Resolution and purification of free primase activity from the DNA primase-polymerase α complex of HeLa cells

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ABSTRACT

DNA primase activity has been resolved from a purified DNA primase-polymerase α complex of HeLa cells by hydrophobic affinity chromatography on phenylSepharose followed by chromatography on hexylagarose. This procedure provides a good yield (55%) of DNA primase that is free from polymerase α . The free DNA primase activity was purified to near homogeneity and its properties characterized. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the purified free DNA primase showed a major protein staining band of M_r 70,000. The native enzyme in velocity sedimentation has an $S_{20,W}^r$ of 5. DNA primase synthesizes RNA oligomers with single-stranded M-13 DNA, poly(dT) and poly(dC) templates that are elongated by the DNA polymerase α in a manner that has already been described for several purified eukaryotic DNA primase-polymerase α complexes. The purified free DNA primase activity is resistant to neutralizing anti-human DNA polymerase α antibodies, BuPdGTP and aphidicolin that specifically inhibit the free DNA polymerase α and also DNA polymerase α complexed with the primase. The free primase activity is more sensitive to monovalent salt concentrations and is more labile than polymerase α . Taken together these results indicate that the DNA primase and polymerase α activities of the DNA primase-polymerase α complex reside on separate polypeptides that associate tightly through hydrophobic interactions.

INTRODUCTION

The principal mechanism for the initiation of synthesis of nascent DNA chains in prokaryotes (1) and eukaryotes (2) occurs through the synthesis of RNA oligomers that are then elongated by the replicative DNA polymerase in concert with other proteins that function in DNA replication. Eliasson and Reichard (3) from their study of polyoma DNA replication proposed the existence of a primase activity in eukaryotes, similar to the dna G gene product in *Escherichia coli* (4,5), the phage T7 gene product (6) and phage T4 gene 41 - gene 61 protein complex (7,8), that functions in the synthesis of iRNA. Following this proposal a primase-like activity was also described for CV-1 cells (9), human lymphocytes (10), *Drosophila* embryos (11) and *Xenopus* eggs (12,13). Surprisingly, and in contrast to prokaryotic systems, a DNA primase

was shown to be tightly associated with DNA polymerase α or its counterpart from a wide variety of eukaryotic cells (14-26). A DNA primase activity has also recently been reported to copurify with presumably DNA polymerase γ activity from mitochondria of mammalian cells (27,28). The general observation from these studies has been that DNA primase and polymerase α activities are tightly associated and cofractionate during several steps of conventional enzyme purification procedures (14,16,18,21,22) and also during immunoaffinity chromatography (19,20). The major exception being the DNA primase associated with DNA polymerase I (the polymerase α counterpart) from yeast cells. These activities have been separated by both conventional (24-26) and immunoaffinity (43) enzyme purification procedures. Also in an unconfirmed report a separation of DNA primase from polymerase α activity of mouse cells was attained by conventional enzyme purification procedures (17). The separation of DNA primase-polymerase α activities from Drosophila embryo extracts (29), however, required the use of 2.8-3.4 M urea and the separation from extracts of mouse (44) and human cells (45) has required chromatography in the presence of relatively high concentrations of ethylene glycol (40-50%). These conditions for dissociation of DNA primase-polymerase α suggest that hydrophobic interactions are involved in the association. Although the separation of DNA primase from polymerase α activity was reported in these studies, the properties of the free DNA primase was not determined.

In this report we describe a procedure for the resolution of DNA primase from a DNA polymerase α complex of HeLa cells by means of hydrophobic affinity chromatography. This procedure provides a good yield of DNA primase that is free from DNA polymerase α activity. The free DNA primase was purified to near homogeneity and its physical and enzymatic properties characterized.

MATERIALS

Chemicals.

Ammonium sulfate, buffers, glycerol, sucrose and urea were ultrapure grade and were purchased from various sources. Aminoacetonitrile-hemisulfate and phenylmethylsulfonyl fluoride (PMSF) were obtained from Aldrich Chemical Co. Triton X-100 was from Sigma and was further purified before use to remove free radicals (30). Alpha amanitin was purchased from Boehringer-Mannheim, aphidicolin was provided by the Natural Products Branch, Division of Cancer Treatment at NCI, and N²-(p-n-butylphenyl)deoxyguano-

sine-5'-triphosphate (BuPGTP) was a gift from Dr. George Wright, University of Massachusetts Medical Center, Worcester MA. Affigel 610, BioGel P-60, Cellex 410, DEAE-BioGel, molecular weight markers and electrophoresis reagents were purchased from Bio-Rad Laboratories. DEAE (DE52) cellulose, phosphocellulose (P11), DE81 paper and glass fiber (GF/C) discs were from Whatman. Native and denatured DNA-celluloses were prepared as previously described (31). Butylagarose was obtained from Miles-Yeda Ltd. and phenylSephadex from Pharmacia. Remazol blue agarose was purchased from Bethesda Research Laboratories. All other chemicals were of reagent grade or better and have been described elsewhere (31,32).

