Purification and characterization of mouse DNA polymerase α devoid of primase activity

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A simple method was developed for the isolation of primase-free DNA polymerase-α from the DNA polymerase-α-primase complex of mouse FM3A cells. The polymerase was separated from primase subunits by chromatography on a single-stranded DNA-cellulose column in the presence of 50% etylene glycol. The primase-free DNA polymerase-α contained two polypeptides with molecular masses of 180000 and 68000. Analysis of the DNA products with poly(dA)-oligo$(dT)_{10}$ as template-primer revealed that both primase-free DNA polymerase-α and the DNA polymerase-α-primase complex predominantly synthesized short DNA with less than 30 nucleotides, but that the DNA polymerase-α-primase complex also synthesized some longer DNA with more than 300–400 nucleotides.

DNA polymerase-α; Primase; DNA replication; Processivity; Mouse FM3A cell

1. INTRODUCTION

The purification of proteins involved in DNA replication and their reconstitution into a replication complex is a promising approach to clarify the mechanism of DNA replication. We have purified two forms of DNA polymerase-α from mouse FM3A cells, one tightly associated with DNA primase activity and the other free of primase activity [1]. The former, DNA polymerase-α-primase complex, was resolved with buffer containing 50% ethylene glycol [2], and the synthesis of RNA primers was studied with isolated primase and a complex of the primase with DNA polymerase-α [3]. Although these studies provided interesting data, the enzymes used were not purified to homogeneity, and so structural-functional relationships at molecular level could not be analyzed. Recently we developed a simple, two-step method for purification of the mouse DNA polymerase-α-primase complex [4]. In this paper we report the purification and characterization of mouse DNA polymerase-α devoid of primase activity.

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2. MATERIALS AND METHODS

2.1. *Buffer and cells*

Unless otherwise stated, buffer 1 contained 20 mM potassium phosphate, pH 7.5, 0.1 mM Na_3EDTA, 1 mM 2-mercaptoethanol, 0.01% Triton X-100, 10 mM sodium bisulfite, 0.25 mM phenylmethylsulfonyl fluoride and 2 μg/ml of antipain. FM3A clone 28 cells, originally established from a spontaneous mammary carcinoma in a C3H/He mouse [5], and the hybridoma line SJK 287-38 [6], which produces SJK 287-38 antibody (anti-human DNA polymerase-α antibody), were grown in suspension culture as described previously [4].

2.2. *Assays of DNA polymerase-α and primase*

DNA polymerase-α activity and primase activity were assayed as described previously [3].

2.3. *Preparation of immunoaffinity-purified DNA polymerase-α-primase*

Immunoaffinity-purified DNA polymerase-α-primase was prepared from mouse FM3A cells as described previously [4].

2.4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Denaturing gel electrophoresis was performed as described by Laemmli [7]. Gels were stained with silver.

2.5. *Analysis of oligonucleotide products synthesized by DNA polymerase-α*

The enzymes were incubated at 37°C for the indicated periods in reaction mixture (30 μl) consisting of 20 mM Tris-HCl, pH 7.5, 3.3 mM dithiothreitol, 8% polyethylene glycol 6000, 7 mM $MgCl_2$, 40 μg/ml bovine serum albumin, 100 μM TTP, 16-32 μCi [α-^{32}P]TTP, and 0.5 μg of poly(dA)-oligo$(dT)_{10}$ (20:1). The reaction was terminated by adding 6 μl of 2.4% SDS-40 mM Na_3EDTA, pH 8.0, and 30 μl of 0.2 mg/ml yeast tRNA-20 mM Na_3EDTA, and cooling the mixture to 0°C. Then 2 vols of ethanol were added to the mixture in the presence of 2 M ammonium acetate, and the precipitate was dissolved in 6 μl of loading buffer containing 90% formamide-0.01% Bromophenol blue-0.01% xylene cyanol, and loaded onto 7 M urea-10% polyacrylamide slab gel. Electrophoresis was carried out at a constant voltage of 700 V.

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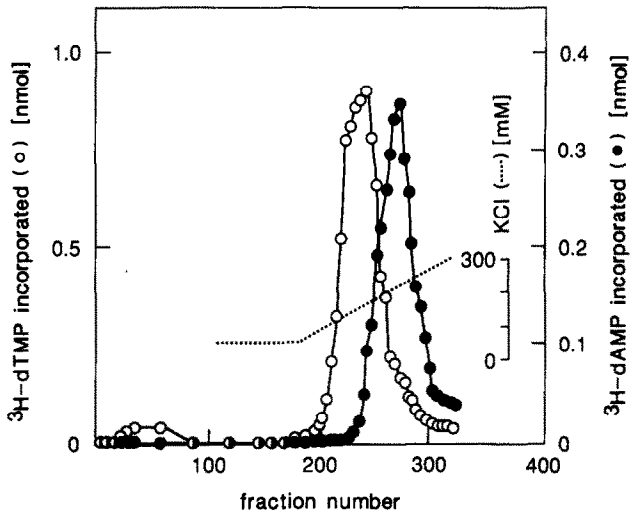

Fig. 1. Separation of polymerase and primase subunits of mouse DNA polymerase-α-primase complex by single-stranded DNA-cellulose column chromatography in the presence of 50% ethylene glycol. The immunoaffinity-purified DNA polymerase-α-primase complex was subjected to single-stranded DNA-cellulose column chromatography as described under section 3. Activities of DNA polymerase-α (○) and primase (●) are plotted.

