Waring, M. (1971) *Prog. Mol. Subcell. Biol. 2*, 216.
Wu, R., Padmanabhan, R., & Bambara, R. (1974) *Methods Enzymol. 29*, 231.
Zunino, F., Gambetta, R., Dimarco, A., & Zaccara, A. (1972) *Biochim. Biophys. Acta 277*, 489.
Zunino, F., Gambetta, R., DiMarco, A., Zaccara, A., & Luoni, G. (1975) *Cancer Res 35*, 754.
Zunino, F., Gambetta, R., DiMarco, A., Velcich, A., Zaccara, A., Quadrifoglia, F., & Crescenzi, V. (1977) *Biochim. Biophys. Acta 476*, 38.

Purification of Deoxyribonucleic Acid Polymerase δ from Calf Thymus: Partial Characterization of Physical Properties†

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ABSTRACT: Deoxyribonucleic acid (DNA) polymerase δ has been purified 7800-fold from calf thymus, to a specific activity of 28 000 units/mg of protein. Similar to DNA polymerase δ from bone marrow [Byrnes, J. J., Downey, K. M., Black, V. L., & So, A. G. (1976) *Biochemistry 15*, 2817], the calf thymus enzyme is associated with 3′- to 5′-exonuclease activity. Both DNA polymerase and 3′- to 5′-exonuclease activities copurify on hydroxylapatite, DNA–cellulose, and molecular sieve chromatography. The ratio of exonuclease activity to polymerase activity is approximately 1:12. When the most highly purified fraction is subjected to polyacrylamide gel electrophoresis under nondenaturing conditions, both DNA polymerase and exonuclease activities have the same mobility at several acrylamide gel concentrations. Isoelectric focusing experiments have shown that both activities have the same p*I*. These data suggest that 3′- to 5′-exonuclease activity is an intrinsic property of DNA polymerase δ. The molecular weight of the enzyme, as estimated from measurements of Stokes radius and sedimentation coefficient, is 152 000.

There are two mechanisms by which procaryotic DNA polymerases maintain the high fidelity of DNA synthesis: (1) the selection of a complementary deoxynucleoside triphosphate for incorporation at the primer terminus by the polymerase activity and (2) the excision of a mismatched nucleotide incorporated at the primer terminus by the 3′- to 5′-exonuclease activity (Brutlag & Kornberg, 1972; Kornberg, 1974; Topal & Fresco, 1976; Alberts & Sternglanz, 1977; Que et al., 1979). Although fidelity of DNA synthesis is also rigidly maintained in eucaryotes, it has generally been believed that eucaryotic DNA polymerases lack an error-correcting 3′- to 5′-exonuclease activity (Bollum, 1975; Chang & Bollum, 1973; Sedwick et al., 1975; Loeb, 1974; Wang et al., 1974; Sarngadharan et al., 1978). However, there have been several recent reports of the association of 3′- to 5′-exonuclease activity with high molecular weight DNA polymerases from lower eucaryotes: *Euglena gracilis* (McLennan & Keir, 1975), yeast (Wintersberger, 1974; Helfman, 1973; Chang, 1977), *Ustilago maydis* (Banks et al., 1976; Yarranton & Banks, 1977), *Cylindrotheca fusiformis* (Okita & Volcani, 1977), and *Chlamydomonas reinhardii* (Ross & Harris, 1978).

We have previously reported the purification of a high molecular weight DNA polymerase with 3′- to 5′-exonuclease activity from rabbit bone marrow, DNA polymerase δ (Byrnes et al., 1976, 1977). The bone marrow enzyme is the only mammalian DNA polymerase thus far reported to be associated with a 3′- to 5′-exonuclease activity (Byrnes et al., 1976; Byrnes & Black, 1978). In this report we present data demonstrating that DNA polymerase δ is also found in calf thymus tissue and is not unique to bone marrow and that the 3′- to 5′-exonuclease activity is an intrinsic property of calf thymus DNA polymerase δ. Some of the physical properties of this enzyme are also presented.

Materials and Methods

Deoxynucleoside triphosphates were obtained from P-L Biochemicals, and [^{3}H]dTTP (50 Ci/mmol) was obtained from Amersham/Searle. Poly(dA–dT) was purchased from Boehringer Corp.; other synthetic polydeoxyribonucleotide and oligodeoxyribonucleotides were from either P-L Biochemicals or Collaborative Research. Fetal calf thymus glands were obtained from Texas Biological Supply Specialists, Inc. Calf thymus DNA was purchased from Worthington Biochemical Corp. Sephacryl S-200 and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals, phosphocellulose (P11) was from Whatman, and hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories. Acrylamide, methylenebis(acrylamide), Temed (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), and Coomassie blue were purchased from Bio-Rad Laboratories. Ampholytes were purchased from LKB.

