PCNA-Dependent DNA Polymerase δ from Rabbit Bone Marrow†

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ABSTRACT: Proliferating cell nuclear antigen (PCNA) and PCNA-dependent DNA polymerase δ were partially purified and characterized from rabbit bone marrow. Rabbit DNA polymerase δ sediments at 8.2 S upon glycerol density gradient centrifugation. Similar to calf thymus PCNA-dependent DNA polymerase δ, a 125–123-kDa doublet and 48-kDa polypeptides correlate with DNA polymerase activity. Western blotting of rabbit DNA polymerase δ with polyclonal antibody to calf thymus PCNA-dependent DNA polymerase δ gives the same results as calf thymus δ; the 125–123-kDa doublet is recognized. PCNA-dependent DNA polymerase δ is resistant to inhibition by dideoxynucleotides and is relatively insensitive to inhibition by N^2-[*p*-(*n*-butyl)phenyl]dGTP. A 3′→5′ exonuclease copurifies with the DNA polymerase. The processivity of DNA polymerase δ alone is very low but greatly increases with the addition of PCNA from rabbit bone marrow or calf thymus. Comparative studies of the original DNA polymerase δ from rabbit bone marrow demonstrate a lack of recognition by antibodies to calf thymus δ and a high degree of processivity in the absence of PCNA. Additionally, the originally described DNA polymerase δ is a single polypeptide of 122 kDa. These features would recategorize the original δ to the ε category by recently proposed convention. PCNA-dependent DNA polymerase δ is a relatively minor component of rabbit bone marrow compared to DNA polymerase α and PCNA-independent DNA polymerase δ (ε), the relative proportions being α, 60%; δ, 7%; and ε, 30%.

DNA polymerase δ was first isolated and characterized from rabbit bone marrow (Byrnes et al., 1976). It was distinguished from DNA polymerase α by its association with a 3′→5′ exonuclease activity. Otherwise, DNA polymerase δ and DNA polymerase α were similar in many respects. Both are sensitive to the inhibitors *N*-ethylmaleimide and aphidicolin (Byrnes, 1984), and both are resistant to inhibition by dideoxynucleoside triphosphate (Lee et al., 1985). However, they are different in that DNA polymerase δ is much less sensitive to the inhibitors BuAdATP[1] and BuPdGTP (Khan et al., 1984; Byrnes, 1985; Lee et al., 1985). Furthermore, DNA polymerase δ is not associated with a primase activity, whereas DNA polymerase α is (Kaguni et al., 1983); and DNA polymerase δ and α are immunologically distinct (Byrnes, 1985; Wong et al., 1989). DNA polymerases with associated 3′ to 5′ exonuclease have been isolated from other sources and categorized as DNA polymerase δ (Lee et al., 1984; Crute et al., 1986; Lee & Toomey, 1987; Bauer et al., 1988; Focher et al., 1988; Boulet et al., 1989; Budd et al., 1989).

Calf thymus is the second tissue from which a DNA polymerase δ was described (Lee et al., 1980). Subsequently, a 37-kDa protein cofactor for calf thymus DNA polymerase δ was described (Tan et al., 1986). Examination of rabbit bone marrow DNA polymerase δ for a similar cofactor requirement gave negative results. The original DNA polymerase δ from rabbit bone marrow was very active on poly(dA)/oligo(dT), whereas calf thymus DNA polymerase δ required the cofactor for activity on this and similar template-primers. Subsequently, it was shown that the cofactor is PCNA (Bravo et al., 1987; Prelich et al., 1987a,b). Thereafter, DNA polymerase δ became separable into two activities by the response to PCNA: one is PCNA-dependent, showing low processivity on poly(dA)/oligo(dT) and other template-primers with long single-stranded regions, but becoming highly processive upon interaction with PCNA (Downey et al., 1988). The other is PCNA-independent, being processive in the absence of PCNA and not significantly affected by PCNA (Burgers, 1989). An exhaustive search for a DNA polymerase δ from rabbit bone marrow which was PCNA-dependent was conducted. Examination of the starting material and various steps of the DNA polymerase purification procedures gave no indication of a PCNA-dependent DNA polymerase. PCNA-neutralizing antibody obtained from Dr. Antero So inhibited the calf thymus but not the bone marrow enzyme. Mixing studies with DNA polymerase from bone marrow and calf thymus PCNA-dependent enzyme did not indicate the presence of the cofactor. We tried numerous variations of reaction conditions and components, including the divalent cation, salt concentration, and the presence or absence of poly(ethylene glycol). Under no conditions was an effect of PCNA demonstrable. Revision of the enzyme purification process from the earliest steps was necessary to identify a PCNA-dependent DNA polymerase. We report here the isolation and characterization of a PCNA-dependent DNA polymerase δ from rabbit bone marrow and compare it to the originally described DNA polymerase δ from bone marrow and to PCNA-dependent DNA polymerase δ from calf thymus.

