Volume 15 Number 24 1987 Nucleic Acids Research

Characterization of a stable, major DNA polymerase α species devoid of DNA primase activity

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Received September 21, 1987; Revised and Accepted November 10, 1987

ABSTRACT

We have purified from *Xenopus laevis* ovaries a major DNA polymerase α species that lacked DNA primase activity. This primase-devoid DNA polymerase α species exhibited the same sensitivity as the DNA polymerase DNA primase α to BuAdATP and BuPdGTP, nucleotide analogs capable of distinguishing between DNA polymerase δ and DNA polymerase DNA primase α. The primase-devoid DNA polymerase α species also lacked significant nuclease activity indicative of the α-like (rather than δ-like) nature of the DNA polymerase. Using a poly(dT) template, the primase-devoid DNA polymerase α species elongated an oligo(rA_{10}) primer up to 51-fold more effectively than an oligo(dA_{10}) primer. In direct contrast, the DNA polymerase DNA primase α complex showed only a 4.6-fold preference for oligoribonucleotide primers at the same template/primer ratio. The catalytic differences between the two DNA polymerase α species were most dramatic at a template/primer ratio of 300. The primase-devoid DNA polymerase α species was found at high levels throughout oocyte and embryonic development. This suggests that the primase-devoid DNA polymerase α species could play a physiological role during DNA chain elongation *in vivo*, even if it is chemically related to DNA polymerase DNA primase α.

INTRODUCTION

DNA polymerase α is generally believed to be the major enzyme involved in eukaryotic chromosomal DNA replication (1, 2, 3). Most previously described DNA polymerase α species are also capable of *de novo* DNA directed RNA primer synthesis. This primer synthesis is postulated to be the main mechanism through which new DNA chains are initiated for subsequent chain elongation by the action of DNA polymerase α (1). In contrast to the separate DNA polymerase and DNA primase enzymes found in prokaryotes (4, 5), however, eukaryotic DNA primase has only been found tightly associated with the DNA polymerase α catalytic activity in a wide variety of eukaryotic organisms (6-14). Separation of DNA primase activity from DNA polymerase α has usually required the use of conditions that disrupt hydrophobic associations and employ urea (8) or ethylene glycol (15, 16; see 10 for an exception). Taking advantage of these apparent DNA primase-DNA polymerase α subunit interactions, Vishwanatha and Baril

(17) have employed hydrophobic chromatography to resolve a 70 kDa DNA primase subunit from highly purified Hela cell DNA polymerase α.

In general, however, characterization of eukaryotic DNA polymerase DNA primase α has proven difficult since its purification has required either lengthy traditional or sophisticated immunoaffinity separation techniques (6-14, 18). Identification of the catalytic peptides has been particularly difficult, in part due to two identifiable regions of micro-heterogeneity in denaturing polyacrylamide gels of even the most purified preparations (18, 19). For example, Wong *et al.* (18) identified the subunit at 180 kDa as the major polymerase catalytic subunit and showed convincingly that minor bands as low as 140 kDa were chemically related, possibly through proteolysis. A second region of heterogeneity was centered at approximately 60 kDa and contained multiple bands that were apparently chemically unrelated to each other (18).

As a further complication, DNA polymerase α apparently also exists as several discrete multi-enzyme forms that have distinct kinetic properties (20, 21), and novel enzymatic activities (3). Among activities that have been reported as complexed with DNA polymerase α are stimulatory cofactors C1, C2 (22, 23, 24), exonuclease (25), and 5',5'''-P^1, P^4-tetraphosphate binding activity (26).

DNA polymerase δ, another high molecular weight enzyme, has recently also been implicated as a possible replicative and repair DNA polymerase (27). DNA polymerase δ is associated with certain activities thought to be required of a replicative DNA polymerase, *e.g.* exonuclease (27, 28, 29, 30). Except for two reports (27, 28), however, DNA polymerase δ has not been found to be associated with primase activity. Moreover, as with DNA polymerase α, DNA polymerase δ has also been isolated in discrete multicomponent forms (31). The relationships between DNA polymerase(s) α and DNA polymerase(s) δ are unclear at present. The enzymes respond differently to the inhibitors BuPdGTP and BuAdATP (32) and to monoclonal antibodies against human KB cell DNA polymerase α (32).

Previous reports from this laboratory have described the existence of an apparent DNA polymerase α activity, designated DNA polymerase α_2, that was devoid of primase activity (11, 33, 34). DNA polymerase α_2 comprises no less than one third of the total DNA polymerase α activity during oogenesis and embryogenesis (33). Moreover, the ratio of DNA polymerase α_2 to DNA polymerase DNA primase α_1 remains constant through oocyte and embryonic development (33). In this study we asked whether DNA polymerase α_2 was a DNA polymerase δ, a DNA polymerase α_1 related enzyme or some previously undescribed protein. We also compared its catalytic properties with those of homogeneous DNA polymerase DNA primase α_1 isolated from the same preparation of *Xenopus laevis* ovaries.

MATERIALS AND METHODS

Frogs

Selected fat fertile *X. laevis* females were obtained from the South African Snake Farm (P. O. Box 6, Fish Hoek, Cape Providence, South Africa).

