofreading function of the polymerase-associated 3′- to 5′-exonuclease in procaryotic DNA replication. DNA polymerase δ, a more recently described enzyme, is unlike previously studied mammalian DNA polymerases in that it is associated with a 3′- to 5′-exonuclease activity. This activity was first described in rabbit bone marrow (Byrnes et al., 1976; Byrnes, 1984) and has also been characterized from calf thymus (Lee et al., 1980, 1981, 1984). The significance of the 3′- to 5′-exonuclease activity of DNA polymerase δ thus lies in the possibility that it has the potential to function as a proofreading mechanism as in the case of the procaryotic DNA polymerases (Brutlag & Kornberg, 1972), and hence plays a significant role in the maintenance of the fidelity of mammalian DNA replication. The possible relationships of DNA polymerase δ to polymerase α have not been clarified.

DNA polymerase δ has not been as extensively studied as have the other mammalian DNA polymerases and has not been previously studied in a human tissue. Previous studies of human DNA polymerases have involved studies of the error frequency of partially purified preparations of human placental polymerase α (Seal et al., 1979; Krauss & Linn, 1980) and studies of purified polymerase α from cultured KB (Wang et al., 1984) and HeLa cells (Vishwana et al., 1986). We report here the first isolation and characterization of DNA polymerase δ from human placenta, with an emphasis on the identification of its catalytic subunit and the issue of its separate identity from DNA polymerase α as determined by immunochemical procedures.

Materials and Methods

Materials. DEAE-cellulose (DE-52) and phosphocellulose (P11) were obtained from Whatman. Phenyl-agarose was obtained from Bethesda Research Laboratories, and Affi-Gel–heparin and Bio-Rex 70 were from Bio-Rad Laboratories. Oligo(dA)–cellulose and terminal deoxynucleotidyl transferase were obtained from Collaborative Research. Hydroxylapatite was prepared as by Muench (1971). All materials were otherwise of the highest grade available.

DNA Polymerase Assays. The standard assay for DNA polymerase δ utilized activated poly(dA-dT) alternating copolymer as the template-primer (Lee et al., 1984). The assay (125 μL) contained 0.025 A_{260} ODU of poly(dA-dT), 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] (pH 7.5), 0.8 mM $MgCl_2$, 40 μM dATP, 4 μM [^{3}H]dTTP (500–1000 cpm/pmol), 5 μg of bovine serum albumin, 2% glycerol, 0.04% Triton X-100, 2 mM dithiothreitol, and 0.2–0.5 unit of DNA polymerase δ. The assay mixture was incubated at 37 °C for 15 min and terminated by the addition of 5% trichloroacetic acid containing 20 mM sodium pyrophosphate.

†Supported by National Institutes of Health Grant GM 31973 and an Established Investigatorship Award of the American Heart Association to M.Y.W.T.L.

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Ap_4A, P^1,P^4-di(adenosine-5′) tetraphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylenimine); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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The precipitate was collected on Whatman GF/C filters, washed, and counted.

When activated calf thymus DNA was used as the template-primer, the reaction mixtures contained 0.03 A_{260} ODU of activated calf thymus DNA, 40 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 40 μM each of dATP, dGTP, and dCTP, 4 μM [^{3}H]dTTP (500–1000 cpm/pmol), 5 μg of bovine serum albuminum, and 2% glycerol in a volume of 125 μL.

When poly(dA)·$(dT)_{12-18}$ was used as the template-primer, the reaction mixtures (125 μL) contained 40 mM HEPES, pH 6.5, 6 mM $MgCl_2$, 10% glycerol, 5 μg of bovine serum albumin, 4 μM [^{3}H]TTP (500–1000 cpm/pmol), and 0.1 A_{260} ODU of poly(dA)·$(dT)_{12-18}$ (ratio of 20:1). One unit of DNA polymerase activity was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of dNTP into DNA per hour.

3′- to 5′-Exonuclease Assays. Routine assays for exonuclease activity during the enzyme purification were performed as previously described (Lee et al., 1984), by 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) was used, the reaction mixtures (50 μL) contained 50 mM HEPES, pH 7.5, and 15 μM poly(dA-dT)–[^{3}H]dTMP (50 cpm/pmol). When [^{3}H]$(dT)_{50}$ was used, the reaction mixtures (50 μL) contained 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 5 μg/mL bovine serum albumin, 0.1–0.2 unit of DNA polymerase δ, and 3 μM [^{3}H]$(dT)_{50}$ (250 cpm/pmol). One unit of exonuclease activity was defined as that amount of enzyme which catalyzed the release of 1 nmol of dTMP per hour.

5′- to 3′-Exonuclease Activity. This was assayed for by the release of acid-soluble [^{32}P]dTMP from 5′ end labeled [^{32}P]$(dT)_1$–$(dT)_{600}$, in the presence and absence of poly(dA). No detectable 5′- to 3′-exonuclease activity was detected in the polymerase δ preparations.

Endonuclease Activity. Endonuclease activity was assayed for by conversion of supercoiled PM2 DNA to relaxed circular or linear forms by agarose gel electrophoresis as described by Johnson and Grossman (1977).

Proofreading Assays. This was done by measurement of polymerase activity and exonuclease activity in the same reaction mixtures, essentially as described by Brutlag and Kornberg (1972) and Yarranton and Banks (1977). The reaction mixtures contained 50 mM HEPES, pH 7.4, 2.5 mM $MgCl_2$, 5 μM [α-^{32}P]dTTP (1.7 Ci/mmol), and 2 units of DNA polymerase δ in a final volume of 125 μL. The experiments were performed by using two template-primers. These were poly$(dT)_{12}$ end labeled at the 3′ termini with either [^{3}H]$(dA)_{0.2}$ or [^{3}H]$(dC)_{0.2}$ and annealed to poly(dA). Samples (10 μL) of the incubation mixtures were taken and counted for ^{32}P incorporation to determine polymerase activity as picomoles of [α-^{32}P]dTMP incorporated, and for ^{3}H end label remaining as an index of the action of exonuclease activity.

Combined Measurement of DNA Polymerase and 3′- to 5′-Exonuclease Activities. The reaction mixtures (50 μL) contained poly(dA-dT) (0.025 A_{260} ODU), 40 μM TTP, 4 μM [α-^{32}P]dATP (70 000 cpm/pmol), 60 mM HEPES, pH 7.5, 0.2 mM $MnCl_2$ (or 1 mM $MgCl_2$), and 0.2–0.4 unit of DNA polymerase δ, in the absence or presence of 2 mM 5′-AMP. The reactions were terminated by the addition of 10 mM EDTA and stored on ice. An aliquot (20 μL) was precipitated with 10% trichloroacetic acid and filtered for measurement of incorporation as for the standard DNA polymerase assay, and a second aliquot (20 μL) was analyzed for labeled 5′-monophosphate generation by thin-layer chromatography on PEI–cellulose plates developed with 1 M sodium formate, pH 3.5 (Randerath & Randerath, 1967).

Purification of DNA Polymerase δ from Human Placental Tissue. All procedures were performed in the cold room unless otherwise stated. The entire purification, from steps 1 to 8, was normally completed in 4 days.