EXPERIMENTAL PROCEDURES

Poly(dA)$_{400}$, oligo(dT)$_{12-18}$, and pd(T)$_{18}$ are purchased from P-L Biochemicals. pBR322-*Hae*III digest, pepstatin, and leupeptin are from Sigma. Poly(dA-dT) and calf intestine

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[1] Abbreviations: PCNA, proliferating cell nuclear antigen; BuPdGTP, N^2-[*p*-(*n*-butyl)phenyl]dGTP; BuAdATP, 2-[*p*-(*n*-butyl)anilino]dATP; PMSF, phenylmethanesulfonyl fluoride; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; TEMED, *N,N,N′,N′*-tetramethyethylenediamine.

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alkaline phosphatase are from Boehringer Mannheim. [^{3}H]-dTTP (78 Ci/mmol) is purchased from Du Pont New England Nuclear. [γ-^{32}P]ATP (7000 Ci/mmol) is from ICN Biochemicals. Phenyl-Sepharose is from Pharmacia Labs, and Heparin–Agarose is from GIBCO BRL. Econo Pac Q and Econo Pac Blue columns are from Bio-Rad Laboratories. T_4 polynucleotide kinase is from Bethesda Research Labs. Vectastain kits are from Vector Labs. Calf thymus PCNA, calf thymus DNA polymerase δ, and the polyclonal antibody against a 20 amino acid peptide of PCNA-dependent calf thymus DNA polymerase δ are from Dr. Antero G. So. BuPdGTP is from Dr. Neal C. Brown.

Purification of PCNA-Dependent DNA Polymerase δ. Erythroid hyperplasia is induced in New Zealand White rabbits (Byrnes et al., 1976). Marrow from the long bones is placed in cold buffer A (122 mM NaCl, 5 mM KCl, 7.4 mM $MgCl_2$) and centrifuged to sediment the cells. Lysis buffer B (50 mM Tris, pH 7.8, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.8 mM PMSF, 5 μg/mL pepstatin, 50 μg/mL leupeptin) is added, and the cells are disrupted by gentle homogenization. The lysate is centrifuged at 200000*g* at 4 °C for 1 h. DNA polymerase δ and PCNA are purified from the supernatant by a series of procedures outlined in Tables I and II. The pH is adjusted to 7.8, and the material is adsorbed to DE 52 cellulose equilibrated with buffer C (50 mM Tris, pH 7.8, 7.5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.8 mM PMSF). The DE 52 cellulose is eluted with a gradient from 100 mM to 600 mM KCl in buffer C. DNA polymerase appears at 0.1–0.2 M KCl and PCNA appears at 0.2–0.25 M KCl (Figure 1). The polymerase fractions are put onto a phosphocellulose column equilibrated in buffer C which is eluted stepwise with buffer C containing 600 mM KCl. The DNA polymerase fractions are adjusted to 55% ammonium sulfate, and the precipitate is collected at 15000*g* for 15 min. The precipitate is resuspended in buffer D (20 mM KP_i, pH 7.0, 7.5% glycerol, 1 mM EDTA, 1 mM DTT, 0.8 mM PMSF) and adjusted to 18% ammonium sulfate saturation. The DNA polymerase is adsorbed to phenyl-Sepharose, equilibrated with buffer E (18% $(NH_4)_2SO_4$ saturation, 20 mM KP_i, pH 7.0, 7.5% glycerol, 1 mM EDTA, 1 mM DTT, 0.8 mM PMSF), and eluted with buffer F (50 mM Tris, pH 8.8. 20% glycerol, 1 mM EDTA, 1 mM DTT, 0.8 mM PMSF). Fractions containing DNA polymerase are adjusted to pH 7.8 with cold 0.1 N HCl and put onto a phosphocellulose column equilibrated with buffer G (50 mM Tris, PH 7.8, 20% glycerol, 0.5 mM EDTA, 1 mM DTT, 0.2 mM PMSF). The column is eluted with a gradient of 100–500 mM KCl in buffer G; DNA polymerase δ appears at 0.1–0.2 M KCl. The DNA polymerase δ fractions are dialyzed against buffer H (20 mM KP_i, pH 7.0, 20% glycerol, 1 mM DTT, 0.2 mM PMSF) and loaded onto a hydroxylapatite column. The PCNA-dependent DNA polymerase δ is eluted by buffer H containing 600 mM KCl, whereas PCNA-independent DNA polymerase elute with 600 mM KP_i. PCNA-dependent DNA polymerase δ is dialyzed against buffer G containing 100 mM KCl and loaded onto a heparin–Agarose column equilibrated with buffer G containing 100 mM KCl. The column is eluted with a gradient from 150 mM to 500 mM KCl in buffer G. DNA polymerase δ appears at 0.3–0.4 M KCl. The DNA polymerase is concentrated on a mini PC column and eluted by 600 mM KCl in buffer G.