Chemicals

Tris, glycerol, and other buffer components were of enzyme grade quality from various sources. Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co. [N-(p-n-butylphenyl)-9-(2-deoxy-D-ribofuranosyl guanine 5' triphosphate)] (BuPdGTP) and [2-(p-n-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl) adenine 5'-triphosphate] (BuAdATP) were generous gifts from Dr. George Wright, University of Massachusetts Medical Center, Worcester, MA 01605. DEAE Cellulose (DE52) and Cellulose Phosphate (P11) ion exchange resins were from Whatman. Hydroxylapatite (HA-Ultrogel) was from LKB. DEAE-Bio-Gel and Silver Stain Kit were from Bio-Rad Laboratories. Sephacryl S-300 resin was from Pharmacia. Electrophoresis reagents were purchased from Bethesda Research Laboratories. Electrophoresis molecular weight markers were from Sigma Chemical Company and Bio-Rad Laboratories.

Nucleotides and Templates

Radioactive ribo- and deoxyribonucleotides were from New England Nuclear Corp. Non-radioactive nucleotides, homopolymers, oligo rA_{10}, oligo dA_{10}, and oligo $dG_{12\text{-}15}$ were all purchased from Pharmacia P-L Biochemicals. Activated calf thymus DNA was prepared using pancreatic DNAse according to Baril *et al.* (35). Single-stranded (ss) and double-stranded (ds) bacteriophage M13mp7 DNA was grown and harvested using standard procedures and subsequently rigorously purified using neutral sucrose gradients (ds and ss) followed by alkaline gradients (ss) according to Bayne and Dumas (36) in order to remove any preexisting primers on the single-stranded template. Synthetic oligodeoxyribonucleotide M13mp7 primer fragments were synthesized on a Biosearch Model 8750 DNA synthesizer and purified via sequential reverse phase and ion exchange HPLC columns on a Waters 840 chromatography system. Synthetic template/primer combinations were prepared by dissolving the appropriate amount of primer and template in 100 μl of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 75 mM NaCl. The mixtures were incubated at 90°C for 10 mins and allowed to cool slowly to room temperature.

Proteins

Nuclease free bovine serum albumin (BSA) was obtained from Boehringer Mannheim. Pancreatic DNAse I was from Sigma. Calibration proteins for gel filtration (ferritin, catalase, bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A) were from Pharmacia.

Table 1
Purification of DNA polymerase α_2 from *Xenopus laevis*

	Fraction	Volume ml	Total Protein mg	Total Units nmol dAMP/hrx10^{-3}	Specific Activity units/mg	Purification - fold
I	Crude Extract	8740	347,000	560(100)	1.61	1
II	Low Speed Supernatant	8040	105,000	551(98)	5.25	3.26
III	High Speed Supernatant	7760	88,000	538(96)	6.11	3.80
IV	DEAE-Cellulose	3322	1,220	186(33)	153	95.0
V	Phosphocellulose	361	290	223(39)	769	478
VI	Hydroxylapatite	20	15.0	98(18)	6530	4060
VII	DEAE-Bio-Gel	2.5	4.70	59(11)	12,600	7830
VIII	Sephacryl S-300	1	0.710	45(8)	63,400	39,400
IX	Glycerol Gradient	1.7	0.095	22(4)	232,000	144,000

Based on 1,018 g of ovaries

METHODS

Isolation of DNA Polymerase α_2

DNA polymerase α_2 (DNA primase free) was purified from ovarian extracts of the frog *X. laevis*. All manipulations were carried at 4°C. Unless otherwise stated all buffers contained 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 10 mM sodium bisulfite, and 25% glycerol (v/v). The entire purification scheme is outlined in Table 1.

Whole ovaries (1018 g) were removed from decapitated frogs, washed several times with 25 mM Tris-HCl (pH 9.3), 5 mM KCl, and homogenized in the same buffer at a concentration of 1 gram wet weight per 8 ml of buffer. The initial buffer pH must be basic since ovary homogenization releases acidic material which can inactivate DNA polymerase α activities. It is also important that the ionic strength be kept low due to the solubility of yolk platelets at high ionic strength. Ovaries were homogenized for a total of 3 mins allowing for 30 second cool down periods (Fraction I). Fraction I was centrifuged at 5,400 x g for 45 mins to remove cell debris, yolk platelets, and most of the black pigment granules. The supernatant (Fraction II) was filtered through 8 layers of cheese cloth to remove a layer of floating lipids. Fraction II was centrifuged at 26,000 x g for 90 mins. Lipid pellicles were removed with cotton tipped applicators. The clear reddish-yellow supernatant (Fraction III) was carefully decanted from the loose pellet. 1 l portions of Fraction III were mixed with 600-700 ml portions of DE 52 equilibrated with 25 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM KCl, and stirred occasionally for 90 mins. Non-adsorbed material was removed by filtration. The cellulose portions were each washed four times (1 l each) and resuspended in 500 ml of the same buffer. All