(*A*) *Step 1.* Term placentas were obtained immediately after Caesarian section in the delivery room and rinsed in ice-cold 25 mM potassium phosphate and 0.15 M potassium chloride, pH 7.0. The tissue (ca. 350 g/placenta) was cut into small pieces and placed in approximately 2 volumes of ice-cold homogenization buffer (50 mM Tris-HCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.25 M sucrose, and 10% glycerol, pH 7.5) containing the following protease inhibitors: 0.25 mg/mL soybean trypsin inhibitor, 10 mM benzamidine, 0.1 mg/mL bacitracin, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 0.8 mM phenylmethanesulfonyl fluoride, and 10 mM sodium bisulfite. The chilled tissue was then removed to the laboratory and homogenized in a Waring Blendor. The homogenate was centrifuged at 25000*g* for 30 min and the supernatant filtered through glass wool. The pellet was resuspended, homogenized, and centrifuged as before, and the two supernatants was combined. Buffers for steps 2–5 contained the same mixture of protease inhibitors as those used in this step.

(*B*) *Step 2*: *Batchwise DEAE-cellulose Adsorption.* The extract was adjusted to pH 7.8 and mixed with 1 L of DEAE-cellulose (Whatman DE-52) equilibrated in 40 mM Tris-HCl, 50 mM KCl, 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol, pH 7.8. The DEAE-cellulose was pelleted by centrifugation at 5000*g* and washed twice by resuspension and centrifugation in the same buffer. The enzyme activity was extracted with 20 mM sodium phosphate, pH 7.0, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 25% saturated (0 °C) ammonium sulfate. The extracts (containing 25% saturated ammonium sulfate) were taken directly to the next step. It may be noted that DNA polymerase activity could only be reliably assayed after this step, because of the presence of endogenous inhibitors.

(*C*) *Step 3*: *Phenyl-agarose Hydrophobic Chromatography.* The extract was loaded onto a phenyl-agarose column (5 × 12 cm) equilibrated in 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 10% glycerol, and 25% saturated ammonium sulfate, pH 7.0. The column was washed with the same buffer. The DNA polymerase δ activity was eluted with 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, and 10% glycerol, pH 7.0.

(*D*) *Step 4*: *Bio-Rex 70 Chromatography.* The pooled fractions were diluted with 50 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM EGTA, 0.1 mM EDTA, and 10% glycerol, pH 7.8, to a conductivity equivalent to that of 50 mM KCl, brought to 20% glycerol, and adsorbed onto a Bio-Rex 70 column (4.5 × 16 cm) previously equilibrated with 50 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM EGTA, 0.1 mM EDTA, and 20% glycerol, pH 7.8. The column was washed with 2 column volumes of equilibrating buffer and then eluted with a gradient (1600 mL) from 0 to 0.6 M KCl. The active fractions eluted at ca. 200 mM KCl and were dialyzed against 20 mM potassium phosphate, 1 mM dithiothreitol, and 30% glycerol, pH 7.0.

(*E*) *Step 5*: *Hydroxylapatite Chromatography.* The dialyzed preparation was chromatographed on a hydroxylapatite column (2.4 × 5 cm) equilibrated in 20 mM potassium phosphate, pH 7.0, 30% glycerol, and 1 mM dithiothreitol. The column was eluted with a linear gradient of KCl (0–0.5 M) in a total of 400 mL of the same buffer. Fractions were

collected in tubes containing a sufficient volume of EDTA and EGTA solutions such that their final concentrations in each tube were 0.5 and 0.1 mM, respectively. (This was done in order to guard against the potential activation of any calcium-dependent protease activity.) The fractions were assayed for polymerase and exonuclease activities. A peak of DNA polymerase activity was routinely eluted at 155 mM KCl. This activity was associated with a 3′- to 5′-exonuclease activity and is the enzyme activity which is the subject of this work. When the column was washed with 20 mM potassium phosphate, pH 7.0, 30% glycerol, and 1 mM dithiothreitol following the KCl gradient elution and then stripped with 0.3 M potassium phosphate, a second peak of DNA polymerase activity was eluted. This fraction was identified as DNA polymerase α activity.

(*F*) *Step 6*: *Affi-Gel–Heparin Chromatography*. The pooled fractions (100 mL) from the KCl gradient were diluted with and chromatographed on an Affi-Gel–heparin column (3 × 7 cm) previously equilibrated with 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 M KCl, and 30% glycerol, pH 7.0. A linear gradient from 0.1 to 0.5 M KCl in 1 L of the same buffer was then applied to the column. The enzyme activity was eluted at 265 mM KCl. Buffers for this and the following steps contained the same mixture of protease inhibitors as for step 1 except for the omission of soybean trypsin inhibitor.

(*G*) *Step 7*: *Phosphocellulose Chromatography*. The pooled fractions (45 mL) were diluted with 30% glycerol and 1 mM dithiothreitol to a conductivity equivalent to that of 100 mM KCl and loaded onto a phosphocellulose column (1.6 × 3 cm) previously equilibrated with 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, and 30% glycerol, pH 7.0. The column was eluted with a gradient of KCl (0.1–0.5 M, total volume 320 mL) in the same buffer as step 6. The DNA polymerase δ activity eluted at 230–240 mM KCl.

(*H*) *Step 8*: *Oligo(dA)–Cellulose Chromatography*. The pooled fractions from step 7 were dialyzed against 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, and 30% glycerol, pH 7.0, and loaded onto an oligo(dA)–cellulose column (1.6 × 5 cm). The enzyme was eluted from the column with 0.3 M KCl in the same buffer. The purified enzyme was stored in small aliquots at −70 °C after the addition of bacitracin to a concentration of 0.1 mg/mL, as the latter was found to enhance the stability of the enzyme. Buffers for this step included the same protease inhibitors as the previous step. The entire procedure was completed in 4 days because of the lability of the enzyme. Except where otherwise stated, characterization of the enzyme was performed with preparations carried to this stage.

(*I*) *Step 9*: *Nondenaturing Disc Gel Electrophoresis*. Portions of step 8 enzyme were dialyzed and electrophoresed on 5% polyacrylamide cylindrical gels using the buffer system described by Fisher and Korn (1977) and then sliced and extracted. The extracts were then assayed for enzyme activity.

SDS–Polyacrylamide Gel Electrophoresis. This was performed by the procedure of Laemmli (1970) in slab gels. Gels were stained either with Coomassie blue or by the silver staining procedure (Merril et al., 1981).

Activity Staining for DNA Polymerase after SDS–Polyacrylamide Gel Electrophoresis and Renaturation. This was performed as described by Spanos et al. (1981). Samples were prepared as described by Blank et al. (1983) by heating for 3 min at 37 °C. These were then electrophoresed in a 7% SDS–polyacrylamide gel containing 6 A_{260} ODU of poly(dA-dT) and 0.75 mg of fibrinogen per 15 mL of acrylamide. Following electrophoresis, the gels were incubated in a series of renaturing buffers (100 mM Tris-HCl, 5 mM 2-mercaptoethanol, and 20% 2-propanol, pH 7.5 for 30 min; 50 mM Tris-HCl and 10 mM 2-mercaptoethanol, pH 7.5 for 30 min; and finally in 50 mM Tris-HCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.5 for 16–18 h). The gels were then incubated at 37 °C for 18 h in the assay mixture for polymerase activity (40 mM Tris-HCl, pH 7.4, 0.8 mM $MgCl_2$, 1 mM mercaptoethanol, 20 μM [α-^{32}P]dTTP containing 180 μCi in a total volume of 45 mL). The gels were washed in 5% trichloroacetic acid containing 20 mM sodium pyrophosphate at 4 °C, with a change of wash liquid every 30 min for 4 h, and then with a final wash for 18 h. The gels were then stained for protein and dried. The dried gels were then autoradiographed at −70 °C with a Cronex "Lightning" screen and preflashed film (Kodak XAR-5). ^{14}C-Methylated protein standards were used as markers. Purified *E. coli* Pol I was included as a control in a separate lane to ensure that the stain was functioning. For samples with weak activity, the gels were incubated in dimethyl sulfoxide for 30 min followed by 22%, 2,5-diphenyloxazole in dimethyl sulfoxide before drying and autoradiography.