Purification of PCNA. The PCNA-containing fractions from step II are adjusted to 60% ammonium sulfate, and the precipitate is collected at 20000*g* for 15 min, resuspended in buffer I (50 mM Tris, pH 7.8, glycerol 7.5%, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF), and then passed over a Sephadex G-50 column equilibrated with buffer I containing 100 mM KCl. The passthrough is applied to a phosphocellulose column, equilibrated with the same buffer. Again, PCNA is in the passthrough. The PCNA is loaded onto a DEAE-cellulose DE 52 column equilibrated with buffer I containing 100 mM KCl and eluted by a gradient to 400 mM KCl in buffer I. The PCNA active fractions are dialyzed against 20 mM KP_i, pH 8.0, and then passed through on Econo Pac Blue column. The passthrough containing the PCNA is adjusted to pH 7.0; glycerol and DTT are added to 20% and 1 mM and then loaded onto a HA column. The column is washed with buffer H, containing 600 mM KCl, and subsequently with a gradient from 20 mM to 300 mM KP_i, pH 7.0, in buffer H; PCNA appears at 50 mM KP_i. The PCNA is adjusted to pH 7.8 and put onto an Econo Pac Q Column. The column is eluted with a gradient from 100 mM KCl to 450 mM KCl in buffer I; PCNA appears between 0.2 and 0.3 M KCl.

Purification of PCNA-Independent DNA Polymerase δ. The enzyme is purified as described (Byrnes et al., 1976) with additions and modifications through step IX (Byrnes, 1984).

Assay of Activities. The PCNA-dependent DNA polymerase reaction contains 40 mM MOPS, pH 6.5, 2 mM $MgCl_2$, 40 μM dATP, 10 μM [^{3}H]dTTP (1 μCi/nmol), 0.04 mg/mL BSA, 1 mM DTT, 7.5% glycerol, and 0.24 A_{260} unit/mL poly(dA-dT). One unit of DNA polymerase activity incorporates 1 nmol of dNMP/h. Alternatively, assay of PCNA-dependent DNA polymerase δ is carried out in 40 mM MOPS, pH 6.5, 6 mM $MgCl_2$, 10 μM [^{3}H]dTTP (1 μCi/nmol), 0.04 mg/mL BSA, 7.5% glycerol, 0.25 A_{260} unit/mL poly(dA)/oligo(dT) (40:1), ±PCNA. The PCNA assay reaction contains 40 mM MOPS, pH 6.5, 6 mM $MgCl_2$, 10 μM [^{3}H]dTTP (1 μCi/nmol), 0.25 A_{260} unit/mL poly(dA)/oligo(dT) (40:1), 0.04 mg/mL BSA, 1 mM DTT, 7.5% glycerol, ± PCNA-dependent DNA polymerase δ. One unit of PCNA activity incorporates 1 nmol of dTMP/h at 37 °C in the presence of 0.25 unit of DNA polymerase δ. The PCNA-independent DNA polymerase reaction contains 40 mM MOPS, pH 6.5, 6 mM $MgCl_2$, 0.04 mg/mL BSA, 1 mM DTT, 10 μM dTTP (1 μCi/nmol), 0.25 A_{260} unit/mL poly(dA)/oligo(dT) (40:1), and 7.5% glycerol. The 3′→5′ exonuclease reaction contains 25 mM MOPS, pH 7.0, 1 mM $MnCl_2$, 90 mM KCl, 0.046 A_{260} unit/mL poly(dA-dT)·[^{3}H]-TMP(6.2 μCi/A_{260} unit). The reaction is stopped by the addition of 1 mg of BSA and 1 mL of cold 5% Cl_3CCOOH. The precipitate is collected at 2600*g* for 15 min and the supernatant is counted in Biofluor.

All reactions generally are carried out in 100 μL and incubated at 37 °C for 15 min.

Ultracentrifugation studies are carried out in a Beckman SW 65K rotor at 60 000 rpm for 16 h at 2 °C; 4.7-mL linear 15–30% (v/v) glycerol gradients contain 50 mM MOPS, pH 7.0, 1 mM DTT, 0.5% poly(ethylene glycol) (6000), 0.1 mM EDTA, and 0.5 mM KCl. Sedimentation standards are catalase, 11.3 S; aldolase, 7.0 S; and BSA, 4.5 S.

SDS–Polyacrylamide Slab Gel Electrophoresis. The stacking gel contains 0.12% bisacrylamide and 4.5% acrylamide, and the separation gel contains 0.21% bisacrylamide and 8% acrylamide. Thirty microliters of sample buffer containing 0.0625 M Tris, pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue is added to 40 μL of sample and heated for 30 s at 95 °C. Gels are run at 15 mA through the stacking gel and at 20 mA through the separating gel. The gel is fixed in 50% methanol, 10% acetic acid for 20 min and 5% methanol, 7% acetic acid

overnight and then stained by the method of Oakley et al. (1980).

Immunological Detection of Proteins on Nitrocellulose. After electrophoresis, proteins are electrophoretically transferred onto a nitrocellulose sheet. The blot is soaked in 3% BSA in TB2S buffer (25 mM Tris, pH 8.0, 1.8% NaCl) for 15 h at 46 °C. The membrane is incubated for 4 h with polyclonal rabbit antiserum to DNA polymerase of δ diluted into 3% BSA in TB2S buffer. Following a 30-min wash in TB2S–0.05% Tween, the membrane is reacted with biotinylated goat anti-rabbit IgG. After being washed in TB2S–0.05% Tween, the membrane is soaked for 30 min in an avidin–biotinylated peroxidase complex (Vectastain ABC system) diluted in TB2S. Peroxidase activity is detected by reaction with 0.5 mg/mL 4-chloro-1-naphthol in a 0.1 M Tris-HCl, pH 7.5, in the presence of 16.7% methanol and 0.018% H_2O_2.