cellulose portions were combined and packed into a 110 x 10 cm diameter glass column. The column was eluted with a 20 l gradient of 0.05 M to 0.4 M Tris-HCl (pH 7.9) at a flow rate of approximately 219 ml/hr. The gradient was followed by a 4 l wash of 0.4 M Tris-HCl. 150 ml fractions were collected. The elution pattern showed two peaks of activity responding to assay 1 at approximately 0.12 M and 0.18 M Tris-HCl. The peak eluting at 0.12 M (DNA-polymerase α_1, containing DNA primase activity) was stored at -70°C for further purification. The peak eluting at 0.18 M was pooled (3322 ml) to give fraction IV. Fraction IV was dialyzed (three changes of seven volumes each over 24 hr.) against 50 mM Tris-HCl (pH 7.2), 1 mM EDTA and finally applied to a 30 x 4 cm glass column containing 500 ml of phosphocellulose equilibrated with the same buffer. After loading at 65 ml/hr. the column was washed until no protein was evident in the flow-through as detected by A_{280} (approximately 5 column volumes). The column was then eluted using a 1 l 0.05 M to 1.0 M KCl gradient in the same buffer. 9.5 ml fractions were collected. DNA polymerase activity (assay 1) eluted at approximately 0.26 M KCl. The peak material was pooled to give 361 ml (Fraction V). Fraction V was dialyzed into 5 mM KPO_4 (pH 7.5) and applied to a 24 x 1.5 cm column containing HA-Ultrogel equilibrated with 5 mM KPO_4 (pH 7.5). The column was washed until the A_{280} reached baseline and subsequently eluted using a 300 ml 5 mM to 0.5 M KPO_4 gradient. 5 ml fractions were collected. DNA polymerase activity (assay 1) eluted at approximately 130 mM KPO_4. The peak fractions were pooled and concentrated to 20 ml (Fraction VI) using an Amicon pressure dialysis cell. Fraction VI was dialyzed against 0.04 M Tris-HCl (pH 7.5) and applied to a 60 x 1.2 cm column containing 45 ml of DEAE Biogel equilibrated in the same buffer. A trace of DNA primase activity (associated with DNA polymerase α_1) flowed through the column (see Fig. 2 below). The vast majority of the DNA polymerase activity was then eluted using a 100 ml 0.04 to 0.5 M KCl gradient in the same buffer. The peak of DNA polymerase α activity was centered at approximately 110 mM KCl. The peak fractions were pooled and concentrated to 2.5 ml (Fraction VII). Fraction VII was applied to a 73 x 2 cm glass column containing Sephacryl S-300 equilibrated with 50 mM Tris-HCl (pH 7.2), 200 mM KCl, and 1 mM EDTA. The column had been previously calibrated with the Pharmacia gel filtration calibration kit. The column was eluted at a flow rate of 6 ml/hr. 2 ml fractions were collected. The combined peak fractions were pooled and concentrated to 3 ml (Fraction VIII). Fraction VIII was dialyzed against 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 mM KCl, and 5% glycerol and layered onto 6 SW41 ultracentrifuge tubes containing 10 to 30% (v/v) glycerol gradients in the same buffer. Centrifugation was at 40,000 rpm for 40 hr. at 4°C. Tubes were fractionated (0.3 ml aliquots), peak fractions pooled, and dialyzed into 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1.0 mM EDTA, 50% glycerol for storage at -70°C. Fraction IX enzyme was stable for at least 3 months at -70°C.

DNA Polymerase Assays

The general DNA polymerase assay (assay 1) using activated DNA was performed in a total volume of 100 μl and contained 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 200 μg/ml BSA, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, 2 mM 2-mercaptoethanol, 20 μCi/ml ^{3}H-dTTP (88 cpm/pmol), and 660 μg/ml activated DNA. Incubations were at 30°C for 30 mins. After incubation, 10% trichloroacetic acid precipitable counts were collected onto Whatman 934-AH filters, dried with an infrared lamp and counted in fluor (Liquifluor, NEN). One unit of DNA polymerase activity is defined as that which incorporates 1 nmol of dNMP into acid insoluble counts in 60 mins at 30°C. Assay 2 measured DNA polymerase α activity employing poly(dT)-oligo(rA), a template preferred by DNA polymerase α (33). Assays contained in a final volume of 50 μl, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 200 μg/ml BSA, 6 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM NaCl, 25 mM KCl, 10% glycerol, 100 μM ^{3}H-dATP (100 cpm/pmol), and 200 μM poly(dT)-50 μM oligo (rA_{10}). Assays were incubated at 30°C for 30 mins after which tubes were processed as for Assay 1. One unit of DNA polymerase α activity is defined as that which incorporates 1 nmol of dAMP into acid insoluble counts in 60 mins at 30°C. Assay 2 was typically 3- to 5-fold more sensitive than assay 1 but was not used routinely for economical reasons.

DNA Primase Assay

DNA primase activity was measured at 30°C for 30 mins in 50 μl containing 50 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 10% glycerol, 50 mM potassium chloride, 5 μg poly(dC), 5 mM GTP, ^{3}H dGTP (400 cpm/pmol), and enzyme. Primase assays on natural M13mp7 templates were performed with the indicated amounts of DNA as described elsewhere (11). Primase activity was also measured in a coupled assay with DNA polymerase I (Klenow fragment) according to Conaway and Lehman (37) as modified by Vishwanatha and Baril (17).