Preparation of Antibodies. Murine antisera to polymerase δ were obtained by immunization of five BALB/c mice with purified polymerase δ (step 8 of the purification scheme). Each mouse was injected intraperitoneally with ca. 200 units of enzyme emulsified with an equal volume of Freund's complete adjuvant. After 21 days, they were injected with the same amount of enzyme emulsified with Freund's incomplete adjuvant. The mice were bled from the tail veins. Positive antisera were obtained from three of the mice: these were partially purified by ammonium sulfate precipitation before use.

Monoclonal antibodies to KB cell DNA polymerase α were prepared from ascites fluids from three cell lines, which were obtained from the American Type Culture Collection as CRL 1640, CRL 1644, and CRL 1645, corresponding to SJK 132-20, SJK 287-38, and SJK 237-71 as originally described by Tanaka et al. (1982). The antibodies were purified to homogeneity from the ascites fluids by ammonium sulfate precipitation and chromatography on Affi-Gel–protein A.

Immunoblotting. Immunoblotting was performed essentially as described by Towbin et al. (1979). Samples were electrophoresed in 5–15% polyacrylamide gradients and then transferred to nitrocellulose paper. Prestained protein standards (BRL) were used as molecular weight standards and also to provide confirmation of efficient transfer. The blots were incubated with 3% bovine serum albumin or nonfat dry milk in phosphate-buffered saline as a blocking agent. The blots were then incubated with a 1:1000 dilution of mouse antisera for 16 h and then washed with the blocking solution. The blots were visualized by incubation with biotinylated sheep anti-mouse IgG, followed by incubation with streptavidin–biotinylated peroxidase preformed complex (Yolken et al., 1983) obtained from Amersham. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

RESULTS

Purification of DNA Polymerase δ. The procedure utilized adsorption on DEAE-cellulose, phenyl-agarose, and Bio-Rex 70 as the first steps (see Materials and Methods). Following this, DNA polymerase δ was further purified by column chromatography on hydroxylapatite, Affi-Gel–heparin, phosphocellulose, and oligo(dA)–cellulose. Polymerase δ ac-

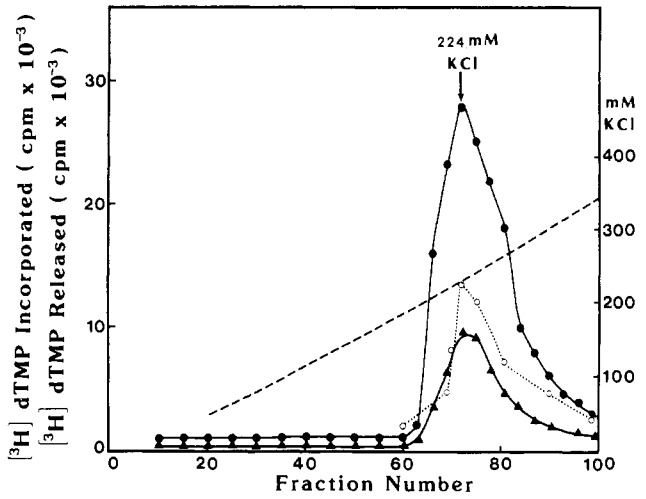

FIGURE 1: Coelution of DNA polymerase and 3′- to 5′-exonuclease activities on phosphocellulose chromatography. DNA polymerase activity was assayed by using either activated poly(dA-dT) (●) or activated calf thymus DNA (▲) as the template-primer. Exonuclease activity was assayed by using [^{3}H](dT)$_{50}$ as the substrate and expressed as [^{3}H]dTMP released (○).

tivity was routinely assayed by incorporation of [^{3}H]dTTP into a poly(dA-dT) template-primer and identified by the presence of associated exonuclease activity. It was observed that polymerase δ could be routinely separated from DNA polymerase α by chromatography on any one of three column supports: phosphocellulose, Affi-Gel–heparin, or hydroxylapatite. On hydroxylapatite, polymerase δ was eluted by a potassium chloride gradient at about 150 mM KCl, whereas polymerase α was eluted by a subsequent potassium phosphate gradient at a concentration of ca. 150 mM phosphate. On Affi-Gel–heparin, the DNA polymerase δ fraction eluted at 250 mM KCl, while polymerase α eluted at 165 mM KCl; on phosphocellulose, polymerase δ eluted at 240 mM KCl while polymerase α eluted at 320 mM KCl (not shown). The human placental DNA polymerase α was also purified to a specific activity of ca. 50 000 units/mg (M. Y. W. T. Lee and L. N. Toomey, unpublished results), a value comparable to those reported for near-homogeneous preparations of human DNA polymerase α (Fischer & Korn, 1977). Our human placental polymerase α preparations were devoid of exonuclease activity, and its properties in general correspond to those reported for mammalian polymerase α. Identification of this enzyme as a form of DNA polymerase α was made on the basis of its apparent molecular size on gel filtration (ca. 400 000), its sensitivity to aphidicolin inhibition (80% inhibition by 3 μg/mL aphidicolin), and a preference for activated calf thymus DNA as a template. It was also found that the bulk of the nonassociated exonuclease activity is separated from DNA polymerase δ during the hydroxylapatite chromatography step, so that association of exonuclease activity with polymerase δ activity could be clearly discerned during the later chromatography steps, as shown, for example, on phosphocellulose (Figure 1).

Data for a typical purification are shown in Table I, starting from 350 g of human placental tissue. The procedure has been used for over 20 such preparations and was found to be reproducible. Protease inhibitors were used in the buffers during all stages of purification. Further steps to minimize protease activity included the use of hydrophobic chromatography at an early stage, as this step was found to lead to separation of endogenous protease activity from polymerase activity.

In the preparation shown in Table I, 15 μg of protein with a specific activity of 64 000 units/mg was obtained after the

Table I: Purification of DNA Polymerase δ from Human Placenta[a]

step	protein (mg)	polymerase act. (units)	sp act. (units/mg)	recovery (%)
(1) 25000g supernatant	13900	17000	1.2	
(2) DEAE-cellulose	6390	28800	4.5	100
(3) phenyl-agarose	900	8580	9.5	29
(4) Bio-Rex 70	79	8100	102	28
(5) hydroxylapatite	22	4850	220	17
(6) Affi-Gel–heparin	0.31	3930	12700	13
(7) phosphocellulose	0.036	1730	48200	6
(8) oligo(dA)–cellulose	0.015	950	63500	3.3

[a] Data are shown for a typical purification starting with one placenta (ca. 350 g of tissue). DNA polymerase activity was assayed by using poly(dA-dT) as the template-primer.