Processivity Studies. pd(T)$_{18}$ and pBR322–*Hae*III digest are dephosphorylated by calf intestine alkaline phosphatase and then labeled at the 5′ end with ^{32}P by T_4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatis et al., 1982). Poly(dA)$_{400}$ and [^{32}P]pd(T)$_{18}$ are annealed in 50 mM Tris, pH 7.6, by heating at 70 °C for 5 min and then slowly cooling to room temperature. The nucleotide ratio is 30:1. The concentration of poly(dA)$_{400}$/[^{32}P]pd(T)$_{18}$ is 1.25 A_{260} units/mL with specific activity of 4.7 × 10^8 cpm/mL. DNA synthesis is carried out in 100 μL of solution containing 40 mM MOPS, pH 6.5, 6 mM $MgCl_2$, 4 μg BSA, 1 mM DTT, 7.5% glycerol, 20 μM dTTP, 0.0125 A_{260} unit/mL poly(dA)$_{400}$/[^{32}P]pd(T)$_{18}$, 0.35–2.4 units of DNA polymerase, 1–2 μg of PCNA, if used, and 1.13 A_{260} units/mL calf thymus DNA. The DNA polymerase, reaction mixture, and activated calf thymus DNA are preincubated separately at 37 °C for 1 min. At 0.2 min after the addition of reaction mixture to enzyme, calf thymus DNA is added. The total incubation time is 5 min. The reaction is terminated by addition of EDTA to 0.03 M. Then, 5 μL of 10% SDS and 5 μL of 1 M Tris-HCl, pH 8.65, are added; samples are then extracted with 20 μL of phenol and then 100 μL of chloroform to remove protein. After centrifugation, 100 μL of the aqueous phase is taken; 10 μL of 5 M potassium acetate, pH 4.8, and 400 μL of ethyl alcohol are added to the aqueous phase which is kept at −20 °C overnight to precipitate DNA. The precipitate is collected at 15 000 rpm for 5 min and washed twice with cold 75% ethyl alcohol and then dissolved into 90% formamide, 1 × TBE, 0.1% bromophenol blue, 0.1% xylene cyanol FF. The samples are heated at 90 °C for 5 min and cooled in an ice bath to denature the DNA. Each sample is subject to 7 M urea, 8% polyacrylamide gel (19:1) electrophoresis at 400 V for 1 h. Denatured 5′-^{32}P-labeled pBR322–*Hae*III digest is used as a size marker. The gel is autoradiographed with X-ray film and an intensifying screen at −70 °C overnight.

Other Procedures. Protein is measured with BSA as the standard (Bradford, 1976). Conductivity is measured with a Yellow Spring apparatus.

RESULTS

Purification of PCNA-Dependent DNA Polymerase δ. The result of each step is shown in Table I. The initial identification and separation of DNA polymerase and PCNA occurs in step II, DEAE-cellulose chromatography (Figure 1). It is not until step VI, hydroxylapatite column chromatography, that the PCNA-dependent DNA polymerase is adequately resolved from the other DNA polymerase to be quantitated. PCNA-dependent DNA polymerase δ elutes from hydroxylapatite with 0.6 M KCl, whereas the rest of the DNA polymerase requires the application of KP_i to elute. The amount of PCNA-dependent DNA polymerase is a small fraction of the starting DNA polymerase, less than 10% at this point. With poly(dA)/oligo(dT) (40:1) as template-primer to assay step VI, approximately 20 times greater activity is obtained in the presence of PCNA. After further purification upon heparin–agarose (step VII) and subsequent concentration on a mini PC column, approximately 50-fold stimulation of DNA polymerase activity is obtained by the addition of PCNA. The specific activity at this point is approximately 150 units/mg protein.

Purification of PCNA. The results of each step is shown in Table II. The specific activity of PCNA at the final step is 301 units/mg. The preparation at this point is free of contaminating DNA polymerase, nuclease, or DNA ligase which could interfere in subsequent studies. The preparation is not homogeneous but is adequately characterized and resolved for functional studies.

Table I: Purification[a] of PCNA-Dependent DNA Polymerase δ

	step	total polymerase (unit)	PCNA-dependent DNA polymerase (unit)	other polymerase (unit)
I	100 000 supernatant	3959		
II	DEAE–cellulose	27 375		
III	phosphocellulose	17 623		
IV	phenyl-Sepharose	16 147		
V	phosphocellulose	12 698		4390
VI	hydroxylapatite		387	5050
VII	heparin–agarose		87	

[a] For this assay, activated poly(dA-dT) was used as template-primer as described in the Experimental Procedures section.

