Purification and Characterization of Two Forms of DNA Polymerase α from Mouse FM3A Cells: A DNA Polymerase α -Primase Complex and a Free DNA Polymerase α

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ABSTRACT. Two forms of DNA polymerase α , $\alpha 1$ and $\alpha 2$, have been partially purified from mouse FM3A cells by discriminating one form from the other on the basis of the association of primase activity. The primase activity in the most purified $\alpha 1$ fraction co-sedimented with the DNA polymerase activity in a glycerol gradient, and almost no primase activity was detected in the most purified $\alpha 2$ fraction. The primase activity associated with DNA polymerase α was assayed indirectly by measuring ATP-dependent DNA synthesis with poly (dT) as template. Characterization of the assay system was performed with the purified $\alpha 1$. The system was absolutely dependent on the presence of ATP and a divalent cation. Mn^{2+} was much more effective than Mg^{2+} , and 5-fold higher activity was observed with Mn^{2+} than with Mg^{2+} at their optimal concentrations. The primase activity assayed by the above system showed sensitivity to $(NH_4)_2SO_4$ very similar to that of free primase reported by Tseng and Ahlem (J. Biol. Chem. **258**, 9845–9849, 1983). The activity was inhibited by more than 50% by 20 mM $(NH_4)_2SO_4$.

$\alpha 1$ and $\alpha 2$ were very similar as DNA polymerases in their sensitivity to several inhibitors and their preference for template-primers, except that $\alpha 1$ had a slightly greater preference for poly (dT)·(rA)₁₀ than $\alpha 2$ did. The major difference between the two forms was observed in their S values, 8.2 and 6.4 S for $\alpha 1$ and $\alpha 2$, respectively.

Mammalian cells contain three major classes of DNA polymerases, α , β , and γ . Among these polymerases, DNA polymerase α has been suggested to be involved in DNA replication of mammalian cells (24). Many studies of DNA polymerase α isolated from various mammalian cells and tissues have indicated the existence of multiple forms of the enzyme, which differ in their chromatographic behavior and in

Abbreviations used: araCTP, 1- β -D-arabinofuranosylcytosine triphosphate; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

sedimentation coefficients. We have also observed that HeLa cells or their isolated nuclei contain two forms of DNA polymerase α which differ in binding affinity to DNA or chromatin (13, 14, 17). Furthermore, it has been suggested by using a salt-treated nuclear system that the form that has higher binding affinity to chromatin is responsible for the synthesis of Okazaki fragments (3, 4). However, more critical experiments are necessary to assess the physiological significance of the existence of two forms of DNA polymerase α and to confirm the roles of the enzymes in the processes of DNA replication.

In prokaryotes, genetic analysis and development of *in vitro* replication systems have permitted functional analysis of many enzymes and proteins participating in DNA replication. The analogous approaches have not yet been well developed in eukaryotes. In recent years we have tried to isolate temperature-sensitive or drug-resistant mutants related to DNA replication from mouse FM3A cells and have already isolated several mutants (11, 19, 27). In addition, we have isolated several enzymes and protein factors such as DNA-dependent ATPases (18, 23) and DNA polymerase α stimulating factors (8, 9) from FM3A cells. These cells are a good source for the isolation of enzymes and protein factors related to DNA replication because it is possible to obtain a large amount of S phase accumulated cells grown in the abdominal cavity of the mouse (6). Therefore, we have concentrated our efforts on the purification of two forms of DNA polymerase α from FM3A cells, but we have not succeeded in separating clearly the two forms by ion exchange column and DNA-cellulose column chromatographies.

Recently it has been observed in a variety of eukaryotic cells that one form of DNA polymerase α is associated with primase activity (2, 5, 12, 15, 16, 20, 22, 25, 26, 28), providing an effective means to distinguish subspecies of DNA polymerase α . In this report, we have separated and partially purified two forms of DNA polymerase α from FM3A cells on the basis of the association of primase activity with one of the forms and compared their properties as DNA polymerases. In addition, we have characterized *de novo* synthesis primed by the primase associated with a DNA polymerase α on poly(dT) as template.