3. RESULTS AND DISCUSSION

To isolate the DNA polymerase-α-primase complex, in which 4 polypeptides form a physical complex, we subjected the immunoaffinity-purified fraction (SJK 287-Sepharose column fraction: 0.36 ml) to glycerol gradient sedimentations as described previously [4]. The active fractions were pooled and dialyzed against buffer 1 containing 50% glycerol and 100 mM KCl, and stored at −20°C until use. This fraction was named α_1.

Primase-free DNA polymerase-α was isolated as follows. One-third of the immunoaffinity-purified fraction (2.5 ml) was diluted with an equal volume of buffer 1, and loaded onto a column (bed vol. 2 ml: 0.29 cm^2 × 7 cm) of single-stranded DNA-cellulose, which had been equilibrated with buffer 1 containing 20% ethylene glycol and 50 mM KCl. The column was washed with buffer 1 containing 50% ethylene glycol and 50 mM KCl, and then the proteins bound to the column were eluted with 12 bed vols of a linear gradient of 50 to 300 mM KCl in buffer 1 containing 50% ethylene glycol. Under these conditions, DNA polymerase-α and primase were eluted separately from the column at 140 mM and 220 mM KCl, respectively (Fig. 1). The DNA polymerase-α-rich fractions were pooled and applied to a second phosphocellulose column (bed vol. 1 ml: 0.18 cm^2 × 5.5 cm) that had been equilibrated with buffer 1 containing 50% ethylene glycol and 150 mM KCl. Material on the column was eluted with 12 bed vols of a linear gradient of 150 mM to 800 mM KCl in buffer 1 containing 50% ethylene glycol. The DNA polymerase-α fractions with virtually no primase activity were pooled, dialyzed against buffer 1 containing 50% glycerol and 100 mM KCl, and stored at −20°C until use. This fraction was named α_2.

The purifications of α_1 and α_2 are summarized in Table I. The steps up to SJK 287-Sepharose column chromatography have been reported [4]. The recovery of DNA polymerase-α activity on glycerol density gradient centrifugation was lower than that of primase activity, reflecting the broader distribution of the former activity in the gradient [4]. Single-stranded DNA-cellulose column chromatography followed by phosphocellulose column chromatography markedly decreased the recovery of DNA polymerase-α activity, but these steps were effective for obtaining primase-free DNA polymerase-α (α_2). The polypeptide structures of α_1 and α_2 were examined by SDS-PAGE (Fig. 2). α_1 was composed essentially of four polypeptides with apparent molecular masses of 180000, 68000, 54000 and 46000, as described previously [4], whereas α_2 consisted of two polypeptides with molecular masses of 180000 and 68000. These results are consistent with previous findings that DNA polymerase activity is associated with a 180-kDa polypeptide [8], whereas primase activi-

Table I

Purifications of α_1 and α_2 from mouse FM3A cells

Purification step	Protein (mg)	Volume (ml)	DNA polymerase α			Primase		
			Total act. (units)	Spec.act. (units/mg)	Yield (%)	Total act. (units)	Spec.act. (units/mg)	Yield (%)
Crude extract	5140	275	218 000	42	100	33 100	6.4	100
1st phosphocellulose	790	340	405 000	513	186	595 000	753	1800
SJK 287-Sepharose	1.0	8.0	131 000	131 000	60	18 300	18 300	55
(I) α_1								
G.D.G. input	0.045	0.36	4680	104 000	100	690	15 300	100
G.D.G.	0.0074	0.83	760	103 000	16	280	37 800	41
II (α_2)								
ssDNA-cellulose input	0.31	5.0	40 900	132 000	100	3710	12 000	100
ssDNA-cellulose	0.020	7.8	5370	269 000	13	4.2	210	0.1
2nd phosphocellulose	0.0032	1.2	870	272 000	2.1	0.2	62.5	0.0