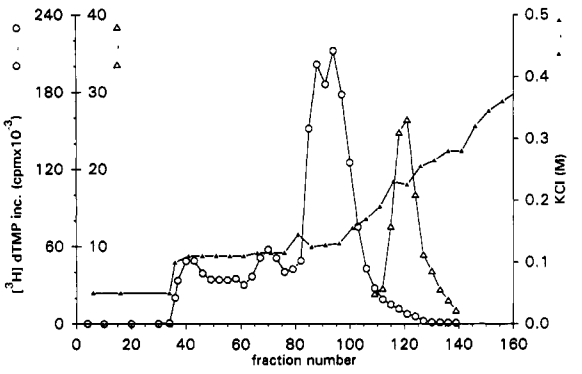

FIGURE 1: DE 52 cellulose chromatography. Rabbit bone marrow extract was applied to a DEAE-cellulose DE52 column and eluted as described in text. Poly(dA)/oligo(dT) (○) was used as template-primer in the DNA polymerases assay. PCNA activity (△) was determined by the difference of [^3H]dTMP incorporation in the presence or absence of PCNA-dependent DNA polymerase δ using poly(dA)/oligo(dT) as template-primer. The KCl concentration (▲) was determined by conductance measurements. Initially, calf thymus PCNA-dependent DNA polymerase from Dr. So was used, and subsequently PCNA-dependent DNA polymerase from bone marrow was used.

Table II: Purification of PCNA from Rabbit Bone Marrow

	step	volume (mL)	protein (mg)	total activity (unit)	sp act. (unit/mg)
I	100 000 supernatant	950	5168		
II	DEAD-cellulose	430	860		
III	phosphocellulose	140	382	14 448	38
IV	DEAE-cellulose	80	100	7424	74
V	Econo Pac Blue	80	83	8000	96
VI	hydroxylapatite	19	9.5	1611	170
VII	Econo Pac Q	20	4.2	1266	301

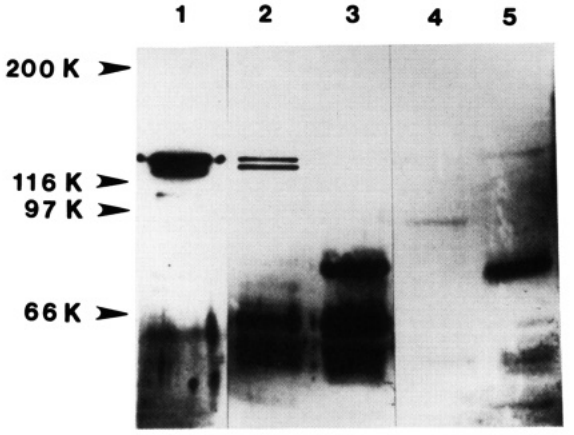

FIGURE 2: Western blotting of PCNA-dependent and PCNA-independent DNA polymerase δ. Three units of calf thymus PCNA-dependent DNA polymerase δ (lane 1), 1.5 units of bone marrow PCNA-dependent DNA polymerase δ (lane 2), and 3.2 units of bone marrow PCNA-independent DNA polymerase δ (lane 3) were applied to 8% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane. In lanes 1–3, the membrane was reacted with the antibody against calf thymus DNA polymerase δ and then detected by biotin-conjugated second antibody followed by avidin-biotinylated peroxidase complex as described in the Experimental Procedures section. A replicate experiment was performed, the nitrocellulose membrane was treated the same except for the omission of the first antibody. Lane 4 is bone marrow PCNA-dependent DNA polymerase, and lane 5 is bone marrow PCNA-independent DNA polymerase δ (ε) under these conditions.

Comparison of Structural Features of PCNA-Dependent and PCNA-Independent DNA Polymerase δ. Upon glycerol density gradient centrifugation, PCNA-dependent DNA polymerase δ sediments at 8.2 S. The detection of DNA polymerase activity is dependent upon the addition of PCNA when poly(dA)/oligo(dT) is used as template-primer. Rabbit bone marrow DNA polymerase δ prepared as previously described (Byrnes, 1984) sediments at 6.5 S. Examination of the glycerol gradient fractions by SDS–polyacrylamide gel electrophoresis and protein staining with silver indicated the enzyme was not pure; however, a 125–123-kDa doublet band and a 48-kDa band are evident in the active fractions and correspond by color density to the activity of PCNA-dependent DNA polymerase δ. Several other silver-staining bands are visible but are discordant to the DNA polymerase activity. The PCNA-independent DNA polymerase is a single 122-kDa polypeptide which appears homogeneous and corresponds to the DNA polymerase activity as previously described (Goscin & Byrnes, 1982a).

Western blotting of both forms of bone marrow DNA polymerase δ and calf thymus PCNA-dependent δ are carried out with antibody to calf thymus PCNA-dependent DNA polymerase δ. The bone marrow PCNA-dependent DNA polymerase manifests as a doublet band of 125 and 123 kDa (Figure 2, lane 2). These bands are not present when the primary antibody is omitted (Figure 2, lane 4). PCNA-dependent DNA polymerase δ from calf thymus gives an identical result; a doublet of 125 and 123 kDa is obtained. The 123-kDa band probably represents degradation of the 125-kDa peptide (Zhang et al., 1991). The original DNA polymerase δ does not react with this antibody; no antibody-dependent reaction pattern is evident; however, some non-specific staining occurs on the nitrocellulose membrane even if the first antibody is not included (Figure 2, lane 5).