Denatured DNA–cellulose was prepared as described by Litman (1968). [^{3}H]Poly(dT)$_{50}$ was synthesized with terminal deoxynucleotidyltransferase using oligo(dT)$_4$ as primer and [^{3}H]dTTP (200 cpm/pmol) as previously described (Que et al., 1978). Poly(dA–dT) labeled at the 3′ terminus with [^{3}H]dTMP was prepared as previously described (Byrnes et al., 1977). Activated calf thymus DNA was prepared according to Fansler & Loeb (1974).

DNA Polymerase Assays. When poly(dA–dT) was used as template/primer, the reaction mixture contained in a final

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volume of 0.125 mL the following: 0.028 A_{260} unit of poly(dA–dT); 40 mM Hepes buffer, pH 6.0; 40 mM KCl; 0.12 mM $MnCl_2$; 0.04 mM dATP; 4.0 μM [^{3}H]dTTP, 1.8 Ci/mmol; 0.2–0.4 unit of DNA polymerase; 0.01 mg of bovine serum albumin (BSA); 2% glycerol. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976).

When activated calf thymus DNA was used as template/primer, the reaction mixture contained in a final volume of 0.125 mL the following: 0.036 A_{260} unit of activated calf thymus DNA; 60 mM KCl; 0.04 mM each of dATP, dGTP, and dCTP; 4.2 μM[^{3}H]dTTP, 1.8 Ci/mmol; 40 mM Hepes buffer, pH 7.0; 0.1 mM $MnCl_2$; 0.2–0.4 unit of DNA polymerase; 0.01 mg of BSA; 2% glycerol. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976).

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

3′- to 5′-Exonuclease Assays. The 3′- to 5′-exonuclease activity of DNA polymerase δ was assayed by measuring the release of [^{3}H]dTMP from either 3′ terminally labeled poly(dA–dT)·[^{3}H]dTMP or [^{3}H]poly(dT)$_{50}$. When poly(dA–dT)·[^{3}H]dTMP was used as the substrate, each reaction mixture contained in a final volume of 0.06 mL the following: 66 mM Hepes buffer, pH 7.4; 0.2 mM $MnCl_2$; 3.3% glycerol; 0.1–0.2 unit of DNA polymerase δ; 4 μg of BSA; 3.9×10^{-3} A_{260} unit of poly(dA–dT)·[^{3}H]dTMP, 2.2×10^6 cpm/A_{260} unit. When [^{3}H]poly(dT)$_{50}$ was used as the substrate, each reaction mixture contained the following: 2 μM [^{3}H]poly(dT)$_{50}$ (150–200 cpm/pmol); 50 mM Hepes buffer, pH 7.4; 5 μg of BSA; 5 mM $MgCl_2$; 0.3 unit of DNA polymerase δ, in a final volume of 60 mL. The reaction mixture was incubated at 37 °C, and 30-μL aliquots were applied to 2.4-cm circles of Whatman DE-81 paper after 30 min of incubation. The circles were washed, dried, and counted as described by Brutlag & Kornberg (1972).

Polyacrylamide Gel Electrophoresis. Samples were dialyzed against 25 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM mercaptoacetic acid, 20% (v/v) ethylene glycol, and 30% (w/v) sucrose. Aliquots of 0.2 mL were layered on polyacrylamide gels (5 × 100 mm) according to the method of Fisher & Korn (1977). The gels were run at 2 mA/tube for 5 h at 0 °C. For recovery of DNA polymerase and 3′- to 5′-exonuclease activities the gels were sectioned into 2-mm slices, and individual slices were eluted by gentle agitation overnight at 4 °C in 0.25 mL of 25 mM Hepes buffer, pH 7.0, 0.2 M KCl, 20% (v/v) glycerol, 5 mM dithiothreitol, and 0.1% (v/v) Triton X-100.

Isoelectric Focusing. Isoelectric focusing was carried out in a LKB 2117 Multiphor flat-bed electrofocusing unit according to the procedure described in LKB Application Note 198 with some minor modifications. A 4% Ultradex solution (100 mL) containing 2% ampholytes (pH 3.5–10) in 15% glycerol was poured into the tray. Approximately 3 mL of DNA polymerase δ (step VII) was dialyzed against 10 mM Hepes buffer, pH 7.0, 15% glycerol, 0.5 mM EDTA, and 5 mM DTT for 4 h and applied as a narrow zone approximately 11 cm from the cathode. The sample was focused for 16 h at 4 °C, and the gel bed was divided into 30 fractions by the use of a fractionating grid. A small portion of each gel fraction was used to determine pH, and the rest was transferred into a small column (1 × 5 cm) and eluted with 5 mL of buffer D containing 0.25 M KCl. Aliquots of each eluate were assayed separately for polymerase and exonuclease activities as described above.

Purification of DNA Polymerase δ. All steps were carried out at 0–4 °C. Unless otherwise indicated, DNA polymerase activity was followed by using poly(dA–dT) as template/primer.