MATERIALS AND METHODS

Materials. [^3H]dATP and [^3H]dTTP were purchased from Amersham (Buckinghamshire, England). Unlabeled deoxyribonucleoside triphosphates were from Boehringer Mannheim (West Germany). AraCTP, NEM, and PMSF were obtained from Sigma (St. Louis, U.S.A.). Sodium bisulfite and aphidicolin were purchased from Wako Chemicals (Osaka). Antipain was kindly supplied by Dr. H. Umezawa (Inst. Microb. Chem., Tokyo). Bovine serum albumin (fraction V) was obtained from Seikagaku Kogyo (Tokyo). Yeast alcohol dehydrogenase and alkaline phosphatase were obtained from Worthington (New Jersey, U.S.A.). Poly(dA), poly(dT), poly[d(A-T)], (rA)₁₀, (dA)₁₂₋₁₈, and (dT)₁₂₋₁₈ were purchased from P-L Biochemicals (Milwaukee, U.S.A.). Single-stranded DNA-cellulose was prepared essentially according to the method of Alberts and Herrick with slight modification as described previously (17). Activated DNA was prepared as described previously (1).

Cell. FM3A cells (1×10^6) were injected into the abdominal cavity of a ddY mouse. On the 5th day after inoculation, cells were synchronized in S phase by intraperitoneal injection of 100 μg of 5-fluoro-2'-deoxyuridine and harvested as described previously (6). The cells were stored at -80°C until use.

Assay for DNA polymerase α activity. The standard reaction mixture (total volume 30 μ l) contained 20 mM Tris-HCl, pH 8.0, 3.3 mM 2-mercaptoethanol, 5 mM MgCl_2 , 0.2 mg/ml bovine serum albumin, 100 μ M each of dATP, dCTP, and dGTP, 50 μ M [^3H]-dTTP (0.1 Ci/mmol), and 500 μ g/ml activated calf thymus DNA. Incubation was carried out for 30 min at 37°C in a plastic immunological microtitration tray. The reaction mixture was transferred onto a Whatman DE81 filter paper disk and the disk was washed by the method of Lindall *et al.* (10).

Assay for primase activity. The standard reaction mixture (total volume 30 μ l) contained 20 mM Tris-HCl, pH 8.0, 3.3 mM 2-mercaptoethanol, 0.2 mg/ml bovine serum albumin, 2 mM ATP, 2 mM MnCl_2 , 100 μ M [^3H]dATP (0.1 Ci/mmol), and 42 μ g/ml poly(dT). Incubation was carried out for 30 min at 37°C. The reaction was terminated by chilling the reaction mixture to 0°C and by adding 0.2 ml of 0.15 M sodium pyrophosphate, 0.3 ml of 1 mg/ml carrier DNA, and 2 ml of 5% TCA containing 1% sodium pyrophosphate. The TCA-insoluble materials were collected on a Whatman GF/C glass-fiber filter. The filter was washed 5 times with 3 ml of cold 5% TCA-1% sodium pyrophosphate and 2 times with ethanol, and dried. The radioactivity was measured with a liquid scintillation counter.

Glycerol gradient centrifugation. Two hundred microliters of sample was loaded on 4.8 ml of 15–35% glycerol gradient in a medium containing 20 mM potassium phosphate buffer, pH 7.5, 0.3 M KCl, 300 μ g/ml bovine serum albumin, 0.1 mM Na_3EDTA , 1 mM 2-mercaptoethanol, 0.25 mM PMSF, and 10 mM sodium bisulfite. Centrifugation was performed for 14.1 h at $258,000 \times g$ at 4°C. Fractions were collected from the bottom of the tube.

Buffers. Buffer 1 contained 20 mM potassium phosphate, pH 7.5, 0.1 mM Na_3EDTA , 1 mM 2-mercaptoethanol, 0.25 mM PMSF, and 10 mM sodium bisulfite. Buffer 2 contained all the components of buffer 1, 20% ethylene glycol, and 0.01% Triton X-100. Buffer 3 was the same as buffer 2 except that Tris-HCl, pH 8.1, was used instead of potassium phosphate, pH 7.5. Buffer 4 was the same as buffer 2 except that the concentration of ethylene glycol was reduced to 10%.