Some Properties of PCNA-Dependent DNA Polymerase δ. The enzyme was examined for features characteristic of previously described PCNA-dependent DNA polymerase δ.

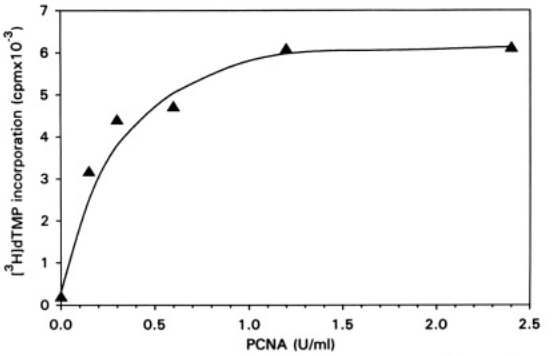

FIGURE 3: Effect of PCNA on rabbit bone marrow PCNA-dependent DNA polymerase δ. Poly(dA)/oligo(dT) (40:1) was used as template-primer as described in the Experimental Procedures section. In this assay, 0.02 unit of DNA polymerase δ (step VII) was used for each reaction and the PCNA was from step V.

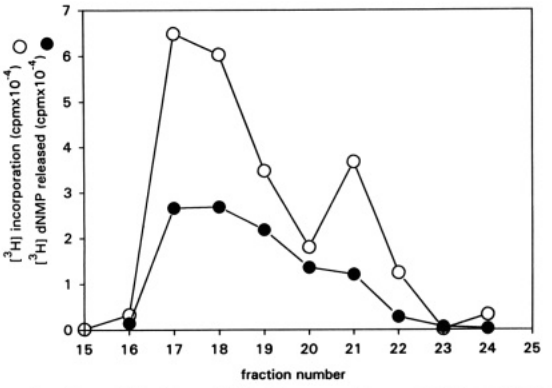

FIGURE 4: Copurification of 3′→5′ exonuclease activity with rabbit bone marrow polymerase activity, step VII. DNA polymerase activity (○) was located by [^{3}H]dTMP incorporation in the presence of PCNA using poly(dA)/oligo(dT) (40:1) as template-primer. 3′→5′ exonuclease activity (●) was assayed by means of measuring the release of [^{3}H]dNMP from the 3′ end of labeled poly(dA-dT).

The effect of increasing concentration of PCNA on DNA synthesis is shown (Figure 3). Maximal stimulation of DNA synthesis occurs at about 0.36 unit/mL PCNA concentration. Clearly this DNA polymerase is a PCNA-dependent enzyme. 3′→5′ exonuclease activity was assayed by the release of dNMP from the 3′ end of ^{3}H labeled poly(dA-dT). A 3′→5′ exonuclease copurifies with the PCNA-dependent DNA polymerase activity. The coelution of DNA polymerase and 3′→5′ exonuclease activity in step VII is shown (Figure 4). PCNA-dependent DNA polymerase δ is resistant to inhibition by ddTTP and is relatively insensitive to BuPdGTP; more than 70% activity remains in 10 μM BuPdGTP.

Processivity Analysis of Rabbit PCNA-Dependent Polymerase δ Compared to Calf Thymus DNA Polymerase δ. Length analysis of nucleic acid products shows (Figure 5) that the processivity of DNA polymerization by rabbit PCNA-dependent DNA polymerase δ is very low (lane 4) but greatly increases with the addition of PCNA, either from rabbit bone marrow (lane 6) or from calf thymus (lane 5); poly(dA)$_{400}$/p(dT)$_{18}$ is elongated almost to the end. Similar results are obtained with calf thymus DNA polymerase δ. PCNA from either calf thymus (lane 2) or rabbit bone marrow (lane 3) greatly increases the processivity of calf thymus DNA polymerase δ (lane 1).

Processivity of DNA Synthesis Catalyzed by the Original Rabbit Bone Marrow DNA Polymerase δ. The products of DNA synthesis catalyzed by the initially described rabbit bone marrow DNA polymerase δ and by calf thymus DNA polymerase δ were compared in the presence and absence of