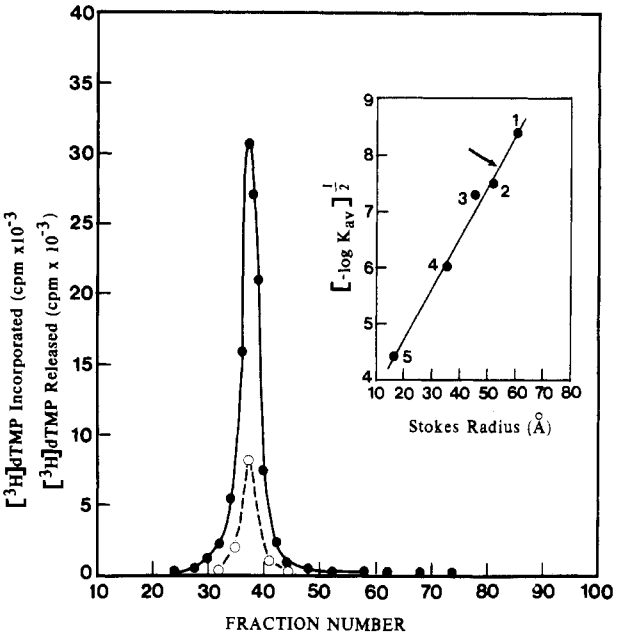

FIGURE 2: Gel filtration chromatography of DNA polymerase δ on Sephacryl S-300. DNA polymerase δ (500 units) was chromatographed on a Sephacryl S-300 column (1.6 × 66 cm) previously calibrated with standard proteins. The column was eluted with 0.3 M KCl, 20 mM potassium phosphate, 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol, pH 7.0, and fractions were assayed for polymerase activity (●) and for 3′- to 5′-exonuclease activity (○). The polymerase and exonuclease activities coeluted at the position shown, corresponding to a Stokes radius of 53.6 Å (see arrow, inset; 1, ferritin; 2, catalase; 3, aldolase; 4, bovine serum albumin; 5, cytochrome *c*). (The apparent molecular weight, according to a plot of log molecular weight against V_e/V_0, was 280 000.)

chromatography procedures (steps 1–8, Materials and Methods). The enzyme could be further purified by nondenaturing polyacrylamide gel electrophoresis, followed by extraction of the gel slices. Typically, a specific activity of 180 000 units/mg was estimated for the enzyme after such a final step (step 9, Materials and Methods).

Physicochemical Properties of DNA Polymerase δ. DNA polymerase δ was examined by gel filtration on Sephacryl S-300. Both DNA polymerase and 3′- to 5′-exonuclease activities coeluted as a single peak, at a position corresponding to a Stokes radius of 53.6 Å (Figure 2). On glycerol gradient ultracentrifugation, the enzyme behaved with a sedimentation coefficient of 7.8 S, with both polymerase and exonuclease activities cosedimenting as a monodisperse species (not shown). From these data, the molecular weight of the enzyme was calculated to be 172 000 as by Siegel and Monty (1966). (The apparent native molecular weight by gel filtration as deter-

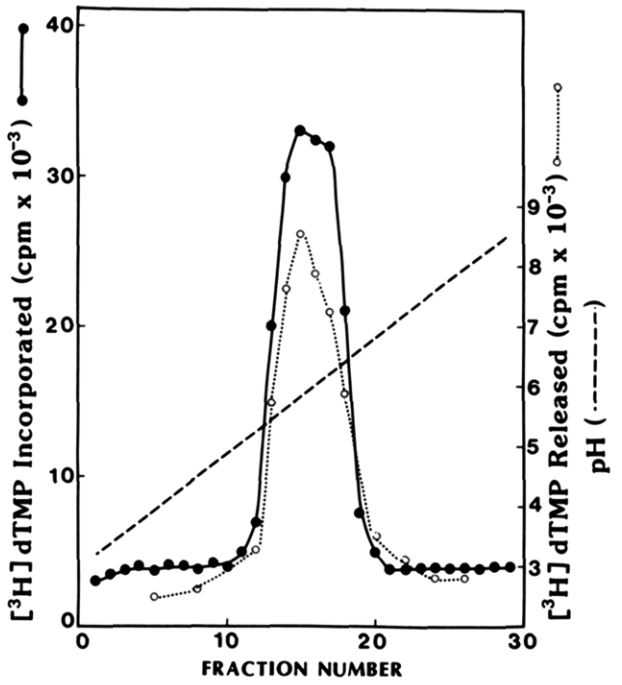

FIGURE 3: Comigration of DNA polymerase and 3′- to 5′-exonuclease activities on isoelectric focusing. DNA polymerase δ (1000 units, step 6 of the enzyme purification, Materials and Methods) was subjected to isoelectric focusing using the LKB Multiphor system. Polymerase activity was assayed by using activated poly(dA-dT) (●) as the template-primer. Exonuclease activity was assayed by using [^{3}H](dT)$_{50}$ as substrate and expressed as [^{3}H]dTMP released (○).

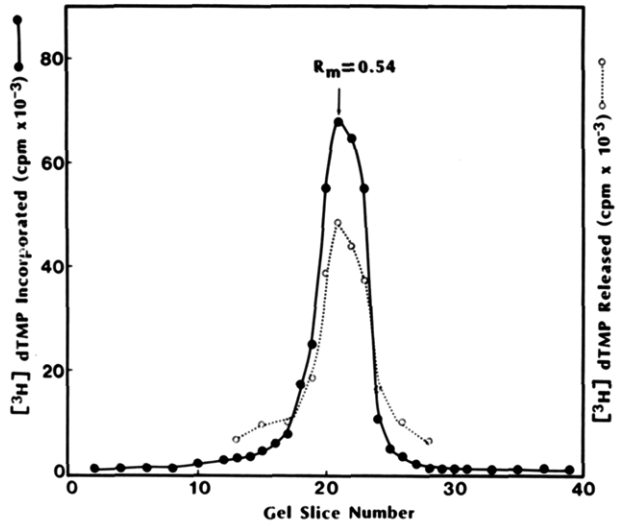

FIGURE 4: Comigration of DNA polymerase and 3′- to 5′-exonuclease activities on polyacrylamide gel electrophoresis. DNA polymerase δ (20 units, step 8 of the purification) was subjected to nondenaturing disc gel electrophoresis. The gel was sliced, and DNA polymerase (●) and exonuclease (○) activities in extracts of the slices were determined.

mined by comparison with standard proteins was 280 000, indicating that the enzyme is asymmetric.) Both the polymerase and exonuclease activities of DNA polymerase δ comigrated as a single species of p*I* 5.9 on isoelectric focusing (Figure 3). This association is also observed on nondenaturing polyacrylamide disc gel electrophoresis (Figure 4). This close association of exonuclease activity with polymerase activity allows for the stringent identification of the purified enzyme as a form of DNA polymerase δ.

Identification of the Molecular Weight of the Polypeptide Responsible for Polymerase Activity. A number of preparations which were carried to the oligo(dA)–cellulose step and

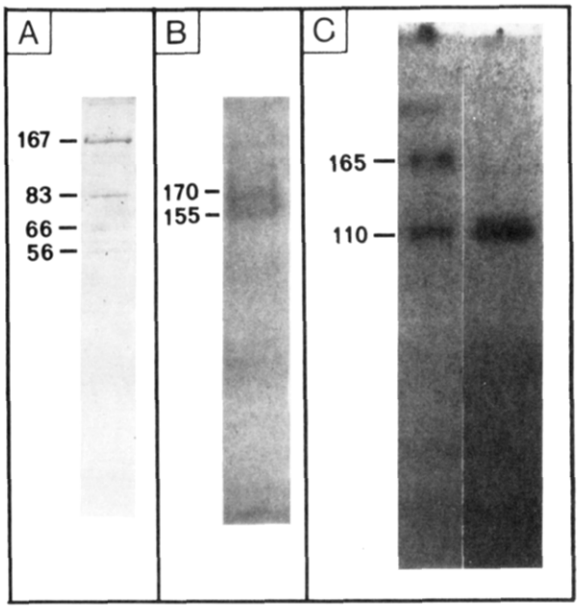

FIGURE 5: Polypeptide compositions of DNA polymerase δ by SDS–polyacrylamide gel electrophoresis. (Panel A) Purified DNA polymerase δ (step 8) was run on a 10% acrylamide slab gel which was then stained for protein by the silver staining procedure. (Panel B) DNA polymerase δ (5 units), purified to step 5 (hydroxylapatite chromatography, Materials and Methods), was run on a 7% acrylamide slab gel and then stained for activity. (Panel C) Purified DNA polymerase δ (7 units, step 9) was run on a 10% acrylamide slab gel and stained for activity, as shown in the left lane. The activity stain for purified *E. coli* Pol I (M_r 110 000), run in parallel on the same gel, is shown in the right lane.