RESULTS

Purification of two forms of DNA polymerase α . All operations were carried out at 0–4°C. A frozen stock of 5×10^{10} FM3A cells was thawed, suspended in 400 ml of buffer 1 which contained 2 μ g/ml antipain, and sonicated 4 times for 10 s each with 20 s intervals by a Branson sonifier model 185 (40 W). The sonicate was made 0.3 M in KCl by the addition of 1/10 volume of buffer 1 containing 3.3 M KCl. Extraction was performed for 30 min with stirring and followed by centrifugation for 1 h at $105,000 \times g$. The supernatant was loaded on a DEAE-cellulose column (500 ml; 4.2×36 cm) equilibrated with 0.3 M KCl in buffer 1. The flow-through fractions were combined and dialyzed against 50 mM KCl in buffer 2 after the addition of Triton X-100 at a final concentration of 0.01%. The dialyzate was loaded on a second DEAE-cellulose column (250 ml; 4.2×18 cm) equilibrated with 50 mM KCl in buffer 2. The column was washed with 2.5 bed volumes of the same buffer, and the proteins adsorbed to the column were eluted with 2.5 bed volumes of 0.4 M KCl in buffer 2. DNA polymerase α and primase activities were recovered in the 0.4 M KCl-eluate.

The active fractions were combined and dialyzed against buffer 2 containing 0.1 M KCl. The dialyzate was loaded on a phosphocellulose column (70 ml; $2 \times$

22 cm) equilibrated with 0.1 M KCl in buffer 2. The column was washed with 2.5 bed volumes of the same buffer and eluted with 560 ml of a linear gradient of KCl from 0.1 to 0.6 M in buffer 2. As shown in Fig. 1, DNA polymerase α was eluted as a broad peak extending from 0.3 to 0.5 M KCl, and the primase activity was eluted at about 0.37 M KCl, forming a rather sharp peak. We designated the DNA polymerase α activity associated with primase activity as α_1 and the activity free from primase activity as α_2 . The two activities were pooled separately as indicated in Fig. 1 and dialyzed against 0.05 M KCl in buffer 2. Each dialyzate was loaded separately on DEAE-cellulose columns (α_1 , 25 ml, 1.2×22 cm; α_2 , 40 ml, 1.5×22 cm) equilibrated with 0.05 M KCl in buffer 2. The columns were washed with 2 bed volumes of the above buffer and eluted with 8 bed volumes of a linear gradient of KCl from 0.05 to 0.5 M in buffer 2. As shown in Fig. 2, α_1 and α_2 were eluted from the columns at 0.16 and 0.21 M KCl, respectively. The cross-contaminated activities in the α_1 and α_2 fractions from the phosphocellulose column were further separated at this stage. The active fractions were pooled as indicated in Fig. 2 and dialyzed against 0.05 M NaCl in buffer 3. The dialyzates were loaded on single-stranded DNA-cellulose columns (α_1 , 16.5 ml, 1.9×5.7 cm; α_2 , 40 ml, 1.5×22 cm) which were equilibrated with 0.05 M NaCl in buffer 3. The columns were washed with 3 bed volumes of the above buffer and eluted with 8 bed volumes of a linear gradient of NaCl from 0.05 to 0.4 M in buffer 3. Bovine serum albumin was added at a concentration of 200 $\mu\text{g}/\text{ml}$ in the elution buffer so as to stabilize the enzyme activity. The α_1 and α_2 activities were eluted from the columns at about 0.15 M NaCl. A portion of the peak fractions

Fig. 1. Phosphocellulose column chromatography. The second DEAE-cellulose column fraction was chromatographed as described in the test. Aliquots of the fractions were assayed for DNA polymerase α (○) and primase (●) activities as described.

