A New Mammalian DNA Polymerase with 3′ to 5′ Exonuclease Activity: DNA Polymerase δ†

John J. Byrnes,*,‡ Kathleen M. Downey,§ Vicky L. Black, and Antero G. So¶

ABSTRACT: A new species of DNA polymerase has been purified more than 10 000-fold from the cytoplasm of erythroid hyperplastic bone marrow. This DNA polymerase, in contrast to previously described eukaryotic DNA polymerases, is associated with a very active 3′ to 5′ exonuclease activity. Similar to the 3′ to 5′ exonuclease activity associated with prokaryotic DNA polymerases, this enzyme catalyzes the removal of 3′-terminal nucleotides from DNA, as well as a template-dependent conversion of deoxyribonucleoside triphosphates to monophosphates. The exonuclease activity is not separable from the DNA polymerase activity by chromatography on DEAE-Sephadex or hydroxylapatite, and upon sucrose density gradient centrifugation the two activities cosediment at 7 S or at 11 S depending on the ionic strength. Both exonuclease and polymerase activities have identical rates of heat inactivation and both are equally sensitive to hemin and Rifamycin AF/013, inhibitors of DNA synthesis that act by binding to DNA polymerase and causing its dissociation from its template/primer. These results are consistent with the coexistence of two enzyme activities in a single protein.

The high fidelity of DNA replication in prokaryotes has been ascribed, in part, to the presence of a 3′ to 5′ exonuclease associated with DNA polymerase. This exonuclease is an integral part of *Escherichia coli* DNA polymerase I and is found to be present in all prokaryotic DNA polymerases thus far studied (Gefter, 1975). The 3′ to 5′ exonuclease has a proofreading function, as it removes mismatched nucleotides incorporated at the primer terminus during DNA polymerization (Brutlag and Kornberg, 1972). Mutations in the gene coding for T_4 DNA polymerase can result in either an increased rate (mutator) of spontaneous mutation (Speyer, 1965) or a decreased rate (antimutator) of spontaneous mutation (Drake et al., 1969). The increased frequency of spontaneous mutation in the mutator strains is due to a much higher ratio of DNA polymerase to exonuclease activity (i.e., the exonuclease activity is low or altered and, thus, unable to remove errors of incorporation) and, conversely, the DNA polymerase purified from the antimutator strain has a much higher nuclease to polymerase ratio (Muzyczka et al., 1972). Thus, this exonuclease activity plays an important role in replication fidelity and the prevention of mutation.

Fidelity of DNA replication is also rigidly maintained in eukaryotes. However, the eukaryotic DNA polymerases thus far studied have been reported not to be associated with any exonuclease activity (Chang, 1973; Chang and Bollum, 1973; Bollum, 1975; Sedwick et al., 1975; Wang et al., 1974; Loeb, 1974), and it is now generally believed that all purified eukaryotic DNA polymerases contain no exonuclease activity (Wang et al., 1974; Sedwick et al., 1975; Bollum, 1975; Loeb, 1974; Loeb et al., 1974; also see reviews by Gefter, 1975; Kornberg, 1975; Keir and Craig, 1973). Since the frequency of spontaneous mutation in eukaryotic cells is not significantly different from that in prokaryotes, it has been suggested that different mechanisms must exist for correcting misincorporation in eukaryotic cells (Bollum, 1975; Loeb, 1974; Wang et al., 1974).

We wish to report the presence of 3′ to 5′ exonuclease activity in a new species of high-molecular-weight DNA polymerase purified from the cytoplasm of erythroid hyperplastic bone marrow. This DNA polymerase (DNA polymerase δ)[1] is similar to the previously reported eukaryotic DNA polymerase α (Byrnes et al., 1973; Spadari et al., 1974) in its sedimentation properties but may be distinguished from this enzyme by its template specificity and its association with a very active 3′ to 5′ exonuclease activity. In this report we will present data establishing that (1) a nucleolytic activity is associated with DNA polymerase δ and (2) this nucleolytic activity is a 3′ to 5′ exonuclease.

Materials and Methods

^{3}H-Labeled deoxyribonucleoside triphosphates were purchased from New England Nuclear Corp. or Amersham-Searle. Unlabeled deoxyribonucleoside triphosphates were obtained from P-L Biochemicals or Calbiochem. ^{3}H-Labeled poly[d(A-T)] ($1-5 \times 10^6$ mol wt) was obtained from General Biochemicals and was dialyzed before use against 0.01 M Tris-HCl buffer,[2] pH 7.4, containing 0.06 M KCl. Poly[d(A-T)] was either purchased from General Biochemicals or synthesized according to the method of Schachman et al. (1960). Rifamycin AF/013 was a gift of Drs. Giancarlo Lancini and Renato Cricchio of Gruppo Lepetit, Milan, Italy. Hemin chloride was obtained from Calbiochem. Poly(rA)·oligo(dT) was obtained from Collaborative Research and 5′-nucleotidase was obtained from Sigma. Activated calf thymus DNA was prepared as described by Aposhian and Kornberg (1962). Protein was quantitated by the method of Lowry (Lowry et al., 1951) after precipitation with 10% trichloroacetic acid. Salt concentrations were determined by conductivity measurements.

† From the Department of Medicine, Veterans Administration Hospital, and the Departments of Medicine and Biochemistry, University of Miami, Miami, Florida 33152. *Received December 8, 1975.* This research was supported by a grant from the National Institutes of Health (NIH AM 09001).