to the final nondenaturing gel electrophoresis step have been examined by SDS–polyacrylamide gel electrophoresis. These experiments invariably revealed the presence of multiple polypeptide components. Nevertheless, preparations of DNA polymerase δ were found to possess a high molecular weight polypeptide of ca. 170K, provided that stringent precautions to avoid proteolysis were observed. An SDS–polyacrylamide gel stained for protein for a typical preparation is shown in Figure 5A. This shows a major polypeptide of 167 kDa and minor polypeptide bands in the 50–80-kDa range.

The identity of the active polypeptide species was determined by a direct procedure based on that described by Spanos et al. (1981) and Blank et al. (1983). This method depends on the renaturation of the enzyme in situ, following gel electrophoresis in the presence of sodium dodecyl sulfate, and visualization of the activity by incubation with [α-^{32}P]dNTP and autoradiography. The results of two such experiments are shown in Figure 5B,C. A preparation at the hydroxylapatite step (step 5, Materials and Methods, Figure 5B) revealed polypeptides of 170 and 155 kDa, while a preparation carried to the final purification stage (extraction after nondenaturing disc gel electrophoresis, step 9) contained active polypeptides of ca. 165 and 110 kDa (Figure 5C). A control containing purified *E. coli* Pol I is also shown in Figure 5C. We have also detected lower smaller active polypeptide species (60–70 kDa) in some of our preparations. The data indicate that the largest polypeptide species with which DNA polymerase δ activity can be identified in the purified enzyme is that of ca. 170 kDa and is consistent with data obtained by protein staining and Western blotting (see below). Our interpretation of these data is that this polypeptide is the native subunit possessing the catalytic site for polymerase activity; the similarity to the native molecular weight suggests that polymerase δ may consist of a single large subunit. The lower molecular weight polypeptides in the preparations may be derived from

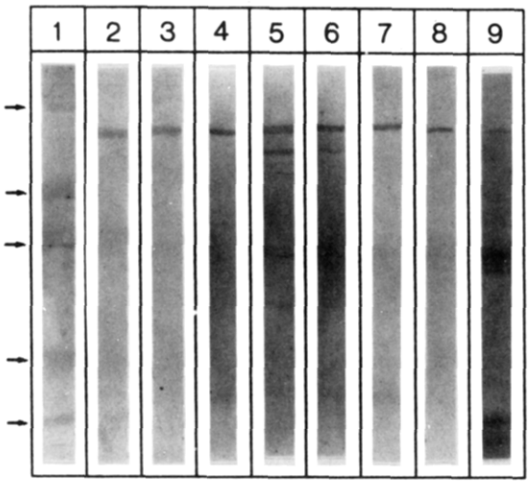

FIGURE 6: Immunoblotting of DNA polymerase δ using a mouse polyclonal antibody. DNA polymerase δ taken from steps 1–7 of the purification procedure (see Materials and Methods) was examined by immunoblotting using a polyclonal mouse antisera against polymerase δ (lanes 2–8). The protein stain of the preparation at step 7, stained with Coomassie blue, is shown in lane 9. The molecular weight of the major high molecular weight band was estimated at 170000. Prestained molecular weight standards (BRL) are shown in lane 1; in descending order of size, as shown by the arrows, these were the following: myosin, 200K; phosphorylase, 93K; bovine serum albumin, 68K; ovalbumin, 43K; and α-chymotrypsinogen, 26K.

proteolytically "nicked" enzymes, or possibly represent "accessory" proteins as has been proposed for polymerase α (Fry, 1982; Hubscher, 1983).

Western Blotting of DNA Polymerase δ with Polyclonal Antiserum. Polyclonal sera against DNA polymerase δ were obtained by immunization of BALB/c mice. Three such antisera were obtained and were used for the immunoblotting of DNA polymerase δ. These antisera immunoprecipitated radioiodinated polymerase δ but did not immunoprecipitate radioiodinated preparations of polymerase α, even when the modified solid-phase immunoadsorption procedure of Tanaka et al. (1982) was used. These antisera also selectively inhibited DNA polymerase δ activity (see below). The results of the immunoblotting experiments are shown in Figure 6, where samples of the enzyme preparation from steps 1–7 (Table I) were immunoblotted. It is seen that a single major immunoreactive polypeptide of ca. 170 kDa is present, starting from the crude homogenate (Figure 6, lane 2); some breakdown is observed at steps 4 and 5, but the lower molecular weight components are largely removed in the subsequent two steps. For comparison, the protein stain of the preparation at step 7 is shown in lane 9 (Figure 6). It is seen that the only high molecular weight component in the preparation at this stage greater than 80 kDa is a 170-kDa polypeptide, despite the presence of significant amounts of material of ca. 60–70 kDa. In experiments where equal units of activity of polymerase α and δ were immunoblotted, only the polymerase δ preparation showed a positive blot.

Enzymatic Properties of DNA Polymerase δ. The template-primer preference of DNA polymerase δ was examined by using a number of template-primers. The synthetic alternating copolymers poly(dA-dT) and poly(dG-dC) were found to be the best template-primers. Both poly(dT)·oligo(rA) (ratio 10:1) and poly(rA)·oligo(dT) (ratio 1:1 or 10:1) were ineffective as template-primers. We have observed that the template-primers, activated calf thymus DNA, and poly(dA)·oligo(dT) (ratio 10:1 or 20:1) gradually lost their efficacy as polymerase δ was purified. It was found that the activity of purified DNA polymerase δ with these template-primers could be markedly stimulated by a protein factor of 36.5 kDa which is separated from the enzyme during the phosphocellulose step (M. Y. W. T. Lee and L. N. Toomey, unpublished observations).

Table II: Inhibitors of Human Placental DNA Polymerase δ[a]

inhibitor	polymerase (% inhibition)	exonuclease (% inhibition)
2 mM *N*-ethylmaleimide	91	87
5 mM glutathione disulfide	72	73
3 μg/mL aphidicolin	73	51

[a] DNA polymerase δ was assayed by using poly(dA-dT) as the template-primer, and 3′- to 5′-exonuclease activities were assayed by using end-labeled poly(dA-dT)·[^{3}H]dTMP under standard conditions as described under Materials and Methods. Data are expressed as percentages of control activities.

DNA polymerase δ is inhibited by sulfhydryl reagents (Table II). *N*-Ethylmaleimide, which is also known to inhibit DNA polymerase α, inhibits both the polymerase and 3′- to 5′-exonuclease activities of DNA polymerase δ, as does glutathione disulfide (Table II). Aphidicolin, an inhibitor of DNA replication in vivo, has been shown to inhibit DNA polymerase α, but not polymerases β and γ, and such inhibition has been used as supporting evidence for replicative function for α (Scovassi et al., 1980). It is significant that DNA polymerase δ is also inhibited by aphidicolin, and human placental DNA polymerase δ appears to be more sensitive than DNA polymerase α (Lee et al., 1985). We observed that the exonuclease activity of human DNA polymerase δ is also inhibited by aphidicolin (Table II). The fact that both activities are inhibited is consistent with, although not confirmatory, the possibility that both activities may well reside in the same protein molecule. Like polymerase α, polymerase δ activity is resistant to inhibition by dideoxy-TTP (not shown), in distinction to polymerases β and γ.