Nucleotides and Templates.

Nucleotides, oligonucleotides, polydeoxycytidylic acid poly(dC) and polydeoxyguanylic acid poly(dG) were purchased from Pharmacia P-L Biochemicals. Polydeoxyadenylic acid poly(dA)1000 and polydeoxythymidylic acid poly(dT)1000 were obtained from Midland Certified Reagents Co. Radioactive ribo- and deoxyribonucleotides were either from Amersham or New England Nuclear Corp. Single-stranded circular bacteriophage M13 DNA was obtained from New England Biolabs. Pancreatic DNase activated calf thymus DNA was prepared by a described procedure (33).

Proteins.

DNA polymerase I (Klenow fragment) was purchased from International Biotechnologies, Inc.; bacterial alkaline phosphatase and pancreatic DNase from Worthington Biochemical Corp.; pancreatic RNase and RNase T1 from Boehringer-Mannheim Corp. Polynucleotide kinase and T4 DNA polymerase were purchased from New England Biolabs. Monoclonal antibodies to chicken oviduct RNA polymerase II was a kind gift from Dr. Bert O'Malley, Baylor College of Medicine, Houston TX, and monoclonal antibody (SJK132-20) to human DNA polymerase α was provided by Dr. Neal Brown, University of Massachusetts Medical School, Worcester, MA. Rabbit polyclonal antibody to HeLa cell DNA polymerase α was produced as described previously (32). Bovine serum albumin (fatty acid free) was purchased from CalBiochem, and stock solutions (1 mg/ml) were prepared in distilled H₂O, heated at 53°C for 3 hours and then stored at -20°C.

METHODS

Cell Culture and Fractionation.

HeLa S₃ cells were propagated in suspension cultures in Joklik's modified Eagle's medium containing 3.5% each of calf serum and fetal calf

serum and harvested as described previously (34,35). Frozen cell pellets were thawed rapidly at 37°C, suspended in 4 volumes of 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM PMSF, and 1 mM aminoacetonitrile-hemisulfate and homogenized by 30 strokes in a loose-fitting (Kontes, pestle B) Dounce homogenizer. This resulted in over 90% breakage of cells but with nuclei remaining intact as determined by light microscopy following staining with 0.1% azure C. The homogenate was centrifuged at 600 x g for 5 minutes to isolate a nuclear pellet and EDTA-Na₃ and EGTA-Na₃ were added to the resulting supernatant solution to a concentration of 5 mM of each. The supernatant solution was centrifuged successively at 15,000 x g for 15 minutes and 105,000 x g for 1 hour to remove mitochondria and microsomes respectively. In the interim the nuclear pellet was suspended in 4 volumes at 50 mM Tris-HCl (pH 7.5), 0.15 M KCl, 1 mM PMSF, 1 mM aminoacetonitrile-hemisulfate, 1 mM EDTA and 1 mM EGTA by gentle homogenization in a Dounce homogenizer and stirred at 4°C for 2 hours. The nuclear suspension was centrifuged at 15,000 x g for 15 minutes and the resulting supernatant (nuclear extract) was combined with the postmicrosomal supernatant solution and served as the starting material for purification of the enzymes.

DNA polymerase assays.

The standard assays for DNA polymerases α and β activity were performed at 35°C using pancreatic DNase activated or heat-denatured calf thymus DNA as described previously (31,32,35). Under these conditions one unit of DNA polymerase activity is equal to the incorporation of 1 nmole of total dNTP into acid-insoluble material in 60 minutes at 35°C. The conditions for the assays with other DNA templates are described with the separate experiments.

DNA primase assay.

DNA primase activity was routinely measured at 30°C for 30 min in a 100 μ l reaction containing 100 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.5, 5 mM magnesium acetate, 1 mM dithiothreitol, 100 μ g of bovine serum albumin (heated at 55°C to inactivate nucleases), 50 μ M [³H]GTP (800-1,000 cpm/pmol), 5 μ g of poly(dC) and enzyme. Following the incubation period, 50 μ l aliquots were removed and processed on DE-81 paper and counted in a toluene based liquid scintillation counting fluid as previously described (35). One unit of DNA primase activity is 1 nmol of NMP incorporated per hour at 30°C.