Preparation of Microsomal Extract. Frozen calf thymus tissue (250 g) was thawed, minced with scissors, and blended with 2 volumes of lysis buffer (10 mM Tris–HCl, pH 7.4, 7.5 mM KCl, 0.25 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride) in a Waring blender at high speed for three 20-s bursts. The suspension was gently homogenized with a loose-fitting glass–Teflon (Potter–Elvejehm) homogenizer and stirred for 20 min. Tissue debris, intact nuclei, and mitochondria were removed by centrifugation at 30000*g* for 15 min. The supernatant was carefully decanted and brought to 5 mM in $MgCl_2$ (fraction I).

Fraction I was centrifuged at 78000*g* for 2 h and the clear supernatant discarded. The pellet and the overlying loosely packed materials were homogenized in 100 mL of lysis buffer containing 1.0 M KCl (microsomal fraction). After centrifugation at 152000*g* for 90 min, the supernatant was decanted and saved (fraction II).

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added slowly to fraction II to 60% saturation. After being stirred for 30 min, the precipitate was collected by centrifugation at 30000*g* for 15 min, dissolved in 30 mL of buffer A (50 mM Tris-HCl, pH 7.8, 5.0 mM dithiothreitol, 20% (v/v) glycerol, 0.5 mM EDTA, and 0.1 mM PMSF) and stored at −76 °C (fraction III). When a total of 1 kg of calf thymus tissue had been processed through step III, the fractions were pooled and dialyzed against 2 L of buffer A for 4 h with two changes of buffer.

Phosphocellulose Chromatography. The dialyzed ammonium sulfate fraction (fraction III) was loaded onto a 4 × 16 cm column of phosphocellulose previously equilibrated with buffer A containing 0.05 M KCl. The column was washed with 3 column volumes of the same buffer, and the enzyme was eluted with a 1-L linear gradient of 0.05–1 M KCl in buffer A. The major peak of polymerase activity was eluted between 0.15 and 0.35 M KCl. Active fractions were pooled and dialyzed against 4 L of buffer A for 4 h with two changes of buffer (fraction IV).

DEAE-Sephadex A-25 Chromatography. Fraction IV was loaded onto a DEAE-Sephadex A-25 column (4.2 × 18 cm) previously equilibrated with buffer A. The column was washed with 3 column volumes of the same buffer, and the enzyme was eluted with an 800-mL linear gradient of KCl (0–0.5 M) in buffer A. The polymerase activity appeared as a broad indistinct peak between 0.08 and 0.2 M KCl. The fractions with polymerase activity were pooled (fraction V).

Hydroxylapatite Chromatography. Fraction V (200 mL) was loaded onto a column of hydroxylapatite (2.2 × 10 cm) that had been equilibrated with buffer B (0.03 M potassium phosphate buffer, pH 7.5, 20% (v/v) glycerol, and 5 mM dithiothreitol). The column was washed with buffer B and developed with a 500-mL linear gradient of 0.02–0.4 M potassium phosphate, pH 7.5. DNA polymerase δ, which prefers poly(dA–dT) as template as compared to activated calf thymus DNA, is eluted at 0.07 M potassium phosphate, whereas DNA polymerase α, which has a preference for activated calf thymus DNA as template, is eluted at 0.15 M potassium phosphate (Figure 1). Fractions containing DNA polymerase δ activity

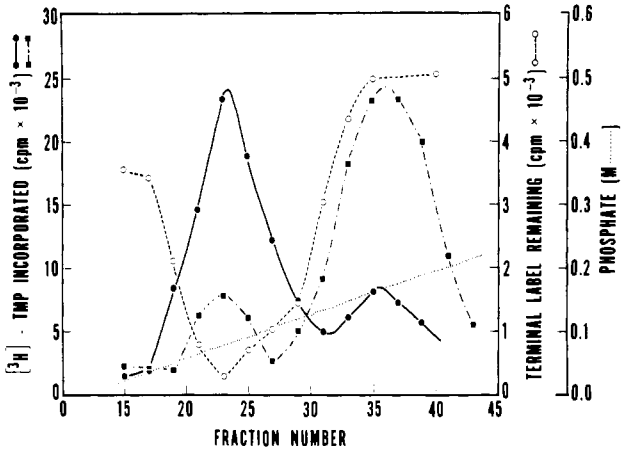

FIGURE 1: Resolution of DNA polymerase δ from DNA polymerase α upon hydroxylapatite chromatography. Experimental details were as described under Materials and Methods except that in the DNA polymerase assays the concentration of KCl was 0.015 M. Polymerase activity assayed with poly(dA–dT) (●); polymerase activity assayed with activated calf thymus DNA (■); exonuclease activity assayed with 3′ terminally labeled poly(dA–dT)·[^{3}H]dTMP as substrate and expressed as cpm remaining (○); potassium phosphate concentration (---).