Nuclease Assays

Single-stranded M13mp7 [^{3}H] DNA was prepared and used intact for single-stranded endonuclease assays as described previously (38). Single-stranded DNA for exonuclease assays was prepared by digesting [^{3}H] M13mp7 with BamHI, an enzyme that will cut the genome once within the self-complimentary linker region. Double-stranded nuclease assays were performed with [^{3}H] pBR322 DNA either intact (ds-endonuclease assay) or digested with BglI (ds-exonuclease assays). One unit of nuclease activity was defined as the amount of enzyme that catalyzed the release of 1 nmol of nucleotide to acid-soluble form in 60 mins at 37°C.

Gel Electrophoresis

Denaturing polyacrylamide gel electrophoresis of proteins was performed using a

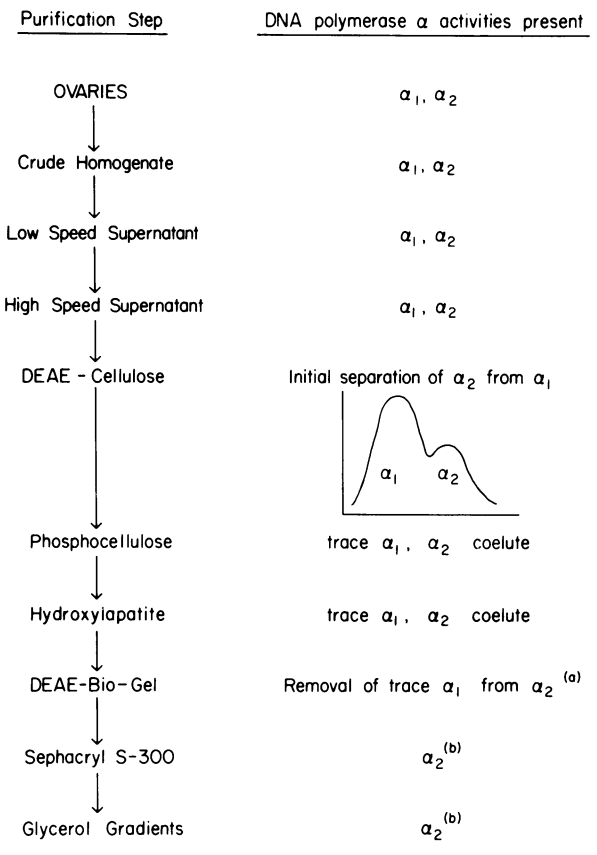

Fig. 1. Flow chart for the separation of DNA polymerase α_2 from DNA polymerase DNA primase α_1. DNA polymerase α activities present at each step are shown on the right.
(a) See Fig. 2.
(b) DNA polymerases α_1 and α_2 copurify during these steps.

3.75% stacking gel and the indicated resolving gel percentage as described by Laemmli (39). Silver staining was performed using the Bio-Rad kit based on the method of Merril (40).

Protein Determinations

Unless otherwise indicated, protein was measured using the procedure of Bradford (41).

RESULTS

Separation of primase-devoid DNA polymerase α_2 from DNA polymerase DNA primase α_1

Previously, this laboratory has described a strategy for the initial separation of

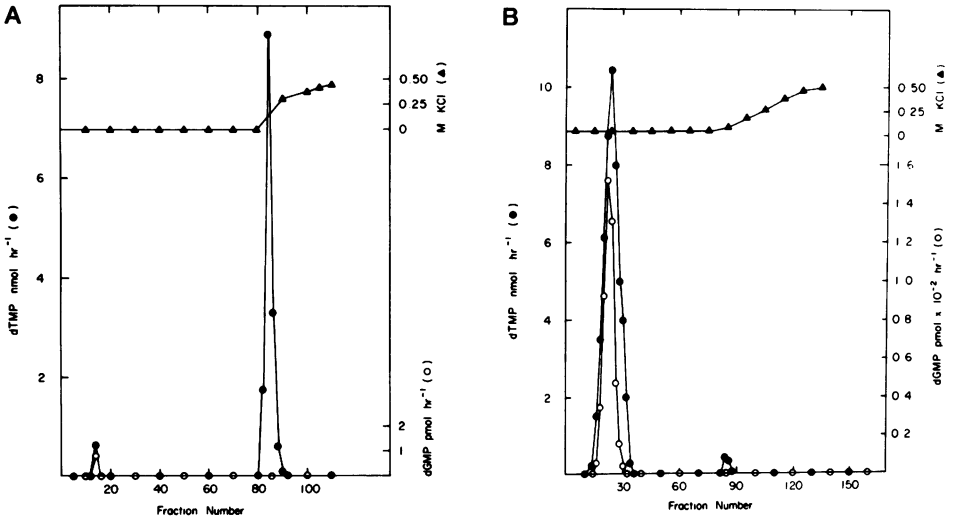

Fig. 2A. Removal of trace DNA polymerase DNA primase α_1 from DNA polymerase α_2 using DEAE-Bio-Gel chromatography. Fraction VI DNA polymerase α_2 was applied to DEAE-Bio-Gel as described in the text. 5 μl aliquots were assayed for DNA primase activity using poly(dC) as template (o open circles). General polymerase activity was measured using activated DNA (assay 1, • closed circles).