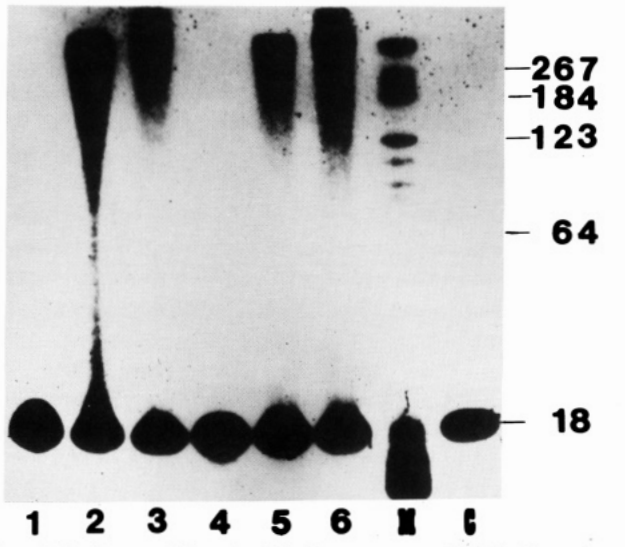

FIGURE 5: Processivity of rabbit bone marrow PCNA-dependent DNA polymerase δ compared to calf thymus DNA polymerase δ. The length of the nucleic acid products catalyzed by rabbit bone marrow DNA polymerase δ or calf thymus DNA polymerase δ were compared in the presence or absence of calf thymus PCNA or rabbit PCNA. The DNA synthesis reaction and sample treatment were as described under the Experimental Procedures section. The enzyme used in lanes 1–3 was 1 unit of calf thymus DNA polymerase δ; the enzyme in lanes 4–6 was 0.35 unit of rabbit bone marrow DNA polymerase δ. Lane 1 and lane 4 were in the absence of PCNA. Lane 2 and lane 5 were in the presence of calf thymus PCNA. Lane 3 and lane 6 were in the presence of rabbit PCNA. Lane M was the ^{32}P labeled pBR322–*Hae*III digest marker. Lane C was a control without enzyme. The length of the fragments in nucleotides is indicated along the margin.

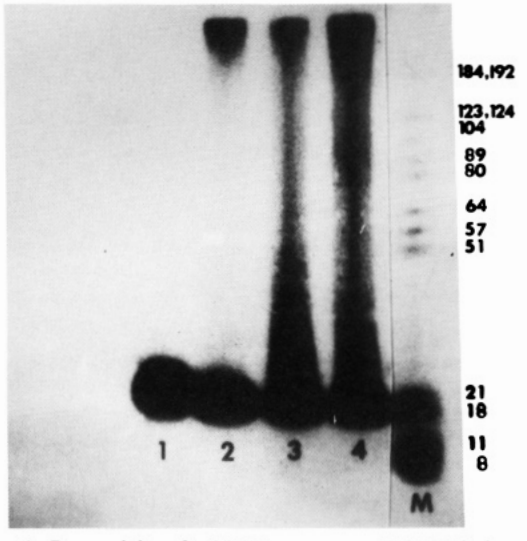

FIGURE 6: Processivity of rabbit bone marrow PCNA-independent DNA polymerase δ (ε). DNA synthesis and sample treatment was as described in the Experimental Procedures section. The enzyme used for lane 1 and 2 was 1 unit of calf thymus DNA polymerase δ, and for lane 3 and 4 it was 2.4 units of rabbit bone marrow PCNA-independent DNA polymerase δ (step VII). Lanes 2 and 3 were in the presence of PCNA. Lanes 1 and 4 were in the absence of PCNA.

PCNA (Figure 6). The original rabbit DNA polymerase δ is highly processive, in either the presence (lane 3) or absence (lane 4) of PCNA. In contrast, the processivity of calf thymus DNA polymerase δ (lane 1) greatly increases with the addition of PCNA (lane 2).

DISCUSSION

We have isolated and characterized a PCNA-dependent DNA polymerase from rabbit bone marrow, and we have obtained PCNA from the same source. The amount of PCNA-dependent DNA polymerase obtained is a small fraction of the total DNA polymerase activity. When it is adequately resolved, step VI, PCNA-dependent DNA polymerase δ is about 7% of the total DNA polymerase recovered. Other DNA polymerase activities encountered in the preparation have characteristics of DNA polymerase α and PCNA-independent DNA polymerase δ.

The PCNA-dependent DNA polymerase δ from rabbit bone marrow is very similar to the DNA polymerase δ initially isolated from calf thymus (Lee et al., 1984) and subsequently found in mouse (Goulian et al., 1990), HeLa cells (Syväoja et al., 1990), and yeast (Bauer et al., 1988). The sedimentation coefficients are not significantly different, and subunit composition is the same. The calf thymus and rabbit bone marrow enzymes sediment at 7.9 S and 8.2 S, respectively, and both are composed of a 125- and a 48-kDa subunit. Both react with an antibody raised against a polypeptide representing a 20-amino-acid sequence of the calf thymus enzyme, and both manifest as a duplex of 125- and 123-kDa bands upon Western blotting with this antibody. Also, their behaviors on column chromatography are similar; the elution from hydroxylapatite by 0.6 M KCl separates and distinguishes both the calf thymus and bone marrow PCNA-dependent DNA polymerase from other DNA polymerases in the preparation. The rabbit bone marrow and calf thymus enzymes share other characteristics, including an associated 3′→5′ exonuclease activity and relative resistance to inhibition by BuPdGTP and dideoxynucleoside triphosphates.

PCNA was shown to act as a cofactor for calf thymus DNA polymerase δ by enhancing processivity, especially on template-primers with long single-stranded regions (Tan et al., 1986). The rabbit bone marrow enzyme likewise requires PCNA for measurable activity on poly(dA)$_{400}$/oligo(dT)$_{18}$, and the increased activity is a result of markedly increased processivity. PCNA from calf thymus is interchangeable with PCNA from rabbit bone marrow. The processivity of the calf thymus or rabbit bone marrow enzyme is increased by PCNA from either tissue. Yeast DNA polymerase III is PCNA-dependent and can be activated by either yeast or calf thymus PCNA (Burgers, 1988). Likewise, calf thymus DNA polymerase δ can interact with yeast PCNA (Bauer & Burgers, 1988). This interchangeability of PCNA from evolutionarily diverse organisms suggests that PCNA and the PCNA-dependent DNA polymerase have been highly conserved, especially the sites of protein–protein interaction.