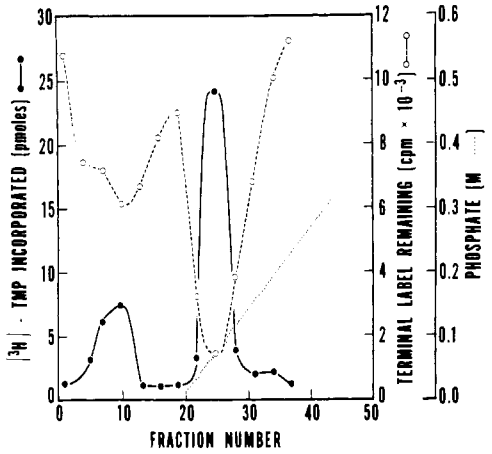

FIGURE 2: Coelution of polymerase and exonuclease activities on DNA–cellulose. Experimental details were as described under Materials and Methods. Polymerase activity assayed with poly(dA–dT) as template/primer (●); exonuclease activity with 3′ terminally labeled poly(dA–dT)·[^{3}H]dTMP as substrate and expressed as cpm remaining (○); potassium phosphate concentration (---).

were pooled (fraction VI) and dialyzed against buffer C. Buffer C is the same as buffer B except the pH of the phosphate buffer is 7.0 instead of 7.5.

DNA–Cellulose Chromatography. Fraction VI was loaded onto a DNA–cellulose column (1.5 × 4.5 cm) previously equilibrated with buffer C containing 0.1 mg/mL BSA. The column was extensively washed with buffer C and eluted with a 100-mL gradient of 0.02–0.4 M potassium phosphate. The enzyme eluted at 80 mM potassium phosphate (Figure 2). The fractions with enzymatic activity were pooled (fraction VII), concentrated by dialysis against solid sucrose, and dialyzed against buffer D (25 mM Hepes buffer, pH 7.0, 0.5 M KCl, 20% (v/v) glycerol, and 5 mM dithiothreitol).

Sephacryl S-200 Chromatography. Two and a half milliliters from a total of 6 mL of fraction VII was layered on a Sephacryl S-200 column (2.5 × 98 cm) previously equilibrated with buffer D and eluted with the same buffer at a rate of 20 mL/h. Four-milliliter fractions were collected. The enzyme activity eluted at 1.13 times the void volume (Figure 3). Fractions containing polymerase activity were pooled, concentrated against solid sucrose, and dialyzed against buffer

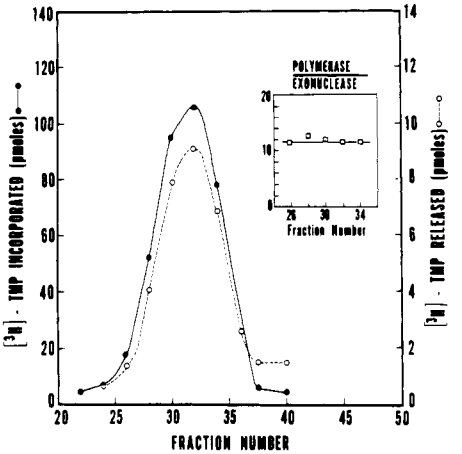

FIGURE 3: Cochromatography of polymerase and exonuclease activities on Sephacryl S-200. See Materials and Methods for experimental details. Polymerase activity assayed with poly(dA–dT) as template/primer (●); exonuclease activity assayed with [^{3}H]poly(dT)$_{50}$ as substrate and expressed as [^{3}H]TMP released (○); ratio of polymerase activity to exonuclease activity (□).

Table I: Purification of DNA Polymerase δ[a]

step	fraction	total act. (units)	total protein (mg)	sp act.	yield (%)
I	crude extract	50 000	14 000	3.6	100
II	microsomal extract	15 000	2 300	6.5	30
III	60% $(NH_4)_2SO_4$	10 000	1 000	10	20
IV	phosphocellulose	8 000	280	29	16
V	DEAE-Sephadex A-25	14 000	46	300	28
VI	hydroxylapatite	2 300	6	380	4.6
VII	DNA–cellulose	1 000	0.18	5 600	2
VIII	Sephacryl S-200	930	0.033	28 000	1.9

[a] Experimental details were as described under Materials and Methods. Poly(dA–dT) was used as template/primer to determine DNA polymerase activity. One unit of DNA polymerase catalyzes the incorporation of 1 nmol of total nucleotide per h at 37 °C. The starting material was 1 kg of fetal calf thymus.

D containing 0.05 M KCl instead of 0.5 M KCl (fraction VIII). BSA was added to a final concentration of 0.1 mg/mL.

Results

Cochromatography of DNA Polymerase with 3′- to 5′-Exonuclease Activity. The purification of DNA polymerase δ from calf thymus is summarized in Table I. From 1 kg of tissue, 33 μg of step VIII enzyme was obtained, representing a yield of 1.9% of the poly(dA–dT) activity. Step VIII enzyme was purified approximately 7800-fold compared to the crude extract.

The initial steps in the purification procedure (steps I–VI) are similar to those used in the purification of DNA polymerase δ from rabbit bone marrow (Byrnes et al., 1976). Although only 30% of the total activity present in the crude extract cosediments with the microsomes at low ionic strength (step II), omission of this step results in a decreased overall yield of DNA polymerase δ (data not shown), possibly because of the presence of proteolytic enzymes in the cytosol.