‡ Veterans Administration Research Associate.

§ Research Career Development Awardee (NIH-K04-HL-00031-01).

¶ Investigator, Howard Hughes Medical Institute.

[1] We have adopted the nomenclature for mammalian DNA polymerase suggested by Baltimore et al., and cited in Gillespie et al. (1975).

[2] Abbreviations used are: DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Preparation of 3′ Terminus-[^{3}H]TMP-Labeled Poly[d(A-T)]. Five units of DNA polymerase δ in a reaction mixture containing: 1.2 A_{260} units of poly[d(A-T)]; 40 μCi of [^{3}H]TTP, 57 Ci/mmol; 40 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (Hepes) buffer, pH 7.0; 20 mM $(NH_4)_2SO_4$; 0.4 mM $MnCl_2$; and 1.6 mM AMP in a volume of 5.0 ml were incubated at 37 °C for 30 min. The reaction was stopped by addition of 12.5 μmol of EDTA and sodium dodecyl sulfate to a final concentration of 1%. The product was chromatographed on a Bio-Gel A 1.5 (100–200 mesh) column (4.2 × 37 cm) previously equilibrated with 0.01 M Tris, pH 7.4, and 0.06 M KCl. The A_{260} peak appearing in the void volume had a specific activity of 16 μCi/A_{260} unit. Assuming that TMP was incorporated at every other 3′-terminus, the calculated chain length of the poly[d(A-T)]·[^{3}H]TMP is approximately 4000 nucleotides (mol wt, 1.4×10^6), which is in reasonable agreement with the known size range.

DNA Polymerase Assay. When poly[d(A-T)] was used as template/primer the reaction mixture contained: 0.06–0.12 A_{260} unit/ml of poly[d(A-T)]; 80 mM Hepes buffer, pH 7.0; 40 mM KCl; 0.4 mM $MnCl_2$; 0.48 mM dATP; 80 μM [^{3}H]-TTP, 100 Ci/mol; and 2–4 units of DNA polymerase in a final volume of 0.25 ml. After incubation for 10 min at 37 °C the reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1973).

When activated calf thymus DNA was used as template/primer the reaction mixture contained: 80 μg/ml activated calf thymus DNA; 120 mM KCl; 0.16 mM each of dATP, dGTP, and dCTP; 80 μM [^{3}H]TTP, 100 Ci/mol; 40 mM Hepes buffer, pH 7.0; 0.4 mM $MnCl_2$ and 2–4 units DNA polymerase in a final volume of 0.25 ml.

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

Assay for 3′ to 5′ Exonuclease Activity. The 3′ to 5′ exonuclease assays were carried out in a final volume of 0.15 ml and contained: 0.017 A_{260} unit/ml of poly[d(A-T)]·[^{3}H]TMP; 80 mM Hepes buffer, pH 7.0; 1 mM $MnCl_2$; 120 mM KCl and 1–2 units of DNA polymerase. The reaction mixture was incubated at 37 °C for 10 min and was terminated by quick chilling and the addition of 2 ml of cold 5% trichloroacetic acid. After remaining on ice for 10 min the mixture was filtered on Millipore HAWP filter disks, 0.45 μm, and washed with 15 ml of distilled water. The filter disks were dried and counted in a toluene–Omnifluor solution in a liquid scintillation spectrometer.

Assay for Template-Dependent Generation of Monophosphates. Highly purified DNA polymerase (step VI) was used to catalyze the template dependent conversion of dNTP to dNMP. Each reaction mixture contained 1.0 μCi of labeled nucleoside triphosphate and the specific activities ranged from 1500 cpm/pmol to 15 cpm/pmol. In addition, the reaction mixture contained: 40 mM Hepes buffer, pH 7.0, 120 mM KCl, 1.0 mM $MnCl_2$, and 0.1 A_{260} unit/ml of poly[d(A-T)] in a final volume of 0.125 ml. The reaction was started by the addition of 2 units of DNA polymerase and incubated at 37 °C for 30 min. The reaction was terminated by rapid chilling and the addition of EDTA to a concentration of 2 mM. Nucleoside monophosphates, diphosphates, and triphosphates were separated by thin-layer chromatography on poly(ethylenimine)–cellulose. Multiple control incubations were run including zero time samples and reaction mixtures that lacked enzyme or template/primer.

Poly(ethylenimine)–Cellulose Thin-Layer Chromatography. Poly(ethylenimine)-impregnated cellulose thin-layer plates 20 × 20 cm (Polygram Cel 300 PEI) purchased from Brinkmann Instruments, Inc. were prerun with distilled water and were stored at 0 °C before use. Lanes of 1.5 cm were formed by scraping channels in the adsorbent. The reaction mixture was applied 2 cm from the bottom in the middle of the lane by the consecutive application of 20 μl aliquots with intervening and subsequent drying. The plates were then immersed in 800 ml of absolute methanol for 15 min. After drying, the plates were developed with 1.0 M LiCl for thymidine and deoxycytidine, 1.1 M LiCl for deoxyadenosine, and 1.2 M LiCl for deoxyguanosine nucleotides. The plates were dried and each lane was cut into 18 1-cm sections, placed in toluene–Omnifluor, and counted in a liquid scintillation spectrometer. Deoxyribonucleoside triphosphate, diphosphate, and monophosphate markers were located by ultraviolet absorption.