Fig. 2. DEAE-cellulose column chromatography. $\alpha 1$ -rich and $\alpha 2$ -rich fractions of the phosphocellulose column as indicated in Fig. 1 were chromatographed separately as described in the text. Aliquots of the fractions were assayed for DNA polymerase α (○) and primase (●) activities as described. (A) $\alpha 1$, (B) $\alpha 2$.

of each activity was pooled and dialyzed against 0.05 M NaCl in buffer 1 containing 50% glycerol. The dialyzates were stored at -80°C until use. Another portion of the peak fractions of each activity was pooled and dialyzed against 0.1 M KCl in buffer 4. The dialyzates were adsorbed to small phosphocellulose columns and concentrated by stepwise elution with 0.6 M KCl in buffer 4. The peak fractions were pooled and a portion was layered on the top of a glycerol gradient. Centrifugation was performed as described under MATERIALS and METHODS. As shown in Fig. 3, $\alpha 1$ and $\alpha 2$ sedimented

Fig. 3. Glycerol gradient centrifugation of purified $\alpha 1$ and $\alpha 2$. The DNA-cellulose column fractions of $\alpha 1$ and $\alpha 2$ were concentrated and sedimented through glycerol gradients as described in the text. Aliquots of the fractions were assayed for DNA polymerase α (○) and primase (●) activities as described. The protein markers run in a parallel gradient were yeast alcohol dehydrogenase (7.4 S) and bacterial alkaline phosphatase (6.1 S). (A) $\alpha 1$, (B) $\alpha 2$.

TABLE 1. PURIFICATION OF DNA POLYMERASE $\alpha 1$ AND $\alpha 2$ FROM FM3A CELLS

Purification Step	DNA Polymerase α Activity				Primase Activity		
	Protein (mg)	Total Act. (units)	Sp. Act. (units/mg)	Yield (%)	Total Act. (units)	Sp. Act. (units/mg)	Yield (%)
Crude Extract	5817	29195	5.0	100	4957	0.8	100
1st DEAE-cellulose	4173	28273	6.8	97	2214	0.5	45
2nd DEAE-cellulose	1114	27256	24.5	93	2010	1.8	41
$\alpha 1$							
Phosphocellulose	94	19940	211	68	2725	29	55
DEAE-cellulose	21	7903	364	27	2207	102	45
ssDNA-cellulose	4.9	2255	461	7.7	820	168	17
$\alpha 2$							
Phosphocellulose	89	18863	213	65	501	5.7	10
DEAE-cellulose	31	9720	314	33	59	1.9	1.2
ssDNA-cellulose	7.6	3255	427	11	5.7	0.7	0.1

DNA polymerase α and primase activities were assayed as described under MATERIALS AND METHODS. One unit of activity is defined as the amount catalyzing the incorporation of 1 nmol of dXTP into acid-insoluble materials in 1 h at 37°C for DNA polymerase activity and 1 nmol of labeled dAMP for primase activity.

as single peaks at 8.2 and 6.4 S, respectively. It must be noted that the sedimentation profile of the primase activity coincided well with that of the DNA polymerase activity in the case of the $\alpha 1$ fraction, and almost no primase activity was detected in the $\alpha 2$ fraction (Fig. 3). A summary of a typical purification experiment for DNA polymerase $\alpha 1$ and $\alpha 2$ is shown in Table 1.

Characterization of de novo synthesis. Primase activity was assayed indirectly by measuring the incorporation of dAMP in the presence of ATP with poly(dT) as

Fig. 4. (A) Effect of divalent cations on the primase activity. DNA primase activity was assayed in the primase assay mixture which contained 42 μ g/ml poly(dT) and 2 mM ATP in the presence of various concentrations of MnCl₂ (○) or MgCl₂ (●). (B) Effect of divalent cations on the DNA polymerase activity with poly(dT)·(rA)₁₀ as template-primer. DNA polymerase activity was assayed in the presence of various concentrations of MnCl₂ (○) or MgCl₂ (●) under the same conditions as the primase assay except that 2 mM ATP was omitted and 8.4 μ g/ml (rA)₁₀ was added.

Fig. 5. Effect of salts on the primase activity. Assays were performed under the conditions for primase assay in the presence of the indicated concentrations of KCl (○), NaCl (●), or $(\text{NH}_4)_2\text{SO}_4$ (▲). In the absence of added salts, the assay mixture contained 3.3 mM NaCl that derived from the buffer in which $\alpha 1$ was dissolved. One hundred percent of the activity corresponds to 631 pmol of dAMP incorporation.