B. Separation of trace DNA polymerase α_2 from DNA polymerase DNA primase α_1. DNA polymerase DNA primase α_1 (15,000 units, assay 1) was applied to DEAE-Bio-Gel and eluted as described for DNA polymerase α_2. Assays were performed as described above.

two high molecular weight DNA polymerase α species, DNA polymerase DNA primase α_1 and DNA polymerase α_2 (primase-devoid) using DEAE-cellulose chromatography (38). We have now extended this protocol to purify DNA polymerase α_2 to near homogeneity. This protocol monitors virtually all of the total DNA polymerase activities in *X. laevis* ovarian extracts (38). The purification scheme of DNA polymerase α_2 is outlined in Fig. 1. Less than 5% of the general polymerase activity (assay 1) flowed through the DEAE-cellulose column and was not pursued here (see 38). The indicated Tris-HCl gradients (rather than monovalent cation) consistently gave the best resolution between the DNA polymerase α_1 and α_2 activities on DEAE-cellulose. In addition, we have also incorporated a subsequent anion exchange step to remove trace contamination by DNA polymerase DNA primase α_1 since DNA polymerases α_1 and α_2 copurify during most procedures (Fig. 1). As shown in Fig. 2 we have been able to successfully resolve even minute amounts of DNA polymerase DNA primase α_1 from our primase-devoid DNA polymerase α_2 using DEAE-Bio-Gel (Fig. 2). Using this purification scheme, we have

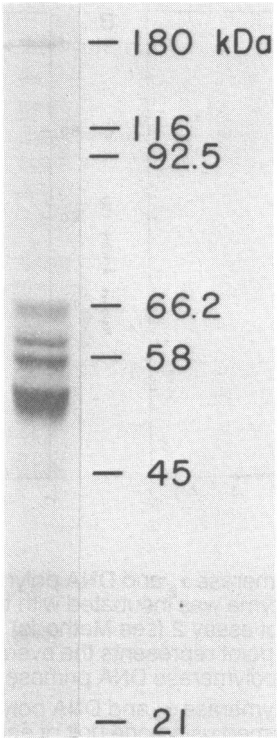

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of fraction IX DNA polymerase α_2. 4 μg of fraction IX DNA polymerase α_2 was applied to a 3.75% stacking gel and 10% resolving gel and electrophoresis performed according to Laemmli (39). Proteins were visualized by silver staining.

also resolved stimulatory cofactors C1 and C2 (23) at the phosphocellulose step and a low molecular weight RNAse H activity at the gel filtration step (Kaiserman and Benbow, unpublished results).

Fraction VIII DNA polymerase α_2 did not contain significant double stranded nuclease (endo- or exo-, <0.5 pmol dNMP/hr) or single-stranded nuclease activities (endo- and exo-, <1 pmol NMP/hr). Additionally, the near-homogeneous DNA polymerase α_2 preparation was devoid of primase activity (Fig. 2A). The final purification yielded an enzyme with a specific activity of 232,000 units/mg protein using 200 μM poly(dT)-50 μM oligo(rA_{10}) as template. The enzyme was entirely dependent on the presence of a pre-formed primer in order to use single-stranded M13 DNA as a template.

Gel Electrophoretic Analysis of DNA Polymerase α_2

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fraction VIII (Fig. 3)

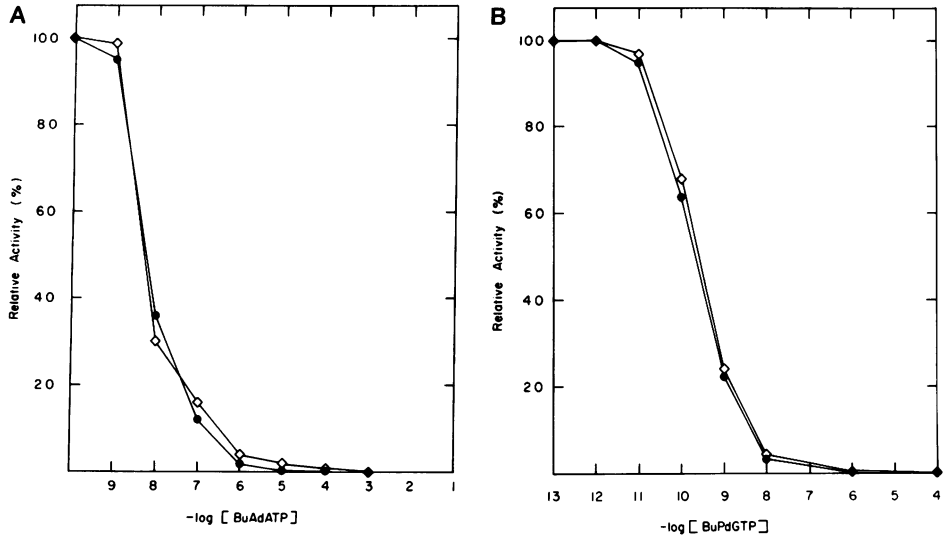

Fig. 4A. Inhibition of DNA polymerase α_2 and DNA polymerase DNA primase α_1 by BudATP. One unit of each enzyme was incubated with the indicated amounts of BudATP under the conditions of assay 2 (see Methods) using 200 μM poly(dT)-50 μM oligo(dA_{10}) as template. Each point represents the average of three determinations. o DNA polymerase α_2, o DNA polymerase DNA primase α_1.