Purification of DNA Polymerase. Erythroid hyperplastic bone marrows were induced in rabbits by the procedure of Borsook et al. (1952). New Zealand rabbits weighing 6–8 lb were given daily injections of 1.0 ml of a 2.5% neutralized phenylhydrazine solution for 4 days. The rabbits received no injections on the 5th and 6th days and were killed on the 7th day. The femur and tibia were removed and immediately placed in ice-cold washing buffer (10 mM Tris-HCl, pH 7.8, 0.13 M NaCl, 5 mM KCl, 7 mM $MgCl_2$). The bones were cracked open and the marrow was removed into washing buffer. Wright stain of the marrow cells revealed an intense erythroid hyperplasia with greater than 70% erythroid precursors. The marrow cells were sedimented at 4000*g* for 10 min and lysed by the addition of 10 volumes of lysing buffer (5 mM Tris-HCl, pH 7.4, 7.5 mM KCl, 10% glycerol, 0.25 mM 2-mercaptoethanol). The suspension was gently dispersed with a loose-fitting glass–Teflon homogenizer and stirred for 20 min. Intact nuclei, mitochondria, and stroma were removed by centrifugation at 30 000*g* for 15 min.

The 30 000*g* supernatant fraction (step I) was brought to 5 mM $MgCl_2$ and centrifuged at 78 000*g* for 2.5 h. The microsomal pellets were suspended in buffer A (50 mM Tris-HCl, pH 7.8, 1.0 mM dithiothreitol, 0.1 mM EDTA, 25% glycerol) containing 1 M KCl by gentle homogenization in a loose-fitting glass homogenizer and the suspension was left overnight at 0 °C. The suspension was centrifuged at 152 000*g* for 75 min and the supernatant was separated from the microsomal fraction. The microsomal extract (step II) was adjusted to 60% saturation with ammonium sulfate and the precipitate was collected by centrifugation at 30 000 g for 15 min. The bones of 12 rabbits were processed, and the ammonium sulfate precipitate obtained was stored in buffer A at −70 °C. Resuspended precipitate from four batches was dialyzed against 2 l. of buffer A for 4 h with two changes of buffer (step III).

Phosphocellulose Chromatography. The dialyzed ammonium sulfate fraction (80 ml) was applied to a phosphocellulose column (3.8 × 12 cm), equilibrated with buffer A containing 0.05 M KCl, and washed with the same buffer. A linear gradient of 0.05–1 M KCl in 1 l. of buffer A was applied, and 8-ml fractions were collected. DNA polymerase activity eluted at 0.24 M KCl. The fractions containing DNA polymerase were pooled and dialyzed against 2 l. of buffer A for 4 h with two changes of buffer (step IV).

DEAE-Sephadex A-25 Chromatography. Approximately 100 ml of the dialyzed phosphocellulose eluate was applied to a column of DEAE-Sephadex A-25 (3.8 × 12 cm) previously equilibrated with buffer A. A linear gradient of 0–0.5 M KCl in 500 ml of buffer A was applied, and 5-ml fractions were collected. Poly[d(A-T)]-directed DNA polymerase activity

TABLE I: Purification of DNA Polymerase δ.[a]

Step	Total Act. (units)	Protein (mg)	Sp Act. (units/mg of protein)	Yield (%)
I, 30 000*g* supernatant	5096	25 200	0.2	100
II, microsomal extract	4994	2 040	2.4	98
III, 60% ammonium sulfate precipitate	4839	1 337	3.6	95
IV, phosphocellulose chromatography	2664	83.7	31.8	52
V, DEAE-Sephadex A-25 chromatography	1878	15.0	125	37
VI, hydroxylapatite chromatography	1283	0.6	2138	25

[a] Experimental details were as described under Materials and Methods. Poly[d(A-T)] was used as template to determine DNA polymerase activity.

eluted at 0.13 M KCl, and active fractions were pooled (step V).

Hydroxylapatite Chromatography. Approximately 50 ml of DEAE eluate containing the DNA polymerase was loaded directly onto a hydroxylapatite column (1.4 × 15 cm) equilibrated with 0.02 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM dithiothreitol (buffer B). The column was washed with buffer B and developed with a linear gradient of 0.02–0.5 M potassium phosphate, pH 7.5, in a total volume of 200 ml. The poly[d(A-T)]-directed DNA polymerase eluted at 0.04 M potassium phosphate. Active fractions were pooled and dialyzed against 25 volumes of buffer A containing 0.125 M KCl for 2 h (step VI).

Sucrose Density Gradient Centrifugation. Sucrose solutions contained 4 mM Hepes buffer, pH 7.0, 0.4 mM $MnCl_2$, 1.0 mM dithiothreitol, and either 50 mM KCl (low salt) or 300 mM KCl (high salt). Twenty-five units of DNA polymerase from step V was dialyzed against 250 ml of 5% sucrose solution containing either high or low salt at 4 °C for 2 h. A 0.20-ml aliquot of the enzyme was layered on a 4.8-ml, 5–20% linear sucrose gradient containing the appropiate KCl concentration and centrifuged in a SW 65 rotor at 43 500 rpm for 16.5 h at 4 °C. The gradients were collected in 0.2-ml fractions and assayed for DNA polymerase and 3′ to 5′ exonuclease activity. Rat liver tRNA was run simultaneously as a 4S marker.