DNA polymerases α and δ are not separated on phosphocellulose chromatography (step IV) or DEAE-Sephadex chromatography (step V), since rather steep gradients were used to elute polymerase activity from these columns. The increase in total activity following chromatography on DEAE-Sephadex (Table I) suggests the removal of an inhibitor. DNA polymerases α and δ are separated on hydroxylapatite chromatography (step VI). As shown in Figure

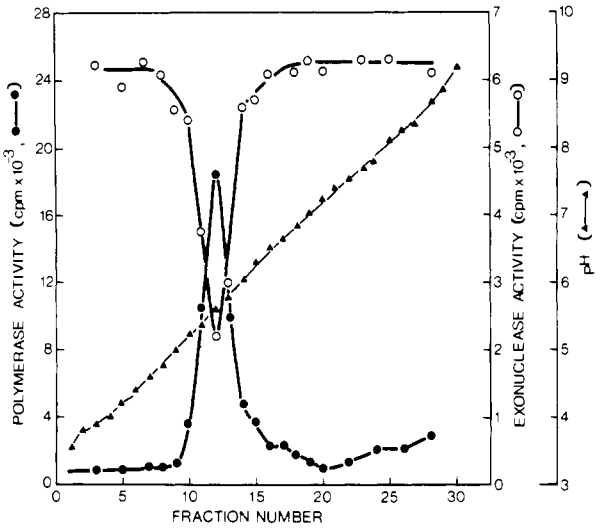

FIGURE 4: Comigration of polymerase and exonuclease activities on isoelectric focusing. Isoelectric focusing was carried out according to the procedure described in LKB Application Note 198 and as described under Materials and Methods. DNA polymerase and 3′- to 5′-exonuclease activities were assayed as described under Materials and Methods. Polymerase activity assayed with poly(dA–dT) as template/primer (●); exonuclease activity assayed with 3′ terminally labeled poly(dA–dT)·[^{3}H]dTMP (○); pH (▲). The recovery of polymerase activity was 70%.

1, the DNA polymerase activity which prefers poly(dA–dT) as template/primer (DNA polymerase δ) copurifies with 3′- to 5′-exonuclease activity on hydroxylapatite chromatography, eluting at 0.07 M potassium phosphate. No exonuclease activity is associated with the DNA polymerase activity which prefers activated calf thymus DNA as template/primer (DNA polymerase α) and elutes at 0.15 M potassium phosphate. The large loss of activity following hydroxylapatite chromatography (Table I) is in part due to the separation of DNA polymerase α at this step.

The 3′- to 5′-exonuclease activity also copurifies with DNA polymerase δ on DNA–cellulose chromatography; both activities are eluted at 80 mM potassium phosphate (Figure 2). DNA–cellulose chromatography has been used successfully to remove contaminating endonuclease and exonuclease from DNA polymerase α (de Recondo et al., 1977).

The elution profile of DNA polymerase δ on Sephacryl S-200 chromatography is shown in Figure 3, and the ratio of polymerase to exonuclease activity is shown in the insert. Again, the elution profile of the 3′- to 5′-exonuclease activity coincides with the elution profile of the polymerase activity. More importantly, the ratio of polymerase activity to exonuclease activity is constant across the peak. At this stage of purification, the ratio of exonuclease activity to polymerase activity is approximately 1:12.

The apparent molecular weight of DNA polymerase δ as determined by gel filtration on Sephacryl S-200 has been estimated to be 210 000 (unpublished experiment). The molecular weight standards used to calibrate the Sephacryl column were catalase (240 000), aldolase (150 000), yeast alcohol dehydrogenase (141 000), creatine kinase (80 000), ovalbumin (45 000), and cytochrome *c* (12 500).

Identical Isoelectric Point for Both Polymerase and 3′- to 5′-Exonuclease Activities. When DNA polymerase δ fraction VII is subjected to isoelectric focusing, both the polymerase and the 3′- to 5′-exonuclease activities were found to have a p*I* of 5.55 (Figure 4).

Coincidence of DNA Polymerase and 3′- to 5′-Exonuclease Activities on Polyacrylamide Gel Electrophoresis. When step

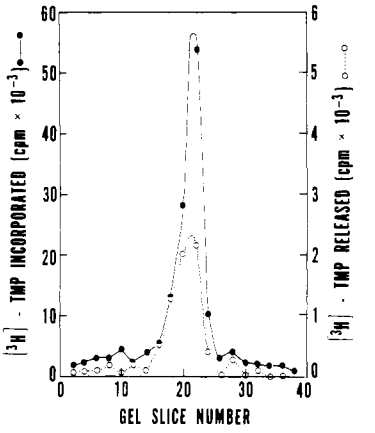

FIGURE 5: Coincidence of polymerase and exonuclease activities on polyacrylamide gel electrophoresis. DNA polymerase δ fraction VIII (4 μg) was layered on a 5% polyacrylamide gel and subjected to electrophoresis according to the method of Fisher & Korn (1977) and as described under Materials and Methods. Polymerase activity assayed with poly(dA–dT) (●); exonuclease activity assayed with poly(dA–dT)·[^{3}H]dTMP as substrate (○). The recovery of DNA polymerase activity following electrophoresis was 72%.