template (*de novo* synthesis). Characterization of the assay system was performed with purified $\alpha 1$. The activity is absolutely dependent on the presence of ATP and a divalent cation. Fig. 4A shows the requirement of *de novo* synthesis for divalent cations in the presence of 2 mM ATP. A sharp optimum is observed at 2 mM with Mn^{2+} , and a broad optimum at 3–4 mM with Mg^{2+} . Mn^{2+} is much more effective than Mg^{2+} , and 5-fold higher activity is observed with Mn^{2+} than with Mg^{2+} at their optimal concentrations. The above requirements are very different from those of DNA polymerase activity with poly(dT)·(rA)₁₀ as template-primer (Fig. 4B). Fig. 5 shows the salt requirements of *de novo* synthesis. The optimal concentrations of KCl, NaCl, and $(\text{NH}_4)_2\text{SO}_4$ are 30, 30, and 5 mM, respectively. Potassium chloride is the most effective among the salts tested. The activity is very sensitive to $(\text{NH}_4)_2\text{SO}_4$. More than 50% inhibition is observed with 20 mM $(\text{NH}_4)_2\text{SO}_4$, distinguishing the activity from known eukaryotic RNA polymerase activities. The sensitivity to $(\text{NH}_4)_2\text{SO}_4$ is very similar to that of the free primase reported by Tseng and Ahlem (21).

The reaction performed under optimal conditions continued linearly for up to 90 min with no lag period. The dose response curve of *de novo* synthesis was sigmoidal, suggesting that the reaction may involve the participation of two or more distinct proteins (not shown).

Comparison of the two forms of DNA polymerase α . The effect of various inhibitors on the activities of DNA polymerase $\alpha 1$ and $\alpha 2$ was investigated. The activities of $\alpha 1$ and $\alpha 2$ are inhibited in a similar manner by NEM and araCTP (Fig. 6A, 6B). Both activities are almost completely inhibited by 5 mM NEM. AraCTP inhibits $\alpha 1$ and $\alpha 2$ by 89% and 94%, respectively, at 1 mM. Aphidicolin also inhibited both activities (Fig. 6C).

Table 2 shows the template-primer preferences of the two forms. Both forms prefer activated DNA and poly(dT)·(rA)₁₀ and exhibit low activity with heat-denatured DNA, native DNA, poly[d(A-T)], and poly(dT)·(dA)_{12–18}. Relatively high activity is

Fig. 6. Effect of various inhibitors on the activities of DNA polymerase $\alpha 1$ and $\alpha 2$. 663 units of $\alpha 1$ (○) and 673 units of $\alpha 2$ (●) were assayed for DNA polymerase activity in the presence of various concentrations of inhibitors. (A) N-ethylmaleimide, (B) AraCTP, (C) Aphidicolin.

obtained by both forms with poly(dA)·(dT)₁₂₋₁₈. No significant difference in template-primer is observed between the two forms except that $\alpha 1$ uses poly(dT)·(rA)₁₀ slightly more efficiently than $\alpha 2$. The major difference observed between the two forms is in their S values, 8.2 and 6.4 S for $\alpha 1$ and $\alpha 2$, respectively (Fig. 3).

Tight association of primase with DNA polymerase α . As described in the previous section, primase activity co-sedimented with DNA polymerase activity in a glycerol gradient which contained 0.3 M KCl, indicating the tight association of the primase with DNA polymerase $\alpha 1$. We next tried to dissociate the primase from the DNA polymerase. DNA polymerase $\alpha 1$ was treated with 2 M urea or 1 M KCl and sedimented through a glycerol gradient containing 2 M urea or 1 M KCl. The primase

TABLE 2. TEMPLATE-PRIMER PREFERENCE OF DNA POLYMERASE $\alpha 1$ AND $\alpha 2$

Template-primer	$\alpha 1$	$\alpha 2$
	pmol(%)	pmol(%)
Activated DNA	616(100)	463(100)
Heat-denatured DNA	11(1.8)	8(2)
Native DNA	26(4.2)	12(2.6)
Poly[d(A-T)]	8(1)	15(3.2)
Poly(dT)·(rA) ₁₀	907(147)	480(104)
Poly(dT)·(dA) ₁₂₋₁₈	30(4.9)	8(2)
Poly(dA)·(dT) ₁₂₋₁₈	103(17.0)	81(17)