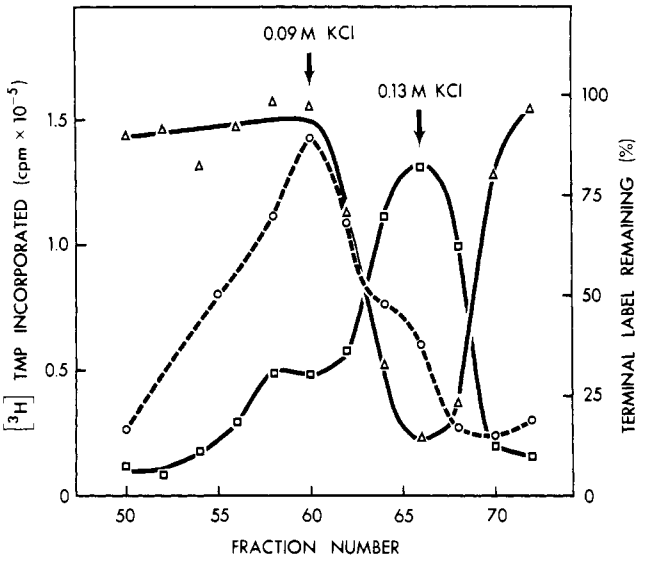

FIGURE 1: DEAE-Sephadex A-25 chromatography of DNA polymerase. Experimental details were as described under Materials and Methods except that in the DNA polymerase assay the concentration of [^{3}H]TTP was 0.16 μM, 50 mCi/μmol. (O - - - O) Polymerase activity assayed with activated calf thymus DNA; (□—□) polymerase activity assayed with poly[d(A-T)]; (△—△) exonuclease activity.

Results

Co-Chromatography of DNA Polymerase with 3′ to 5′ Exonuclease Activity. The purification of DNA polymerase δ from the cytoplasm of erythroid hyperplastic bone marrow is summarized in Table I. As shown in Figure 1, two DNA polymerase activities are partially separated on DEAE-Sephadex chromatography. The polymerase activity that elutes at 0.09 M KCl prefers activated calf thymus DNA over poly[d(A-t)] as template, while the polymerase activity that elutes at 0.13 M KCl has greater preference for poly[d(A-T)] as template. The most active fractions with poly[d(A-T)] as template appear as a trailing shoulder in the activated calf thymus DNA-directed DNA polymerase elution profile. The elution profile of 3′ to 5′ exonuclease activity, measured by the release of [^{3}H]TMP from poly[d(A-T)]·[^{3}H]TMP, corresponds to that of the poly[d(A-T)]-directed DNA polymerase activity, while activated calf thymus directed-DNA polymerase contains little or no associated 3′ to 5′ exonuclease activity. For this reason, we have used poly[d(A-T)] as template in the purification scheme summarized in Table I.

The two DNA polymerase activities have been further resolved on hydroxylapatite chromatography, as shown in Figure 2. Both the polymerase activity that is active with poly[d(A-T)] (DNA polymerase δ) and the exonuclease activity elute together at 0.04 M potassium phosphate, whereas the peak of activated calf thymus DNA-directed activity (DNA polymerase α) elutes at 0.075 M potassium phosphate and contains no exonuclease activity. Thus, the use of activated calf thymus DNA as template to assay for DNA polymerase activity during purification leads to selective purification of DNA polymerase containing no exonuclease activity. DNA polymerase δ activity is associated with a small protein peak, while DNA polymerase α is associated with the major protein fraction. At this stage of purification, DNA polymerase δ has been purified greater than 10 000-fold and has a specific activity in excess of 2000 U/mg of protein.

The possibility that the activated calf thymus DNA-directed polymerase contains an exonuclease activity for activated calf thymus DNA was considered. Activated calf thymus DNA labeled at the 3′ terminus with [^{3}H]TMP was used to assay for exonuclease activity with both DNA polymerase activities. The pattern of exonuclease activity with activated calf thymus DNA·[^{3}H]TMP is similar to that with poly[d(A-T)]·[^{3}H]-TMP and is associated with DNA polymerase δ and not with DNA polymerase α.

Co-Sedimentation of DNA Polymerase δ and Its Associated

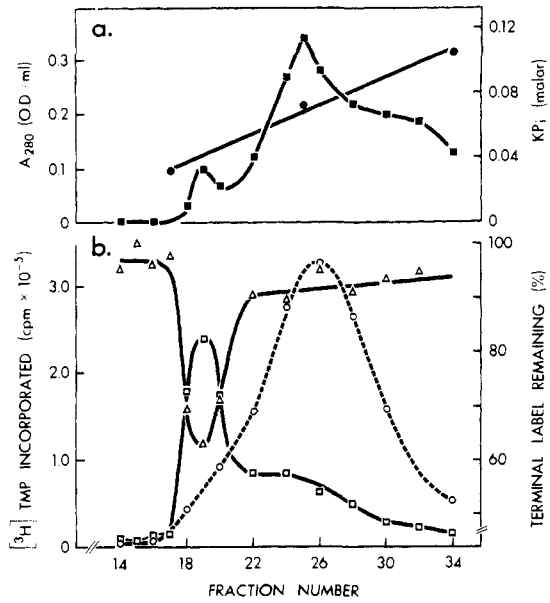

FIGURE 2: Resolution of two DNA polymerase activities by hydroxylapatite chromatography. Experimental details were as described under Materials and Methods except that in the DNA polymerase assays the concentration of [^{3}H]TTP was 0.16 μM, 50 mCi/μmol. (A) (■ — ■) Absorbance at 280 nm; (●—●) potassium phosphate concentration. (B) (□—□) Polymerase activity assayed with poly[d(A-T)]; (○ - - - ○) polymerase activity assayed with activated calf thymus DNA; (△—△) exonuclease activity.