Properties of 3′- to 5′-Exonuclease Activity. Previous studies of partially purified high molecular weight human placental DNA polymerases have shown these to be devoid of exonuclease activity. Moreover, the placenta is a rich source of several exonuclease activities from which DNA polymerases can be separated (Chen & Grossman, 1985). Under the conditions of Krauss and Linn (1980) for the assay of exonuclease activity, our DNA polymerase δ preparations were apparently free of exonuclease activity. However, this is due to the fact that the 3′- to 5′-exonuclease activity of polymerase δ is strongly inhibited by KCl (data not shown) at the concentrations used by Krauss and Linn (1980). Using the assay conditions of Krauss and Lin (1980), we have also noted that the nonassociated fractions of exonuclease activity are in fact separated from DNA polymerase δ at an early stage in our preparation. No detectable 5′- to 3′-exonuclease or endonuclease activities were found in our polymerase δ preparations.

Important criteria for the intrinsic nature of the 3′- to 5′-exonuclease of DNA polymerase δ are that these two activities are present in a relatively constant ratio. This was determined during the final purification steps and during several analytical procedures, as shown in Table III. The relative constancy of the ratios of exonuclease to polymerase activity is significant, since it argues strongly against a simple failure to completely separate a trace of exonucleolytic activity.

The enzyme is capable of the template-dependent generation of monophosphate from dNTPs, as shown in Table IV. The exonuclease activity is strongly inhibited by 5′-AMP (not shown), and the template-dependent generation of 5′-dAMP is also inhibited by 5′-AMP (Table IV). This experiment shows that the polymerase and exonuclease activities are ca-

Table III: Ratio of 3′- to 5′-Exonuclease Activity to DNA Polymerase Activity[a]

procedure	exonuclease: polymerase ratio
(A) last two chromatography steps	
step 7 (phosphocellulose)	1:1.7
step 8 [oligo(dA)–cellulose]	1:1.6
(B) analysis of purified enzyme by	
isoelectric focusing (LKB Multiphor)	1:1.3
nondenaturing disc gel electrophoresis	1:1.4
glycerol gradient ultracentrifugation	1:1.2

[a] Ratios were determined by separate assays of exonuclease activities and of polymerase activities expressed as nanomoles of nucleotide released or incorporated per hour per milligram of protein, as described under Materials and Methods. [^{3}H]Poly(dT)$_{50}$ was used for the assay of exonuclease activity.

Table IV: Template-Dependent Generation of Deoxynucleoside Monophosphate from Deoxynucleotide Triphosphate by DNA Polymerases δ and α[a]

	5′-dAMP (pmol)		DNA synthesis (pmol)	
	δ	α	δ	α
incubn with poly(dA-dT), dTTP, [α-^{32}P]dATP	14.4	0	19	56
as above, with 2 mM 5′-AMP	7.6	ND[b]	25	ND

[a] Labeled 5′-dAMP formation was measured after thin-layer chromatographic separation, and DNA synthesis was measured as the incorporation of α-^{32}P-labeled dATP into poly(dA-dT) template-primer from the same reaction mixtures as described under Materials and Methods. Values given are rates expressed as picomoles of 5′-dAMP released in 10 min or incorporated in 10 min. [b] Not determined.

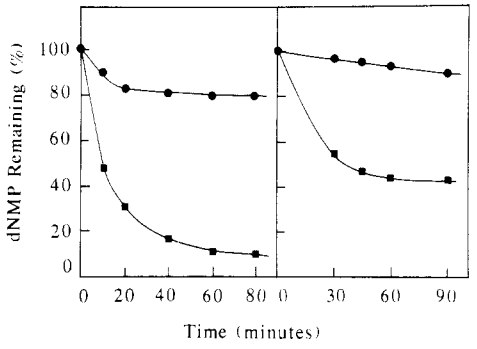

FIGURE 7: Hydrolysis of single-stranded and double-stranded substrates by the 3′- to 5′-exonuclease activity of DNA polymerase δ. (Left panel) The 3′- to 5′-exonuclease activity of purified DNA polymerase δ was determined as described under Materials and Methods. The hydrolysis of single-stranded [^{3}H](dT)$_{50}$ (3 μM) is shown as (■), and the hydrolysis of double-stranded (dA)$_n$·[^{3}H](dT)$_{50}$ (5 μM) is shown as (●). Results are shown as the percentage of label remaining in the substrates. (Right panel) The hydrolysis of the end-labeled template-primer (dA)$_n$·(dT)$_{50}$[^{3}H](dT)$_{2.2}$ (5 μM) is shown as (●), and that of the corresponding template-primer end labeled with dC, (dA)$_n$·(dT)$_{50}$[^{3}H](dO)$_{2.3}$ is shown as (■).

pable of acting in concert on the same template-primer.

The substrate specificity of the 3′- to 5′-exonuclease activity of polymerase δ was examined. Single-stranded substrates were more rapidly hydrolyzed than double-stranded substrates, as shown for [^{3}H](dT)$_{50}$ and dA$_n$·[^{3}H](dT)$_{50}$ (Figure 7, left panel). A comparison of the rates of hydrolysis of a matched vs. an unmatched primer terminus is shown in the right panel of Figure 7, using poly(dA) annealed with poly(dT), the latter being end labeled with either [^{3}H]dT or [^{3}H]dC. The enzyme displays a clear preference for the mismatched primer terminus.

A more direct assessment of an in vitro proofreading capacity of DNA polymerase δ is shown in Figure 8. Two

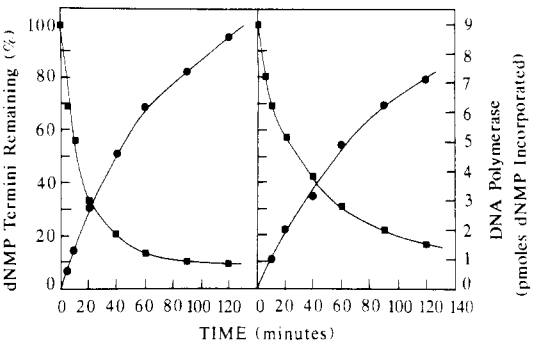

FIGURE 8: Concurrent DNA synthesis and mismatched termini excision by DNA polymerase δ. DNA polymerase δ was incubated with a ^{3}H end labeled polynucleotides and an α-^{32}P-labeled nucleoside triphosphate (dTTP) as described under Materials and Methods; loss of the tritium label (■) and incorporation of [^{32}P]dTMP (●) were measured. Two template-primers were used, either terminally labeled poly(dA)·(dT)$_{12}$[^{3}H](dA)$_{0.2}$ (left panel) or poly(dA)·(dT)$_{12}$[^{3}H](dC)$_{0.2}$ (right panel). Concentrations of template-primer were 30 μM. In the 10-μL samples taken for assay, at 100% the amounts of [^{3}H]dA and [^{3}H]dC were 1.5 and 1.24 pmol, respectively.