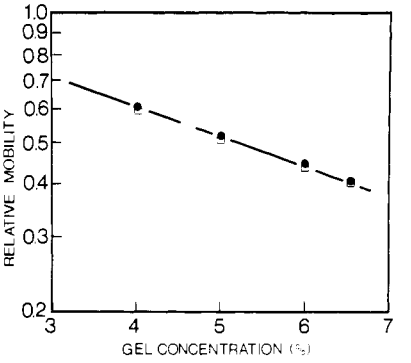

FIGURE 6: Plot of mobility of DNA polymerase δ as a function of gel concentration. The relative mobilities (R_m) of polymerase and 3′- to 5′-exonuclease activities of DNA polymerase δ were determined at the concentrations of polyacrylamide gel indicated: 4, 5, 6, and 6.5%. Electrophoresis was carried out as described in Figure 6. The polymerase and exonuclease activities were assayed as described under Materials and Methods. Polymerase activity assayed with poly(dA–dT) (●); exonuclease activity assayed with poly(dA–dT)·[^{3}H]dTMP (□).

VIII enzyme is analyzed in a discontinuous 5% nondenaturing polyacrylamide gel electrophoretic system, both DNA polymerase and 3′- to 5′-exonuclease activities exhibit the same relative mobility with an R_m of 0.525 (Figure 5). Analytical polyacrylamide gel electrophoresis under nondenaturing conditions was also run at three additional gel concentrations to investigate the possibility of fortuitous comigration of the DNA polymerase with a contaminating 3′- to 5′-exonuclease activity. The exact coincidence of the polymerase activity with the exonuclease activity is shown in Figure 6, where the log R_m values of both the polymerase and the exonuclease activities are plotted as a function of gel concentration. The exact linear relationship obtained in Figure 6 establishes the identity of the polymerase activity with the 3′- to 5′-exonuclease activity (Fisher & Korn, 1977; Robard & Chrambach, 1974).

Molecular Weight. A better estimate of the molecular weight of DNA polymerase δ has been determined from the sedimentation coefficient (Martin & Ames, 1961) and the Stokes radius (Siegel & Monty, 1966). A sedimentation coefficient of 7.6 was determined by centrifugation of fraction VIII enzyme in a 15–30% linear glycerol gradient containing 0.5 M KCl (data not shown). Bovine serum albumin, adolase, and catalase were used as standards. The Stokes radius was

Table II: Physical Properties of DNA Polymerase δ

app mol wt	210 000
Stokes radius	48.3
$s_{20,w}$	7.6
calcd mol wt	152 000
f/f_0	1.37
isoelectric point	5.55

determined by native polyacrylamide gel electrophoresis of DNA polymerase δ by using standards of known Stokes radius (data not presented). The standards used were ovalbumin (26 Å), bovine serum albumin (37.5 Å), catalase (52 Å), and ferritin (79 Å). The molecular radius of DNA polymerase δ was calculated to be 48.3 Å. Assuming a partial specific volume of 0.725, the molecular weight of DNA polymerase δ is estimated to be 152 000. The physical properties of the enzyme are summarized in Table II.

Discussion

We have previously reported the purification of a novel species of high molecular weight DNA polymerase, DNA polymerase δ from rabbit bone marrow (Byrnes et al., 1976, 1977). This enzyme, in contrast to other mammalian DNA polymerases (α, β, and γ) (Weissbach et al., 1975; Holmes & Johnston, 1975; Bollum, 1975; Falaschi & Spadari, 1977; Sarngadharan et al., 1978; Korn et al., 1977), is associated with a 3′- to 5′-exonuclease activity. The present study confirms and extends our previous observations on this enzyme and demonstrates the presence of DNA polymerase δ in calf thymus tissue. We have purified calf thymus DNA polymerase δ approximately 7800-fold to a specific activity of 28 000 units/mg. This is comparable to the reported specific activities of other highly purified eucaryotic DNA polymerases (Hasslewood et al., 1978; Sedwick et al., 1975; Fisher & Korn, 1977).

The data presented here indicate that purified DNA polymerase δ from calf thymus has an associated 3′- to 5′-exonuclease activity, as evidenced by the constant association of both polymerase and exonuclease activities throughout various purification and analytical procedures, e.g., ion-exchange chromatography, gel filtration, affinity chromatography, and, most importantly, disc gel electrophoresis at several gel concentrations. At the final stage of purification the ratio of exonuclease activity to polymerase activity of DNA polymerase δ is approximately 1:12. This is comparable to the ratio of activities found in *Escherichia coli* DNA polymerase I (Setlow, 1974; Que et al., 1978).