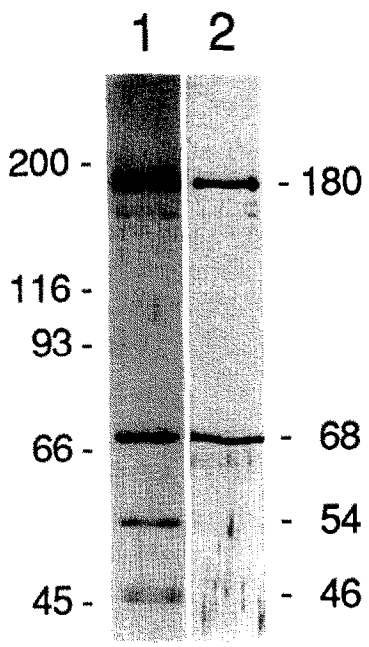

Fig. 2. Polypeptide structures of α_1 and α_2. Aliquots of protein fractions were subjected to SDS-PAGE and stained with silver. Lane 1, α_1 (glycerol density gradient fraction); lane 2, α_2 (second phosphocellulose column eluate).

ty is associated with polypeptides of 55 and 49 kDa [9]. A function of the 68-kDa polypeptide is unknown, except that removal of this subunit from the DNA polymerase-primase complex had been reported to unmask 3′-5′ exonuclease activity associated with a 182-kDa polypeptide in the *Drosophila* DNA polymerase-primase complex [10].

To determine whether the primase subunits influence the processivity of the polymerase, we examined the lengths of the products synthesized by α_1 and α_2 on $(dT)_{10}$-initiated poly(dA) in the absence of ribonucleoside triphosphates. Since the primer was in large excess (molar ratio of primer to enzyme; >500:1), a large proportion of the primer remained unused during this short period of incubation. The lengths of the products should therefore correspond to a single elongation event and reflect the processivity of the enzyme. As shown in Fig. 3, products with lengths of less than 30 nucleotides were formed in the reaction of α_2, while longer products of more than 300–400 nucleotides were synthesized by α_1 in addition to short products of less than 20 nucleotides. Under the reaction conditions used in this experiment, no primer RNA synthesis occurred. These results, therefore, indicate that the primase subunits partially increased the processivity of DNA polymerase-α. Okazaki fragments of eukaryotic organisms have been demonstrated to be between 100 and 200 nucleotides long [11]. Therefore, α_1 might synthesize Okazaki fragments by itself. Judging from the lengths and the amounts of the products of α_1, however, the ratio between longer products and short DNA was very low (Fig. 3). This indicates that α_1 probably requires further protein factors to act as a holoenzyme.

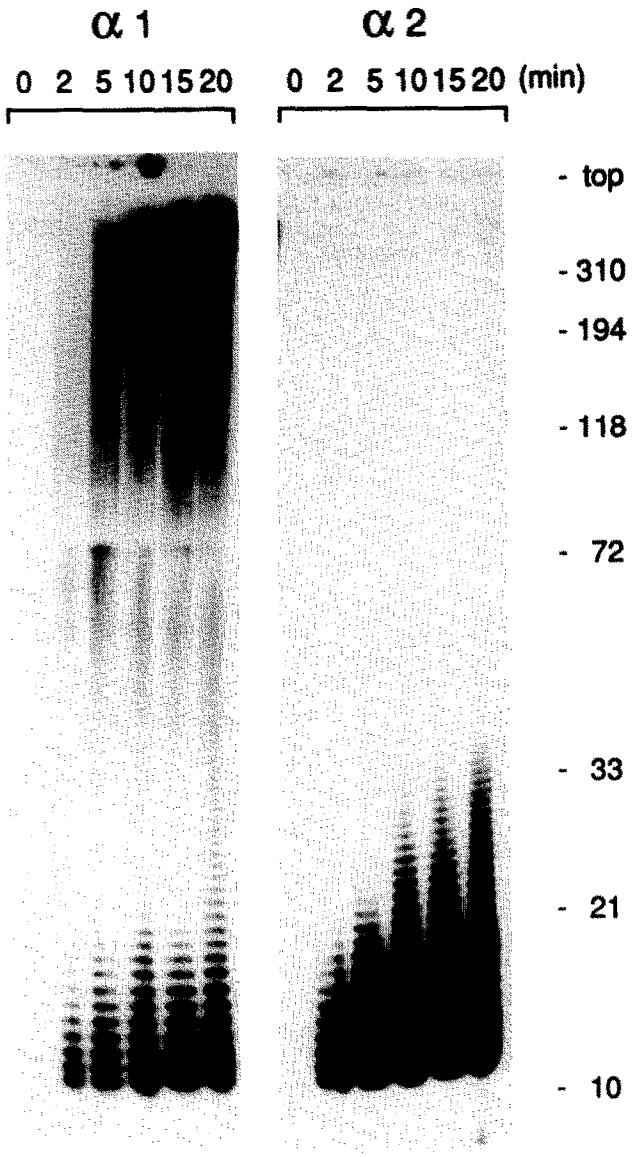

Fig. 3. Processivities of α_1 and α_2. Oligonucleotide products on poly(dA)-oligo$(dT)_{10}$ synthesized by α_1 (0.22 units*) and α_2 (0.15 units*) were analyzed on 10% polyacrylamide slab gel in the presence of 7 M urea as described in section 2. Various incubation times were employed.
*These units of DNA polymerase-α were determined using activated DNA as a template-primer [3].

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