Fifteen micrograms of activated, heat-denatured, native DNA, and 1.25 μ g of the synthetic polymers were used. DNA polymerase activity with activated, heat-denatured, or native DNA was assayed by the α assay system as described under MATERIALS AND METHODS. The activities with the synthetic polymers were assayed under the conditions of the α assay except that the nucleoside triphosphates in the reaction mixture were changed as follows: 50 μ M [³H]-dTTP (0.1 Ci/mmol) and 50 μ M dATP for poly[d(A-T)], 50 μ M [³H]-dATP (0.1 Ci/mmol) for poly(dT)·(rA)₁₀ and poly(dT)·(dA)₁₂₋₁₈, 50 μ M [³H]-dTTP (0.1 Ci/mmol) for poly(dA)·(dT)₁₂₋₁₈.

activity was assayed in the presence of 0.3 units of *E. coli* DNA polymerase I. Under these conditions, the primase still co-sedimented with the DNA polymerase activity. Exposure of $\alpha 1$ to more severe conditions such as 3 M urea resulted in the loss of both enzyme activities.

DISCUSSION

In this study, we have partially purified two forms of DNA polymerase α , one of which is associated with a primase activity, from FM3A cells. The two forms showed almost the same properties as DNA polymerase α in their sensitivity to several inhibitors and their preference for template-primers except that $\alpha 1$ preferred poly-(dT)·(rA)₁₀ slightly more than $\alpha 2$ did. The major difference between the two forms was observed in their sedimentation coefficients in glycerol gradients. The above observations seem to suggest the possibility that $\alpha 1$ consists of $\alpha 2$ and the primase. However, attempts to dissociate the primase from DNA polymerase $\alpha 1$ by treatment with 1 M KCl or 2 M urea were not successful. Kaguni *et al.* (7) reported the separation of the primase from *Drosophila* DNA polymerase α in a glycerol gradient containing 2.8 M urea after the treatment of the enzyme with 3.4 M urea. We also tried to dissociate the primase from $\alpha 1$ under the same conditions but we could not detect primase and DNA polymerase activities under such severe conditions. Recently Tseng and Ahlem (21) have reported that primase which is not associated with DNA polymerase activity can be purified from mouse hybridoma cells by using a medium which contains 20% dimethylsulfoxide, 10% ethylene glycol, and 0.01% Nonidet P-40. Experiments to determine the conditions for dissociating and re-constituting the FM3A DNA polymerase α -primase complex are now in progress.

The primase activity reported here was assayed indirectly by measuring ATP-dependent DNA synthesis with poly(dT) as template. It is reported that a factor that greatly stimulates primase activity exists in Ehrlich ascites tumor cells, and that in the absence of the factor it is very hard to detect primase activity (25, 26). The attempts to detect such a factor in FM3A cells have not succeeded yet. However, even in the absence of the stimulating factor, we could easily detect primase activity. The primary reason for this easy detection may be the assay system used here, although the possibility that a stimulating factor is co-purified with $\alpha 1$ cannot be excluded completely.

The reaction mixture used here for primase assay contained Mn²⁺ instead of Mg²⁺, and 5-fold higher activity was observed with Mn²⁺ than with Mg²⁺ at their optimal concentrations. This preference for Mn²⁺ may be primarily due to the properties of the primase because in the presence of primer, (rA)₁₀, the levels of DNA polymerase activity of $\alpha 1$ in the presence of Mg²⁺ are higher than in the presence of Mn²⁺ (Fig. 4B). In addition, the sensitivity of the primase assay system to (NH₄)₂SO₄ may also be due to the properties of the primase itself because similar sensitivity is observed with primase which is not associated with DNA polymerase α (21). Therefore, the system using poly(dT) and Mn²⁺ is very useful for the detection of primase activity.

It has been suggested from several lines of evidence that the primase associated with DNA polymerase α is responsible for the synthesis of RNA primers of Okazaki fragments. In previous studies with HeLa cells, we suggested that HeLa nuclei contained two forms of DNA polymerase α , form I and form II, and that form I was responsible for the synthesis of Okazaki fragments (3, 4). At present it is not clear how $\alpha 1$ and $\alpha 2$

of FM3A cells correspond to form I and form II of HeLa cells. This question is currently under investigation.

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