From measurements of the Stokes radius and the sedimentation coefficient, a molecular weight of 152 000 has been calculated for calf thymus DNA polymerase δ. The apparent molecular weight as estimated by gel filtration is 210 000, suggesting that DNA polymerase δ is an asymmetric molecule. A relative frictional coefficient of 1.37 is also consistent with this suggestion.

The association of 3′- to 5′-exonuclease activity with high molecular weight DNA polymerase has recently been reported in several lower eucaryotes (McLennan & Keir, 1975; Wintersberger, 1974; Helfman, 1973; Chang, 1977; Banks et al., 1976; Yarranton & Banks, 1977; Okita & Volcani, 1977; Ross & Harris, 1978). Whether DNA polymerase δ is related to any of these enzymes is not known at present.

In the nomenclature of eucaryotic DNA polymerases established in 1975 (Weissbach et al., 1975), DNA polymerase α was operationally defined as a high molecular weight enzyme (>100 000) and an acidic protein, sensitive to sulfhydryl blocking reagents, preferring "activated" double-stranded DNA as template, unable to copy poly(rA)/oligo(dT), and devoid of nuclease activity. Similar to DNA polymerase α, DNA polymerase δ is also a high molecular weight protein (152 000), extremely sensitive to inhibitors that block sulfhydryl groups, being inhibited approximately 50% by 0.2 mM *N*-ethylmaleimide (unpublished experiments), and an acidic protein (p*I* = 5.55). However, in contrast to DNA polymerase α, DNA polymerase δ prefers poly(dA–dT) to activated calf thymus DNA as template/primer and, most distinctively, is associated with 3′- to 5′-exonuclease activity.

Several high molecular weight species of DNA polymerase from calf thymus have previously been resolved by chromatographic procedures (Holmes & Johnston, 1975; Hasslewood et al., 1978; Yoshida et al., 1974). These DNA polymerases have been classified as forms of DNA polymerase α because of their molecular size. Whether any of these species of DNA polymerase is identical with or related to DNA polymerase δ will have to await further characterization of these enzymes and immunological studies.

References

Alberts, B., & Sternglanz, R. (1977) *Nature (London) 269*, 655.

Banks, G. R., Holloman, W. K., Kairis, M. V., Yarranton, G. T., & Spanos, A. (1976) *Eur. J. Biochem. 62*, 131.

Bollum, F. J. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 15*, 109.

Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 241.

Byrnes, J. J., & Black, V. L. (1978) *Biochemistry 17*, 4226.

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

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

Chang, L. M. S. (1977) *J. Biol. Chem. 252*, 1873.

Chang, L. M. S., & Bollum, F. J. (1973) *J. Biol. Chem. 248*, 3398.

de Recondo, A. M., Rossignol, J. M., Mechali, M., & Duguet, M. (1977) in *DNA Synthesis Present and Future* (Molineux, I., & Kohiyama, M., Eds.) p 599, Plenum Press, New York.

Falaschi, A., & Spadari, S. (1977) in *DNA Synthesis Present and Future* (Molineux, I., & Kohiyama, M., Eds.) p 487, Plenum Press, New York.

Fansler, B. S., & Loeb, L. A. (1974) *Methods Enzymol. 29*, 53.

Fisher, P. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 6528.

Hasslewood, I. P., Holmes, A. M., Wakeling, W. F., & Johnston, I. R. (1978) *Eur. J. Biochem. 84*, 123.

Helfman, W. B. (1973) *Eur. J. Biochem. 32*, 42.

Holmes, A. M., & Johnston, I. R. (1975) *FEBS Lett. 60*, 233.

Korn, D., Eichler, D. C., Fisher, P. A., & Wang, T. S.-F. (1977) in *DNA Synthesis Present and Future* (Molineux, I., & Kohiyama, M., Eds.) p 517, Plenum Press, New York.

Kornberg, A. (1974) in *DNA Synthesis*, W. H. Freeman, New York.

Litman, R. M. (1968) *J. Biol. Chem. 243*, 6222.

Loeb, L. A. (1974) *Enzymes, 3rd Ed. 10*, 173.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.

McLennan, A. G., & Keir, H. M. (1975) *Biochem. J. 151*, 227.

Okita, T. W., & Volcani, B. E. (1977) *Biochem. J. 167*, 611.

Que, B. G., Downey, K. M., & So, A. G. (1978) *Biochemistry 17*, 1603.

Que, B. G., Downey, K. M., & So, A. G. (1979) *Biochemistry 18*, 2064.