B. Inhibition of DNA polymerase α_2 and DNA polymerase DNA primase α_1 by BuPdGTP. Assays were performed using one unit of each enzyme, the indicated amount of BuPdGTP, and 200 μM poly(dC)-50 μM oligo-dG_{12-15} as template under the conditions of assay 2. DNA polymerase α_2, o DNA polymerase DNA primase α_1.

revealed one major large polypeptide of 178 kDa and a cluster of major bands of molecular weights 64, 59, 56, and 51 kDa. This electrophoretic pattern is similar to that of purified DNA polymerase DNA primase α_1 (Kaiserman and Benbow, unpublished results). Minor bands barely visible on the gel were observed at 145, 125, 43, and 28 kDa. All of the major bands were present at earlier steps in the purification procedure (data not shown).

Inhibitors of DNA Polymerase α_2.

Purified DNA polymerase α_2 and DNA polymerase DNA primase α_1 were subjected to inhibitor titrations with BuAdATP and BuPdGTP. These two inhibitors have been shown to have differential effects on DNA polymerases α and δ *in vitro* (32). As shown in Fig. 4 both enzymes exhibited almost identical inactivation curves with BuAdATP (left panel) and BuPdGTP (right panel). The $I_{0.5}$ values are 2×10^{-10} M and 3×10^{-9} M for BuPdGTP and BuAdATP respectively. This is in agreement with previous observations that BuPdGTP is a more potent DNA polymerase α inhibitor than BuAdATP on the respective complementary templates (42).

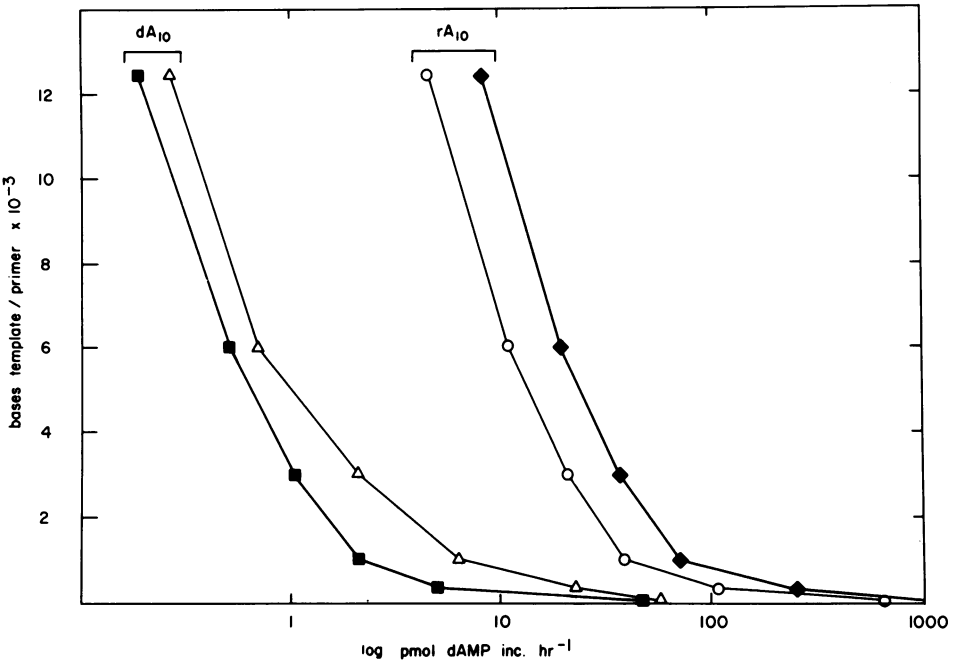

Fig. 5. DNA polymerase α_2 and DNA polymerase DNA primase α_1 activities as a function of the number of template bases/primer using a poly(dT) template and oligo(dA_{10}) or oligo(rA_{10}) primers. Poly(dT) and the indicated primer were annealed in varying weight proportions as described under "Methods" to give template/primer ratios of 30, 300, 1500, 3000, 6000, and 12000. One unit of DNA polymerase α_2 or DNA polymerase DNA primase α_1 was incubated with the indicated template under the conditions of assay 2. The rate of DNA synthesis (pmol dAMP incorporated per hour) *versus* template primer ratio (bases template/primer 3'OH group) is plotted semi-logarithmically.
poly(dT)-oligo(dA_{10}) template: ■ DNA polymerase α_2
△ DNA polymerase DNA primase α_1
poly(dT)-oligo(rA_{10}) template: ◆ DNA polymerase α_2
o DNA polymerase DNA primase α_1

We attempted DNA polymerase titrations with neutralizing antibody SJK 132-20 raised against human DNA polymerase α (43). We were not able to neutralize either DNA polymerase α_2 or DNA polymerase DNA primase α_1 to less than 60% of its full activity using assay 1 (data not shown). This was presumably due to lack of antibody cross-reactivity between human and *X. laevis* DNA polymerase α species.