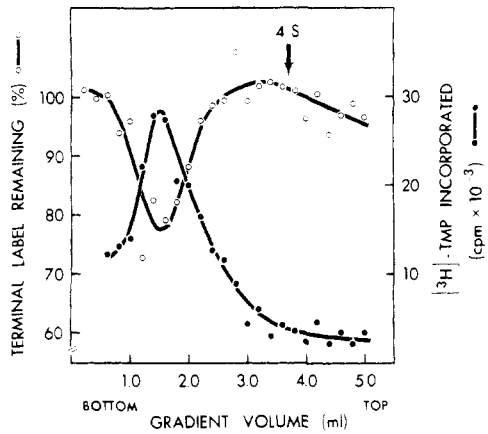

FIGURE 3: Cosedimentation of DNA polymerase δ and exonuclease at low ionic strength. The experimental details were as described under Materials and Methods. The concentration of KCl was 0.05 M. DNA polymerase activity was determined with poly[d(A-T)] as template as described in Materials and Methods except that the concentration of [^{3}H]TTP was 0.16 μM, 50 mCi/μmol. (●—●) DNA polymerase; (○—○) exonuclease.

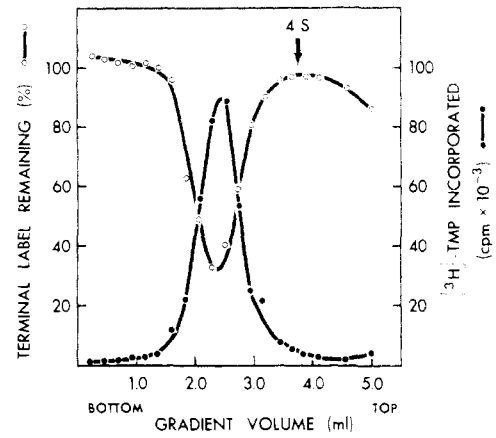

FIGURE 4: Cosedimentation of DNA polymerase δ and exonuclease at high ionic strength. See Materials and Methods for experimental details. The concentration of KCl was 0.3 M. DNA polymerase δ activity was determined with poly[d(A-T)] as template, as described under Materials and Methods except that the concentration of [^{3}H]TTP was 0.16 μM, 50 mCl/μmol. (● — ●) DNA polymerase; (○—○) exonuclease.

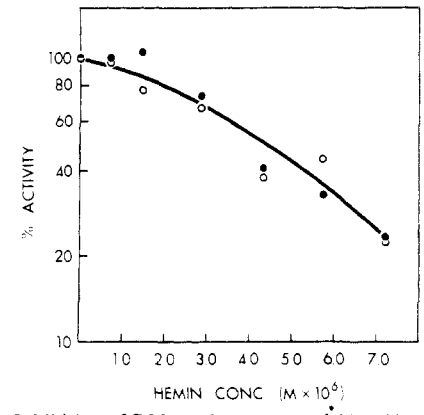

FIGURE 5: Inhibition of DNA polymerase and 3′ to 5′ exonuclease activities by hemin. Assay conditions were as described under Materials and Methods except for the addition of hemin and: (1) in the DNA polymerase assay the [^{3}H]TTP concentration was 4 μM, 4 Ci/mmol; (2) in the exonuclease assay the concentration of poly[d(A-T)]·[^{3}H]TMP was 0.034 A_{260} units/ml. (●—●) DNA polymerase activity; (○ — ○) exonuclease activity.

Exonuclease. To further establish that the 3′ to 5′ exonuclease activity is an inherent property of DNA polymerase δ, we have further purified the DNA polymerase by sedimentation on sucrose density gradients both at low and high ionic strength. Similar to DNA polymerase α (Byrnes et al., 1973; Spadari et al., 1974; Bollum, 1975), DNA polymerase δ also exists either as a monomer of 7 S at high-ionic strength or as a dimer of 11 S at low-ionic strength. As shown in Figure 3, in the presence of 0.05 M KCl both DNA polymerase and exonuclease activities sediment together at 11 S, while at 0.3 M KCl both DNA polymerase and exonuclease activities cosediment at 7 S in a very sharp peak (Figure 4). This finding strongly suggests that both DNA polymerase and exonuclease activities are catalyzed by the same protein. The further purification of the DNA polymerase by sedimentation has not been quantitated because of the small amount of very dilute protein and the instability of the enzyme under the conditions of sedimentation.

Further Evidence That the Exonuclease Is an Integral Part of DNA Polymerase δ. We have previously found that hemin inhibits DNA synthesis with bone marrow DNA polymerase by binding to the enzyme and causing its dissociation from the template/primer (Byrnes et al., 1973, 1975). Since the template/primer for the DNA polymerase reaction is the substrate for the exonuclease reaction, hemin should inhibit both activities concomitantly if the exonuclease is an integral part of the DNA polymerase molecule. The inhibition of both DNA polymerase activity and exonuclease activity by increasing concentrations of hemin is shown in Figure 5. The inactivation curves for both activities are identical. Similar results were obtained when Rifamycin AF/013 was used to inhibit both DNA polymerase and exonuclease activities (data not shown). Since Rifamycin AF/013, analogous to hemin, inhibits DNA synthesis by binding to the enzyme and causing the dissociation of the enzyme-DNA complex (Byrnes et al., 1975), these re-