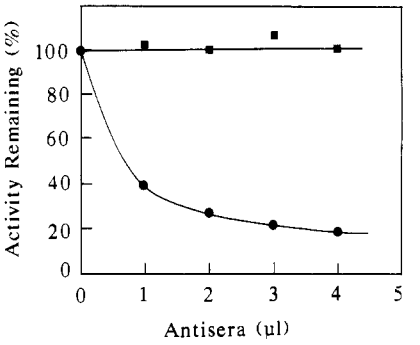

FIGURE 9: Effects of polyclonal mouse antisera against human polymerase δ on DNA polymerase δ and polymerase α activities. Purified human placental DNA polymerase δ (●) and purified polymerase α (■) were incubated with the indicated concentrations of mouse antisera for 2 h at 0 °C, and then assayed for DNA polymerase activity using activated poly(dA·dT) as the template. (Each incubation mixture contained 0.2 unit of DNA polymerase activity.) The data are shown as the percentage of the activities in control incubations in the presence of equivalent amounts of nonimmune mouse serum.

template-primers were used, either terminally labeled poly(dA)·(dT)$_{12}$[^{3}H](dA)$_{0.2}$ (Figure 8, left panel) or poly(dA)·(dT)$_{12}$[^{3}H](dC)$_{0.2}$ (Figure 8, right panel). The loss of terminal ^{3}H label was measured at the same time as the incorporation of ^{32}P from [α-^{32}P]dTTP. As is shown in Figure 8, DNA polymerase activity proceeds with the concurrent removal of the mismatched end terminus. If end-labeled oligo(dT) is used as the primer in a directly analogous experiment, it is observed that less than 5% of the end label is lost during concurrent synthesis. This experiment indicates that the 3′- to 5′-exonuclease which we find in physical association with polymerase δ is capable of functioning in a proofreading manner.

Immunochemical Comparisons of Polymerases δ and α. We have obtained polyclonal antisera to polymerase δ in three of five mice which were immunized (Materials and Methods). These antisera inhibited polymerase δ activity, as is shown for one of the three (Figure 9); none of these three inhibited placental DNA polymerase α activity. We have also examined the effects of two monoclonal antibodies to DNA polymerase α from the human-derived cell line (KB cells) described by Tanaka et al. (1982). Antibodies CRL 1640 and CRL 1644 are inhibitory and appear to recognize distinct epitopes (Miller et al., 1985). CRL 1640 inhibited human placental DNA polymerase α (Figure 10) but, significantly, did not inhibit

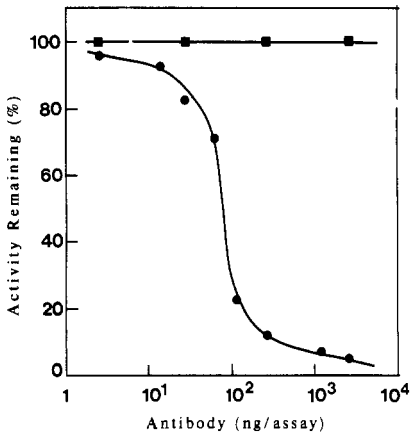

FIGURE 10: Effects of a monoclonal antibody against KB cell polymerase α on DNA polymerase δ and polymerase α activities. Purified human placental DNA polymerase δ or purified polymerase α (0.2 unit) was incubated with the indicated concentrations of purified mouse monoclonal antibody (CRL 1640, American Type Culture Collection) and then assayed for DNA polymerase activity [polymerase δ (■); polymerase α (●)].

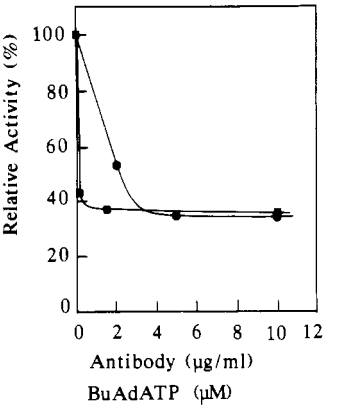

FIGURE 11: Levels of polymerases α and δ in placental extracts as determined by inhibition with a monoclonal antibody and BuAdATP. A placental extract prepared as described in step 2 of the purification was incubated with increasing concentrations of purified monoclonal antibody against polymerase α (CRL 1640, see Materials and Methods) and then assayed for DNA polymerase activity using activated poly(dA-dT) as the template-primer (■). The extract was also incubated with the indicated concentrations of BuAdATP in the assay (●). Concentrations of antibody and BuAdATP refer to those present in the actual assay mixture.

DNA polymerase δ even at antibody concentrations 2 orders of magnitude greater than that which produced a significant inhibition of DNA polymerase α. Similar results were obtained with antibody CRL 1644 (not shown). Antibody CRL 1645, which does not inhibit KB cell DNA polymerase α, did not inhibit either placental DNA polymerase α or placental DNA polymerase δ.

Relative Amounts of Polymerase δ in Placenta. On the basis of the recoveries of total activities during purification (Table I), it can be estimated that roughly 17% of the activity measured in our routine DNA polymerase assay is due to polymerase δ, based on the recovery after hydroxylapatite, the first step at which DNA polymerase δ is separated from DNA polymerase α. This is a minimal estimate, since it assumes no loss of activity. Estimates of the relative contributions of polymerases δ and α to the activity measured in a crude placental homogenate were also made by using the anti-α monoclonal antibody CRL 1640 and BuAdATP [2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate], a potent inhibitor of polymerase α (Khan et al., 1984), which has little effect on human (Lee et al., 1985), rabbit reticulocyte (Byrnes, 1985), or calf thymus (Crute et al., 1986) DNA polymerase δ. In both cases, a maximum inhibition of ca. 38% was observed, indicating that only 62% of the measured activity could be attributed to α (Figure 11). The contribution of polymerases β and γ to the assay was estimated to be less than 5% on the basis of measurements of activity in the presence of dideoxy-TTP (not shown). Thus, it may be estimated that 40% of the assayable activity in placenta can be attributed to polymerase δ. In this context, it is noted that Miller et al. (1985) have titrated the polymerase α activity in human diploid fibroblasts using an inhibitory monoclonal antibody to polymerase α and have found that even at very high antibody concentrations no more than 80% of the apparent α activity could be inhibited.

Discussion

The properties of human placental DNA polymerase δ, in terms of size, specific activity, and enzymatic properties, are generally similar to those of the enzymes isolated from calf thymus (Lee et al., 1980, 1981, 1984) and rabbit reticulocytes (Byrnes et al., 1976; Byrnes, 1984). Our findings, in which we show the association of the exonuclease with polymerase activities of polymerase δ throughout a number of analytical procedures, are consistent with previous studies of the reticulocyte and calf thumus enzymes (Byrnes, 1984; Lee et al., 1984) and support the view that the two activities are more than associated, but are intrinsic to a single protein. The possibility that our results can be explained by contamination with a highly active exonuclease seems unlikely, even bearing in mind the fact that polymerase α has been described in a number of high molecular weight forms, often reported to be contaminated with exonuclease activity which is removed on further purification (Seal et al., 1979; Krauss & Linn, 1980; Skarnes et al., 1986). Our observations that the 3′- to 5′-exonuclease of DNA polymerase δ is almost inactive under the assay conditions previously used for the nonassociated exonuclease may also account for previous failures to detect the associated exonuclease activity.