Catalytic activities of DNA polymerases α_1 and α_2 on substrates of varying template/primer ratios

To compare the template utilization of the two DNA polymerase α species, we annealed poly(dT)-oligo (dA_{10}) in various weight proportions to provide substrates of different template/primer ratios (average number of bases template per number of

Table 2

	dA_{10}	rA_{10}	rA_{10}/dA_{10}	
Template/Primer	α_1/α_2	α_2/α_1	α_1/α_1	α_2/α_2
12,000	1.4	1.8	17.0	44.2
6,000	1.4	1.8	15.7	38.2
3,000	2.0	1.8	10.0	35.2
1,000	3.0	1.9	6.0	33.8
300	4.6	2.4	4.6	51.0
50	1.2	2.2	11.2	30.7

primer 3' OH groups). Figure 5 shows DNA polymerase DNA primase α_1 elongated oligo(dA_{10}) primers more efficiently than DNA polymerase α_2. This difference in chain elongation rate was most exaggerated (4.6 fold) at a template/primer ratio of 300 (Table 2). When template/primer ratios were high (> 6000) or below 300 the two DNA polymerase α species became more similar in catalytic behavior, but still differed somewhat.

To determine if the nature of the primer could affect the chain elongation rate, we annealed poly(dT)-oligo(rA_{10}) in various weight proportions as above for its deoxyribonucleotide analog. As shown in Fig. 5, DNA polymerase α_2 elongated the RNA primer more effectively than DNA polymerase DNA primase α_1, a result exactly opposite to that using a DNA primer. Once again the maximum effect was seen at a template/primer ratio of 300 (Table 2).

Comparing the preference of each enzyme for a ribo- versus a deoxyoligoribonucleotide primer, DNA polymerase α_2 elongated oligoribonucleotide primers by an average of 39-fold more efficiently than their oligodeoxyribonucleotide analogs (Table 2). This preference reached a maximum of 51 fold at a template/primer ratio of 300 for DNA polymerase α_2. In contrast, DNA polymerase DNA primase α_1 elongated oligoribonucleotide primers only approximately 11 fold more efficiently than oligodeoxyribonucleotide primers. This preference for oligoribonucleotide primers fell to a minimum of 4.6 at a template/primer of 300. Figure 5 and Table 2 also clearly show the chain elongation rates of the two DNA polymerase α species to be most disparate at a template/primer ratio of 300.

DISCUSSION

We have purified a stable, major, primase-devoid DNA polymerase α species

(referred to in this paper as DNA polymerase α_2) as a discrete entity separate from DNA polymerase DNA primase α (α_1). This species has been consistently observed by our laboratory in extracts of *X. laevis* oocytes, eggs, and embryos (11, 33, 34, 38, 44). DNA polymerase α_2 exists as a multi-subunit complex (Fig. 3) similar in composition to that of DNA polymerase DNA primase α_1 (Kaiserman and Benbow, unpublished results). Minor electrophoretic pattern differences between DNA polymerases α_1 and α_2 are the complete absence in DNA polymerase α_2 preparations of even a trace band at 70 kDa and the occasional accentuation of a band at approximately 120kDa. In contrast, a band at 70 kDa was always present and the band at 120 kDa was never present in our DNA polymerase DNA primase α_1 preparations (Kaiserman and Benbow, unpublished results). In *X. laevis* DNA polymerase α preparations, a band at 105 kDa has been shown by Konig *et al.* (45) to be a DNA polymerizing polypeptide and may be identical with the band occasionally observed at 120 kDa in our DNA polymerase α_2 preparations. Both bands could be degradation products of larger DNA polymerizing subunits, but were found even in the presence of protease inhibitors.

Vishwanatha and Baril (17) purified and identified a 70 kDa polypeptide as a primase moiety in a DNA polymerase-DNA primase α preparation from HeLa cells. The absence of a 70 kDa subunit could explain the lack of primase activity in *X. laevis* DNA polymerase α_2. DNA polymerase α_2 could be a product of dissociation of DNA polymerase DNA primase α_1 or, conversely, DNA polymerase α_2 could be a not yet fully assembled intermediate of the DNA polymerase DNA primase α_1 complex. The fact that both DNA polymerase α species were found in approximately equal proportions throughout development, however, indicates the possible existence of an *in vivo* interconversion process. Alternatively, DNA polymerase α_2 could be a species chemically unrelated to DNA polymerase DNA primase α_1. In any event DNA polymerase α_2 differs from DNA polymerase DNA primase α_1 catalytically (Fig. 5 and Table 2) as well as the absence of DNA primase activity.