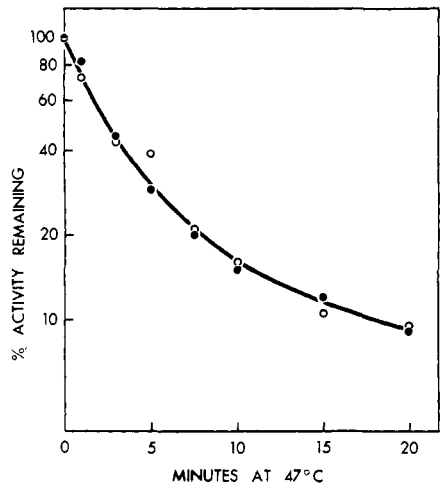

FIGURE 6: Heat inactivation of DNA polymerase and 3′ to 5′ exonuclease. Fraction VI enzyme, at a concentration of 50 μg/ml, was incubated at 47 °C in 0.05 M Tris-HCl (pH 7.8), 0.125 M KCl, 1.0 mM dithiothreitol, 0.05 mM EDTA, and 12.5% glycerol. At the times indicated aliquots were removed and assayed for DNA polymerase and exonuclease activity, as described in Figure 5. Under these conditions both assays were linear with enzyme concentration. (● — ●) DNA polymerase activity; (○ — ○) exonuclease activity.

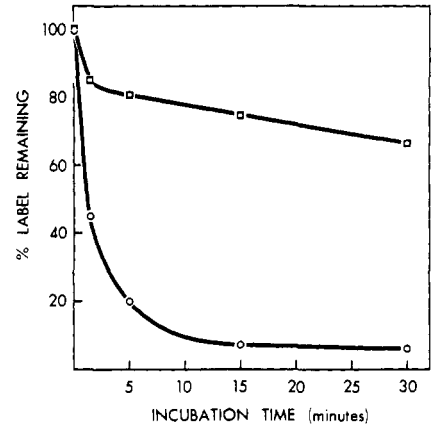

FIGURE 7: Hydrolysis of [^{3}H]TMP labeled poly[d(A-T)]. Assay conditions were as under Materials and Methods: (○ — ○) terminally labeled poly[d(A-T)]·[^{3}H]TMP; (□—□) uniformly labeled [^{3}H]TMP·poly[d(A-T)].

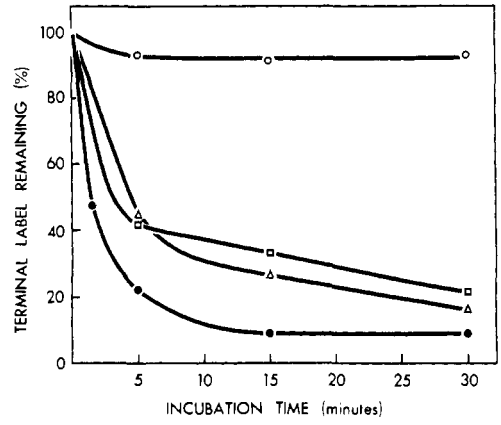

FIGURE 8: Effect of chain elongation on hydrolysis of terminally labeled poly[d(A-T)]·[^{3}H]TMP. Assay conditions were as under Materials and Methods except: (● — ●) no dATP or TTP; (□ — □) 0.16 mM dATP, (△—△) 0.16 mM TTP; (○—○) 0.08 mM each of dATP and TTP.

sults suggest that both polymerase and exonuclease activities reside on the same protein.

Further evidence for the association of these two activities with the same protein comes from the rates of heat inactivation of both the DNA polymerase and exonuclease activities. The rate of heat inactivation of the polymerase activity is indistinguishable from that of the exonuclease activity (Figure 6). This is additional evidence for the integral association of the two activities.

Characterization of the Nuclease Activity. The exonuclease activity, as measured by the release of [^{3}H]TMP from acid precipitable poly[d(A-T)]·[^{3}H]TMP, shows an absolute requirement for a divalent cation, which may be satisfied by either Mn^{2+} or Mg^{2+}, and is also markedly dependent on the monovalent cation concentration. At pH 7.0 the optimal KCl concentration is 120 mM.

That the nuclease activity is directed at the 3′ terminus is demonstrated by the rapid release of the labeled 3′-terminal nucleotide from poly[d(A-T)]·[^{3}H]TMP compared to the relatively slow conversion of uniformly labeled [^{3}H]-poly[d(A-T)] to acid solubility (Figure 7). Incubation of 2 units of DNA polymerase with 0.003 A_{260} unit of poly[d(A-T)]·[^{3}H]TMP or an equal amount of uniformly labeled [^{3}H]poly[d(A-T)] resulted in 85% release of [^{3}H]TMP from poly[d(A-T)]·[^{3}H]TMP after 5 min at 37 °C, whereas after 30 min only 20% of uniformly labeled [^{3}H]poly[d(A-T)] was rendered acid soluble. The released product was identified as TMP in both instances by chromatography of the reaction products on poly(ethylenimine)-cellulose.