Calf thymus DNA polymerase δ has been reported to have a native molecular weight of 173 000, and preparations of this enzyme have been reported to have subunits of 60 and 49 kDa (Lee et al., 1981) and 125 and 48 kDa (Lee et al., 1984), respectively. The rabbit reticulocyte enzyme has been obtained in a form having a single subunit of 120 kDa (Byrnes, 1984). We have identified the catalytic polypeptide of DNA polymerase δ for the first time by activity staining for the polymerase activity. Our data are consistent with the view that placental DNA polymerase δ consists of a native polypeptide of 165–170 kDa to which the catalytic site for polymerase activity is localized.

The number of polypeptide species and their variability from preparation to preparation suggest that despite the precautions taken some proteolytic "nicking" of the protein occurs during purification. The properties of DNA polymerase δ, insofar as its polypeptide composition is concerned, show similarities to those reported for DNA polymerase α. A survey of the literature on mammalian DNA polymerase α preparations shows that they possess multiple polypeptides which can be arbitrarily divided into three groups, 140–180, 110–125, and 30–80 kDa. Current studies of DNA polymerase α strongly favor a native polypeptide of ca. 180 kDa, with forms of 140–120 kDa and lower being possibily the result of proteolytic modification. Evidence for a 180-kDa DNA polymerase α polypeptide comes, for example, from studies of the enzymes of calf thymus (Wahl et al., 1984; Chang et al., 1984; Holmes

et al., 1986), KB cell (Wang et al., 1984), *Drosophila melanogaster* embryos (Kaguni et al., 1982), African green monkey kidney cells (Yamaguchi et al., 1985; Karawaya et al., 1984), and yeast cells (Plevani et al., 1985). However, the largest catalytically active polypeptides so far identified in purified mammalian polymerase α preparations by activity staining are about 120 kDa (Detera-Wadleigh et al., 1984; Karawaya & Wilson, 1982; Albert et al., 1982), although a 190-kDa active polypeptide has been identified after immunoprecipitation of ^{35}S-labeled monkey cell extracts (Karawaya et al., 1984). In the latter study, the 190-kDa species could not be stained by the activity stain procedure, and identification was made by direct enzyme assay after electroelution and renaturation. In yeast, DNA polymerase I has been shown by activity staining to have catalytic polypeptides of 180 and 140 kDa (Plevani et al., 1985).

If, as indicated by our data, the polymerase activity is associated with a ca. 170-kDa polypeptide, the fact that both polymerase and exonuclease activities behave with a native molecular weight of similar size is consistent with both catalytic sites being localized to the same polypeptide. This suggests that the native enzyme might be expected to consist of a single polypeptide of 165–170 kDa possessing both polymerase and exonuclease activities. In this respect, polymerase δ may be regarded as being the mammalian counterpart of *E. coli* Pol I. However, there remains the alternate possibility that the exonuclease may be localized to a small but as yet undetected subunit, in analogy to *E. coli* Pol III where the 3′- to 5′-exonuclease activity resides on the ϵ subunit (Scheuermann & Echols, 1985). Further investigations are necessary to directly establish the localization of the 3′- to 5′-exonuclease catalytic site.

The question of whether DNA polymerase δ should be viewed as a distinct type of mammalian polymerase on the basis of an intrinsic 3′- to 5′-exonuclease activity, and an assessment of its potential roles in replication, is complicated by the recent findings that polymerase α preparations may be obtained in putative holoenzyme forms which contain other proteins with potential replicative functions [e.g., see Hübscher (1983) and Vishwanatha et al. (1986)]. This includes the existence of replication factors C1 and C2 (Pritchard & De-Pamphilis, 1983; Pritchard et al., 1983), an Ap_4A binding protein (Baril et al., 1983), and primase (Wang et al., 1984; Gross & Krauss, 1985; Plevani et al., 1984), and preliminary evidence that eucaryotic cells may possess a replicative complex involving DNA polymerase α in analogy to the procaryotic replication systems (Hübscher, 1983). Several reports have in fact provided evidence for mammalian DNA polymerase α activities with associated 3′- to 5′-exonuclease activities (Chen et al., 1979; Hübscher, 1984; Vishwanatha et al., 1986). However, polymerase δ is clearly distinct because of the close association of the exonuclease activity, which to date has not been separable from the polymerase activity. In addition, the preparations of Chen et al. and Vishwanatha et al. (1986) displayed both 3′- to 5′- and 5′- to 3′-exonuclease activities, which in the latter case could be resolved from polymerase activity (Skarnes et al., 1986). Crute et al. (1986) have recently studied preparations of two forms of polymerase δ from calf thymus. These preparations contained as many as eight or nine polypeptide components and are clearly not homogeneous, but like polymerase α, are associated with primase activity as well as a 3′- to 5′-exonuclease activity. In our hands, purified DNA polymerase δ from human placenta is devoid of primase activity, although it is detectable in the earlier stages of purification.

Although DNA polymerases δ and α display a number of similar properties, current evidence indicates that polymerase δ is distinct from polymerase α since monoclonal antibodies to KB cell DNA polymerase α do not inhibit human, rabbit reticulocyte, or calf thymus DNA polymerase δ (Byrnes, 1985; Lee et al., 1985; Crute et al., 1986) and polyclonal antibodies to δ, reported here for the first time, do not inhibit α. However, these studies do not preclude the possibility that these two enzymes may yet have some structural homology. Other indirect evidence also points to a structural difference of the two proteins: polymerase δ is poorly inhibited by the dNTP analogues BuPdGTP [N^2-(*p-n*-butylphenyl)-9-(2-deoxy-β-D-ribofuranosyl)guanine 5′-triphosphate] and BuAdATP [2-(*p-n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate], while polymerase α is potently inhibited (Lee et al., 1985); polymerase δ activity is activated in the presence of dimethyl sulfoxide while polymerase α is inhibited (Lee & Toomey, 1986). The resolution of the questions of the potential structural relationships of polymerase δ to polymerase α, as well their respective functions in the replicative process, will require more detailed investigations of the properties of these two proteins. Several possibilities may be entertained. First, it is possible that polymerase α is a degraded form of δ, having lost its exonucleolytic function in the process, and implying an identity of the core polymerase polypeptide. Second, polymerase δ may be simply a form of polymerase α, with an associated subunit conferring the exonuclease activity, in analogy to the ϵ subunit of Pol III (Scheuermann & Echols, 1985) or the primase function of the β/γ subunits of the *Drosophila* enzyme (Kaguni et al., 1985). A third possibility is that both polymerases α and δ may be part of the replicative machinery necessary for cellular function and are distinct proteins, with the added possibility of an evolutionary relationship. The latter hypothesis is favored by our studies, in particular the failure of specific antibodies to cross-inhibit the two enzymes.

The importance of the existence of DNA polymerase δ in mammalian tissues and the study of its properties lie in the potential role of this enzyme as one of the means for maintaining the fidelity of DNA replication in eucaryotic cells. In this work, we have also shown a functional association of a 3′- to 5′-exonuclease activity with polymerase activity, in that it is capable of concomitant DNA synthesis with excision of a mismatched primer end terminus. In the human placenta, our data show that polymerase δ is present in a very significant proportion of the measurable DNA polymerase activity. The presence of DNA polymerase δ in mammalian systems therefore raises significant questions regarding its potential role in DNA replication and/or repair, as well as an interesting parallel with the procaryotic polymerases of which *E. coli* Pol I and Pol III (Kornberg, 1984) are archetypical examples. Further investigations of this DNA polymerase, which has been the subject of relatively few investigations by comparison with polymerase α, are likely to provide new insights into the mechanisms of mammalian DNA replication.

Registry No. DNA polymerase, 9012-90-2; 3′- to 5′-exonuclease, 62683-21-0.

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