DNA polymerase α_2 probably is analogous to the DNA polymerase species observed by Yagura *et al.* (46) to elute later than the DNA polymerase-DNA primase complex during DEAE-cellulose chromatography of extracts from a number of organisms. Similarly Yamaguchi *et al.* (6) used DEAE-Bio-Gel chromatography to remove traces of a DNA polymerase devoid of primase activity from their DNA polymerase DNA primase α preparation from simian cells. We note that careful homogenization conditions (*i.e.* mildly basic pH 9.3) were necessary in order to reproducibly obtain an initial separation of the two *X. laevis* DNA polymerase α species. We also purposely excluded from our purification scheme any procedures that seemed liable to disrupt the hydrophobic association between the DNA polymerase and DNA primase subunits. Our laboratory has not observed any interconversion of the two DNA polymerase α forms *in*

vitro (38). We do not mention this to imply that DNA polymerase α_2 could not be derived from DNA polymerase DNA primase α_1. We merely wish to emphasize that primase-devoid DNA polymerase α_2 was repeatedly isolated from ovarian extracts as a discrete complex in the absence of any apparent interconversion or proteolytic process. Since our preparation lacked any gross proteolytic artifacts as judged by comparison to SDS gel electrophoretic patterns of DNA polymerase DNA primase α from a variety of sources (6-14), we now propose that DNA polymerase α_2 may exist as a discrete entity *in vivo*. Consistent with this, Zierler *et al.* (33) showed previously that the relative proportions of DNA polymerases α_1 and α_2 remain constant throughout oogenesis and embryogenesis. The two *X. laevis* DNA polymerase α species responded almost identically to the inhibitors BuAdATP and BuPdGTP. This response was identical to the inhibition of mammalian DNA polymerase α species (42) and seemingly rules out any apparent similarity between *X. laevis* DNA polymerase α_2 and mammalian DNA polymerase δ. Nelson *et al.* showed previously that DNA polymerases α_1 and α_2 responded similarly to the more common DNA polymerase α inhibitors (38).

In addition, during hydroxylapatite chromatography, *X. laevis* DNA polymerase α_2 elutes at approximately 130 mM KPO_4 whereas mammalian DNA polymerase δ has been reported to elute at 70 mM KPO_4 (47). Both *X. laevis* DNA polymerase α species reacted similarly toward the human KB cell DNA polymerase α neutralizing antibody SJK 132-20 (albeit equally poor). DNA polymerase δ has been reported to have an exonuclease activity equal to approximately 10% of its polymerizing activity (28). In contrast, the exonuclease activity of our near homogeneous DNA polymerase α_2 preparation was much less than 1% of its DNA polymerase activity (see Table 1 and nuclease assay in Methods). We cannot, however, rule out at this point the possibility that some sort of cryptic nuclease activity is present but masked as reported recently by Cotteril *et al.* (48). For the above reasons we believe the species described in this paper as DNA polymerase α_2 is most likely an α-like activity previously undescribed in its near homogeneous form.

The most striking catalytic difference between the two DNA polymerase α species is the preference of each enzyme for RNA versus DNA primers. Primase-devoid DNA polymerase α_2 elongates RNA primers an average of approximately 39-fold more efficiently than DNA primers. This preference for RNA primers increases to 51-fold at a template/primer ratio equal to 300. In contrast, DNA polymerase DNA primase α_1 elongates RNA primers an average of only 11-fold more efficiently than DNA primers. The preference of DNA polymerase DNA primase α_1 for RNA primers falls to a minimum of 4.6-fold at a template/primer ratio of 300. At a template-primer ratio of 300 the primer elongation preferences of the two DNA polymerase α species are most exaggerated, differing by as much as 10-fold. Less striking is the rate of primer extension by DNA

polymerase α_2 versus DNA polymerase DNA primase α_1. DNA polymerase α_2 elongates RNA primers a maximum of 2.4-fold more efficiently than DNA polymerase DNA primase α_1 at a template/primer ratio of 300. In contrast, DNA polymerase DNA primase α_1 elongates DNA primers a maximum of 4.6-fold more effectively than DNA polymerase α_2 at a template/primer ratio of 300.

Hockensmith and Bambara described two DNA polymerase α species distinguishable by their kinetic properties (20). Using DNA templates containing varying gap sizes, they also showed the two enzymes to differ dependent upon gap length, an observation in agreement with our own. Although it is difficult to know the exact relationship between the DNA polymerase α species used in their study and the DNA polymerase α species described above, the catalytic differences are similarly dependent on the template/primer ratio. Also of potential interest is the fact that a template/primer ratio of 300 (when the two DNA polymerase α species are catalytically most distinct) is similar (within a factor of 2) to the size of Okazaki fragments spanning a nucleosome during DNA replication. Also, the influence of primer length on the template preferences of the two enzymes may be dramatic and remains to be examined. To postulate distinct physiological roles for the two *X. laevis* DNA polymerase α species is highly speculative at this point. Should DNA polymerase α_2 exist *in vivo* as a fully mature primase-devoid entity, however, it would almost certainly play a role in subsequent DNA chain elongation since it is incapable of *de novo* primer synthesis.

Acknowledgments

We thank Diny Poll and K. T. Hiriyanna for helpful discussions during this work. We also thank Nick Marini and Charisse Buising for comments on the manuscript, and Mary Nims for excellent secretarial assistance. We are grateful to Deborah Stowers for outstanding technical assistance and graphic arts. This work was supported by funds from the National Institutes of Health and the National Science Foundation. HBK gratefully acknowledges the support of the Office of Biotechnology at Iowa State University and NRSA postdoctoral fellowship GM10565 from the National Institutes of Health.

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