Protection of 3′ Terminus by Complementary Deoxyribonucleoside Triphosphates. The nucleolytic release of the 3′-terminal [^{3}H]TMP from poly[d(A-T)]·[^{3}H]TMP is prevented by the presence of the deoxyribonucleoside triphosphates which promote DNA synthesis. As illustrated in Figure 8, in the absence of dATP and TTP, the rate of hydrolysis of [^{3}H]TMP from the 3′ terminus of poly[d(A-T)]·[^{3}H]TMP is very rapid, approximately 80% being hydrolyzed in 5 min, and complete removal is attained in 15 min. Either dATP or TTP alone provides very little protection, since neither nucleotide by itself can extend the DNA chain by more than one nucleotide. However, in the presence of both dATP and TTP, extensive chain elongation can occur and the [^{3}H]TMP is almost completely protected from nucleolytic attack by the 3′ to 5′ exonuclease. Protection of the 3′ terminal nucleotide by DNA synthesis confirms the 3′-exonuclease character of the enzyme and also suggests that endonuclease activity is absent.

Template-Dependent Conversion of Nucleoside Triphosphates to Free Nucleoside Monophosphates. Similar to the 3′ to 5′ exonuclease of *E. coli* DNA polymerase I and T_4 DNA polymerase (Deutscher and Kornberg, 1969; Hershfield and Nossal, 1972; Muzyczka et al., 1972), the exonuclease activity associated with marrow DNA polymerase catalyzes the template-dependent conversion of deoxyribonucleoside triphosphates to monophosphates, as shown in Table II. In no case was there conversion of nucleoside tri- to monophosphate in the absence of template, and the specificity of the reaction for substrates complementary to the template was high. Deoxyribonucleoside triphosphates noncomplementary to the template (dGTP and dCTP) were converted to monophosphates at a much reduced rate, and ATP was likewise converted to AMP to a very limited extent.

The inclusion of 5 units of 5′-nucleotidase in either the exonuclease reaction, in which the 3′-terminal nucleotide is released as free monophosphate, or in the template-dependent generation of monophosphate changed the labeled product to

TABLE II: Template Requirements and Specificity for Conversion of Nucleoside Triphosphate to Nucleoside Monophosphate[a]

Template	^{3}H-Labeled Triphosphate	^{3}H-Labeled Nucleotide Incorporated into Polymer (pmol)	Free ^{3}H-Labeled Nucleoside Monophosphate Formed pmol
Poly[d(A-T)]	dTTP	43	189
None	dTTP	<2	<2
Poly[d(A-T)]	dATP	45	475
None	dATP	<2	<2
Poly[d(A-T)]	dGTP	3.0	13
None	dGTP	<1	<1
Poly[d(A-T)]	dCTP	3.0	3.2
None	dCTP	1.8	<1
Poly[d(A-T)]	rATP	<1	4.7
None	rATP	<1	2.0

[a] Assays were performed as under Materials and Methods. Values reported with TTP as substrate were obtained at a concentration of 3.2×10^{-5} M and a specific activity of 19 cpm/pmol; with dATP, 3.2×10^{-5} M and 15 cpm/pmol; with dGTP, 0.4×10^{-5} M and 138 cpm/pmol; with dCTP, 0.12×10^{-5} M and 168 cpm/pmol; with rATP, 0.8×10^{-5} M and 71 cpm/pmol.

nucleoside ([^{3}H]thymidine) as shown by thin-layer chromatography. Therefore the product of both reactions is a nucleoside 5′-monophosphate.

Discussion

A previously undescribed species of mammalian DNA polymerase, DNA polymerase δ, has been purified over 10 000-fold from the cytoplasm of erythroid hyperplastic bone marrow. This DNA polymerase, in contrast to all other eukaryotic DNA polymerases thus far described, is associated with a very active 3′ to 5′ exonuclease activity. The 3′ to 5′ exonucleolytic nature of this enzyme activity is illustrated by the rapid hydrolysis of 3′-terminally labeled poly[d(A-T)]·[^{3}H]TMP compared to the slow rate of hydrolysis of uniformly labeled [^{3}H]poly[d(A-T)] and the demonstration that the hydrolytic product is a 5′-deoxymononucleotide. That the enzyme is a 3′-exonuclease is further supported by the observation that DNA chain elongation on a 3′-terminally labeled template/primer protects the labeled nucleotide from exonuclease attack, since the labeled nucleotide becomes internally located following DNA chain elongation (Hershfield and Nossal, 1972; Brutlag and Kornberg, 1972). DNA polymerase δ, similar to the *E. coli* and T_4 DNA polymerases (Deutscher and Kornberg, 1969; Hershfield and Nossal, 1972; Muzyczka et al., 1972), also catalyzes a template-dependent conversion of deoxyribonucleoside triphosphate to deoxyribonucleoside monophosphate, a characteristic of DNA polymerase associated 3′ to 5′ exonuclease activity which reflects alternate incorporation and hydrolysis of the 3′ terminal nucleotide of the template/primer (Hershfield and Nossal, 1972). Purified DNA polymerase δ (step VI) has a specific DNA polymerase activity of 2300 nmol of TMP incorporated mg^{-1} h^{-1}, and a specific exonuclease activity, as measured by the template-dependent generation of monophosphates, of 380 nmol mg^{-1} h^{-1} with TTP as substrate and 950 nmol mg^{-1} h^{-1} with dATP as substrate.

The data suggest that the 3′ to 5′ exonuclease activity is an integral part of the DNA polymerase. The DNA polymerase is not separable from the exonuclease by various chromatographic procedures, nor by sedimentation in sucrose density gradients either at high- or low-ionic strength, which gives two molecular weight species of the polymerase activity.