Robard, D., & Chrambach, A. (1974) in *Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel* (Allen, R. C., & Maurer, H. R., Eds.) de Gruyter, New York.
Ross, C. A., & Harris, W. J. (1978) *Biochem. J. 171*, 241.
Sarngadharan, M. G., Robert-Guroff, M., & Gallo, R. C. (1978) *Biochim. Biophys. Acta 516*, 419.
Sedwick, W. D., Wang, T. S.-F., & Korn, D. (1975) *J. Biol. Chem. 250*, 7045.
Setlow, P. (1974) *Methods Enzymol. 29*, 3.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346.
Topal, M. D., & Fresco, J. R. (1976) *Nature (London) 263*, 285.
Wang, T. S.-F., Sedwick, D., & Korn, D. (1974) *J. Biol. Chem 249*, 841.
Weissbach, A., Baltimore, D., Bollum, F., Gallo, R., & Korn, D. (1975) *Science 190*, 401.
Wintersberger, E. (1974) *Eur. J. Biochem. 50*, 41.
Yarranton, G. Y., & Banks, G. R. (1977) *Eur. J. Biochem. 77*, 521.
Yoshida, S., Kondo, T., & Ando, T. (1974) *Biochim. Biophys. Acta 353*, 463.

Accumulation of Methyl-Deficient Rat Liver Messenger Ribonucleic Acid on Ethionine Administration†

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ABSTRACT: Highly purified poly(adenylic acid)-containing RNA isolated from livers of rats fed 0.25% DL-ethionine in the diet for 7 days accepted methyl groups from *S*-adenosyl[*methyl*-^{3}H]methionine, when incubated in vitro with mRNA methyltransferases from vaccinia virus or Ehrlich ascites cells, whereas RNA from control rats had no such activity. Nuclease digestion followed by chromatographic analyses of mRNA methylated in vitro revealed that the methyl groups were incorporated at the 5′ end into cap 1 structures (m^7GpppNmp...) by the viral enzyme, whereas both cap 0 (m^7GpppNp...) and cap 1 (m^7Gpppm^{6}Am...) structures were formed by the Ehrlich ascites cell enzymes. The methyl-deficient mRNA isolated from the liver of ethionine-fed rats differed in its translational properties from mRNA isolated from control animals in an in vitro protein synthesizing system from wheat germ.

Several eukaryotic viral and cellular mRNAs as well as heterogeneous nuclear RNA (hnRNA) contain at their 5′ terminus a blocked methylated structure (often referred to as "cap") of the type m^7GpppN′mpN″mp....[1] In this structure, the 7-methylguanosine and the penultimate nucleoside are joined by the 5′-hydroxyl groups through a triphosphate bridge. Nucleosides N′ and N″ are often methylated in the 2′-O position and an N^6,2′-*O*-dimethyladenosine may be found in position N′ (Shatkin, 1976). Certain mammalian, low molecular weight nuclear RNAs, whose functions are not known, have 5′ termini of a similar configuration (Busch, 1976). Additional methylation of several mRNAs occurs internally between its 5′ cap and the 3′-poly(A) end, yielding 6-methyladenosine (Shatkin, 1976) and 5-methylcytosine (Dubin & Stollar, 1975).

The 5′-terminal 7-methylguanosine in mRNAs is apparently required for their efficient translation in vitro (Both et al., 1975; Muthukrishnan et al., 1975, 1978), and is also implicated in protection against degradation by exonucleases (Furuichi et al., 1977). The physiological significance of ribose methylation and internal methylation remains obscure at present.

Considerable information about the enzymatic mechanisms involved in the posttranscriptional modifications of mRNA has been obtained mainly through the use of coupled viral transcription and methylation systems (Wei & Moss, 1974; Furuichi et al., 1976; Furuichi & Shatkin, 1977), or of isolated nuclei, capable of carrying out some of these modifications (Winicov & Perry, 1976). Methyl-deficient RNA synthesized in vitro by viral cores has been used to study reactions catalyzed by enzymes solubilized from purified virus by detergent treatment (Ensinger et al., 1975; Martin et al., 1975) or by purifying some of the modifying enzymes (Ensinger & Moss, 1976; Barbosa & Moss, 1978; Keith et al., 1978). Partially modified mRNAs isolated from purified virus particles have also been used as substrates (Moss, 1977). A homologous substrate for studying these modification reactions in vitro has been isolated from a methionine auxotroph of *Neurospora crassa* under conditions of methionine starvation (Germershausen et al., 1978). However, attempts to produce homologous methyl-deficient mRNA by methionine starvation from mammalian cells have not been successful (Kaehler et al., 1977).

Administration of the hepatocarcinogen ethionine to rats is known to result in the accumulation in liver of undermethylated tRNAs, which are capable of accepting methyl groups from *S*-adenosyl[*methyl*-^{3}H]methionine in vitro by homologous enzymes (Rajalakshmi, 1973; Kerr, 1975; Friedman, 1977; Wainfan et al., 1977).

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[1] Abbreviations used: m^7G, 7-methylguanosine; N, ribonucleoside; Nm, 2′-O-methylated ribonucleoside; m^6A, N^6-methyladenosine; m^6Am, N^6,2′-*O*-dimethyladenosine; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; hnRNA, heterogeneous ribonucleic acid; NETS buffer, 10 mM Tris-HCl, 1 mM EDTA, 0.4 M NaCl, and 0.5% $NaDodSO_4$; ETS buffer, 10 mM Tris-HCl, 1 mM EDTA, and 0.5% $NaDodSO_4$; Cl_3AcOH, trichloroacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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