Although similar in features such as the associated 3′→5′ exonuclease activity and resistance to inhibition by dideoxynucleotides and by BuPdGTP, the bone marrow PCNA-dependent DNA polymerase δ is notably distinct from the originally described DNA polymerase δ. The most significant difference is that the originally described DNA polymerase δ is processive in the absence of PCNA. Furthermore, the PCNA-independent δ, in contrast to the PCNA-dependent enzyme, is not recognized by an antibody raised to the calf thymus PCNA-dependent enzyme. Because the PCNA-independent DNA polymerase δ is a monomer of 122 kDa, and because the antibody reacts with an internal segment of the 125–123-kDa component of the PCNA-dependent DNA polymerase δ, a derivative relationship is excluded.

Other laboratories have described a PCNA-independent form of DNA polymerase δ (Crute et al., 1986; Lee & Toomey, 1987; Focher et al., 1988; Syväoja & Linn, 1989; Hamatake et al., 1990). Burgers et al. (1990) suggested that yeast DNA polymerase II is analogous to the PCNA-independent form

of DNA polymerase δ and that yeast PCNA-dependent DNA polymerase (pol III) is analogous to PCNA-dependent δ. Yeast pol III is substantially more abundant than pol II. However, the converse appears to be the case with rabbit bone marrow in which the PCNA-independent DNA polymerase was approximately 40% of the total DNA polymerase (Byrnes & Black, 1978) compared to the PCNA-dependent form described here, which constitutes approximately 7%.

After DNA polymerase δ from rabbit bone marrow was described (Byrnes et al., 1976), a similar enzyme was found in calf thymus extracts (Lee et al., 1980). The feature which warranted distinction of bone marrow DNA polymerase δ from DNA polymerase α was the associated 3′→5′ exonuclease. When a cofactor (PCNA) was described for DNA polymerase δ from calf thymus, it was not realized that this enzyme was intrinsically different from the originally described DNA polymerase δ. Subsequently, it was realized that there are two forms of DNA polymerase associated with 3′→5′ exonuclease: PCNA-independent and PCNA-dependent. The initially described DNA polymerase δ is clearly PCNA-independent. Here we describe from rabbit bone marrow a PCNA-dependent form which is virtually identical to the PCNA-dependent DNA polymerase δ from calf thymus and other tissues. A consensus conference on the nomenclature of eukaryotic DNA polymerases did not take into account that the PCNA-dependent form was distinct from the initially described DNA polymerase δ (Burgers et al., 1990). Now the predicament arises that the originally described δ does not have a property that was subsequently conferred upon that category, i.e., PCNA dependence. Furthermore, the PCNA-independent forms of δ have been recategorized as DNA polymerase ε. Consequently, the initially described DNA polymerase δ now would be recategorized as DNA polymerase ε. Recently, DNA polymerase ε from HeLa cells has been described to consist of >200- and 55-kDa polypeptides. The catalytic polypeptide is cleavable by trypsin into two fragments with molecular masses of 122 and 136 kDa. The 122-kDa fragment is resistant to further proteolysis and possesses the polymerase and exonuclease activities of the enzyme 2–3-fold. Whether this cleavage represents an in vivo or in vitro process is unresolved. The higher activity of the cleaved form may mean that the cleavage is a biologically meaningful process. We have not observed a higher molecular mass PCNA-independent DNA polymerase δ (ε), only the 122-kDa form is evident (Byrnes & Black, 1978; Goscin & Byrnes, 1982a). Thus, from rabbit bone marrow we have isolated DNA polymerase δ and DNA polymerase ε according to the current convention of nomenclature. In other studies, we have characterized DNA polymerase α from rabbit bone marrow (Byrnes & Black, 1978; Goscin & Byrnes, 1982b). DNA polymerase α is the most abundant, approximately 60%; DNA polymerase ε is approximately 30%; and DNA polymerase δ is approximately 7% of initially extracted activity. This relative proportion is similar to results obtained from HeLa cell extracts where DNA polymerase δ constituted approximately 10% of the combined DNA polymerase α, δ, and ε activities (Saväoja et al., 1990). Several models of DNA replication in eukaryotes have been proposed. In one, DNA polymerase δ would be responsible for leading strand synthesis, while Okasaki fragments would be started by DNA polymerase α-primase but elongated by DNA polymerase ε (Burgers, 1991). Another proposal also based upon evidence in yeast suggests that DNA polymerase II (ε) instead of DNA polymerase III (δ) synthesizes the leading strand (Morrison et al., 1990). Furthermore, recent studies also suggest that under some conditions DNA polymerase ε may interact with PCNA (Lee et al., 1991). The precise tasks of DNA polymerase α, δ, and ε and PCNA require further investigation. Their interaction with each other and with additional accessory proteins is just unfolding and was recently reviewed (So & Downey, 1992).

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Registry No. DNA polymerase, 9012-90-2; 3′→5′-exodeoxyribonuclease, 79393-91-2.