Further evidence that the exonuclease activity is an inherent property of the DNA polymerase is the coordinate inhibition of both polymerase and exonuclease activities by hemin and Rifamycin AF/013. These compounds inhibit DNA synthesis by binding to DNA polymerase and causing it to dissociate from the template/primer. Since the template/primer for the DNA polymerase activity is the substrate for the exonuclease activity, inhibitors which affect the formation of the DNA polymerase–template complex should inhibit both activities simultaneously. Furthermore, both polymerase and exonuclease activities have identical rates of heat inactivation. Taken together, all these observations are excellent evidence that the two activities reside on the same protein.

DNA polymerase δ has been resolved from DNA polymerase α by chromatography on DEAE-Sephadex and hydroxylapatite and can be further distinguished from DNA polymerase α by their respective DNA template preferences. The best template for DNA polymerase δ is poly[d(A-T)], while activated calf thymus DNA is a rather poor template. In contrast the most active template for DNA polymerase α is activated calf thymus DNA and poly[d(A-T)] is a relatively poor template. The preference of DNA polymerase δ for poly[d(A-T)] as template and the use of activated calf thymus DNA as template to assay for DNA polymerase activity during purification procedures might explain why DNA polymerase δ was not previously detected. DNA polymerase δ can be distinguished from DNA polymerase β by its molecular weight and from DNA polymerase γ by its inability to utilize poly(rA)·oligo(dT) as template (unpublished observation) for DNA synthesis (Fridlender et al., 1972). The most distinctive feature of DNA polymerase δ is its association with a very active 3′ to 5′ exonuclease, while all other eukaryotic DNA polymerases thus far reported are found not to contain any exonuclease activity.

Analysis on sucrose density gradients suggests that the physical properties of DNA polymerase δ are similar to those of DNA polymerase α, as determined by their sedimentation coefficients at high- and low-ionic strength. In the presence of 0.3 M KCl, both DNA polymerases sediment at 7 S, while in the presence of 0.05 M KCl, both sediment at 11 S. However, whether this enzyme is related to DNA polymerase α has not been determined, and further study is needed to establish the differences and similarities between these two DNA polymerases.

Although the role of the 3′ to 5′ exonuclease activity associated with DNA polymerase δ has not been established, the observation that this enzyme, like the exonucleolytic proofreading activity of T_4 DNA polymerase, consistently catalyzes a small but significant conversion of dGTP and dCTP to their nucleoside monophosphates with poly[d(A-T)] as a template, suggests that the role of this enzymatic activity is to correct errors during DNA synthesis.

It has been suggested that eukaryotic DNA polymerases have no capacity to excise errors when mispaired deoxyribonucleoside monophosphates are introduced during replication and that error correction must be attributed to other enzymes in the cell (Bollum, 1975; Loeb, 1974). However, it has been pointed out by Kornberg (1975) that repair systems that excise a mismatched nucleotide in DNA cannot determine which of the two strands contains the mismatched member of an incorrect pair, whereas the excision of a mismatched nucleotide at the primer terminus by the 3′ to 5′ exonuclease activity of DNA polymerase precludes the necessity for such

a determination. The discovery that DNA polymerase δ is associated with an active 3′ to 5′ exonuclease suggests that this mammalian DNA polymerase is able to correct mistakes made during DNA polymerization and that one of the mechanisms whereby replication fidelity is maintained in eukaryotic cells may be similar to that of the prokaryotes.

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Complexity and Specificity of Polysomal Poly(A+) RNA in Mouse Tissues†

Bryan D. Young,* George D. Birnie, and John Paul

ABSTRACT: Base sequence complexities of polysomal poly(A+) RNA from mouse embryo, brain, and liver have been estimated by hybridization to homologous cDNA to be approximately 7×10^9, 1.5×10^{10}, and 7×10^9 daltons, respectively. By annealing each cDNA with a large excess of total mouse embryo DNA, the genes coding for the polysomal poly(A+) sequences were shown to be unique. Heterologous hybridization experiments showed that the high abundance class of poly(A+) sequences in one tissue is not identical with the high abundance class in other tissues. However, at least 55%, and possibly more, of the poly(A+) RNA in one tissue is present in the poly(A+) RNA of another tissue.

There is now a variety of techniques which can be used to examine heterogeneous populations of messenger RNA (mRNA) prepared from eukaryotic cells. A question of immediate interest is the number of different sequences present in such a population. Galau et al. (1974) hybridized excess polysomal RNA prepared from sea urchin embryos to purified unique DNA and demonstrated that approximately 14 000 different mRNA sequences were present on the polysomes. An alternative approach is to use a viral reverse transcriptase to prepare a complementary DNA (cDNA) to the polyadenylated [poly(A+)] RNA sequences present in the cytoplasm or on the polysomes. By following the hybridization kinetics of the cDNA with its homologous RNA, it has been possible to estimate the number of different RNA sequences present in a number of cell types, e.g., 40 000 cytoplasmic sequences in HeLa cells (Bishop et al., 1974), 8000 polysomal sequences in mouse Friend cells (Birnie et al., 1974) and 8000 cytoplasmic sequences in mouse L cells (Ryffel and McCarthy, 1975). All these studies indicate that there is a wide range in abundance in such RNA populations.

As reviewed by Lewin (1975), DNA reannealing experiments indicate that most mRNA sequences are transcribed from unique DNA. Hence, the total proportion of unique eukaryotic DNA which codes for structural genes will be related to the degree of specificity of mRNA populations in different cell types. Ryffel and McCarthy (1975) demonstrated that cDNA from L cell cytoplasmic poly(A+) RNA can be hybridized efficiently to cytoplasmic poly(A+) RNA from mouse

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