When single-stranded circular M13 phage DNA was used as template the

standard reactions contained in a 100 μ l volume: 50 mM HEPES buffer, pH 7.8, 13 mM magnesium acetate, 1 mM dithiothreitol, 15% glycerol, 0.1 mM each dCTP, dGTP, [3 H]dTTP (200 to 300 cpm/pmol) or [3 H]dATP (200 to 300 cpm/pmol), 0.2 mM each CTP, UTP, GTP and [α - 32 P]ATP or [γ - 32 P]ATP (2,000 to 5,000 cpm/pmol) or 4 mM ATP, 1 μ g of DNA and 10 μ g of bovine serum albumin (heated at 55 $^\circ$ to inactivate nucleases). The products formed in the reaction were processed for counting as previously described (32,35).

During purification, primase activity was measured in a reaction coupled to *E. coli* DNA polymerase I (14). The reaction mixture (25 μ l) was a modification of that used by Yamaguchi *et al.* (23) and contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM magnesium acetate, 50 mM sodium acetate, 1 mM dithiothreitol, 15% glycerol, 4 mM ATP, 0.1 mM [3 H]dATP (100 cpm/pmol), 0.5 μ g poly(dT)1000, 12.5 μ g bovine serum albumin (heat-inactivated) and 0.5 units of *E. coli* DNA polymerase I (Klenow fragment) and primase-DNA polymerase α fractions as indicated. Reactions were terminated after 15 to 30 min by the addition of 100 μ l each of solutions of 0.1 M sodium pyrophosphate, 1 mg/ml of calf thymus DNA, 50% trichloroacetic acid and 3 ml of 0.1 N HCl. The acid-insoluble products formed in the reaction were filtered on glass fiber filters and processed for counting as previously described (35).

DNases.

The assays for endo- and exodeoxyribonuclease activities were carried out separately under optimal conditions for DNA primase and DNA polymerase α activities. Double-stranded and single-stranded specific endonuclease activities were assayed with [14 C]SV40 supercoiled DNA and [3 H]fd DNA respectively as previously described (26). Exodeoxyribonuclease activity was measured by the conversion of native and heat-denatured *E. coli* [3 H]DNA to acid-soluble material as previously described (36).

DNA-dependent ATPase.

ATPase activity was measured as the conversion of [γ - 32 P]ATP to charcoal non-adsorbable form following a 30 minute incubation at 30 $^\circ$ C in the presence and absence of 1 μ g of ϕ X 174 virion DNA, 1 mM ATP and under the optimal assay conditions for DNA primase-polymerase α .

Purification of DNA polymerase α_2 from HeLa cells.

DNA polymerase α_2 (pol α_2) was purified from the combined nuclear extract and postmicrosomal supernatant solution of HeLa cells by a modification of a published procedure (32). All operations were carried out at 4 $^\circ$ C. The combined nuclear extract-postmicrosomal supernatant, usually

obtained from 25 to 50 g of cells, was dialyzed overnight against 4 liters of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA- Na_3 , 1 mM dithiothreitol (DTT) (TDE) buffer and 10% glycerol. The dialyzed extract was clarified by centrifugation at 10,000 x g for 20 minutes and the supernatant solution was loaded at a flow-rate of 1 ml/5 min onto a DEAE (DE52)-cellulose column (2.5 X 10 cm) equilibrated with TDE-buffer. After washing the column with 10 column volumes of TDE buffer - 10% glycerol, the DEAE-cellulose column was coupled to 3 columns arranged in a series in descending order; native DNA-cellulose (1 X 10 cm), denatured DNA-cellulose (1 X 10 cm) and DEAE-BioGel (1 X 10 cm). The matrices had all been equilibrated with TDE-buffer - 10% glycerol. The DEAE-cellulose column was eluted at a flow rate of 1 ml/5 min with 10 column volumes of TDE-buffer containing 10% glycerol. The 0.15 M KCl eluate from the DEAE-cellulose column passed successively through the native and denatured DNA-cellulose and DEAE-BioGel columns. The 0.15 M KCl eluate from the DEAE-BioGel column contains the pol α_2 activity (31,32). This is a M_r 640,000 multiprotein form of polymerase α with associated primase, 3'-->5' exonuclease and Ap4A binding activity, as well as the primer recognition proteins C1, C2 (31,32,36,37). The column fractions containing pol α_2 activity were pooled; dialyzed against 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.15 M KCl, 10% glycerol; concentrated by dialysis against solid sucrose and stored at -80°C.

Analysis of DNA and RNA products synthesized by DNA primase and the DNA primase - polymerase α complex.

DNA primase was incubated in a 100 μ l total volume containing 50 mM Tris-HCl, pH 8, 10 mM magnesium acetate, 10 mM sodium acetate, 500 μ g/ml heat inactivated bovine serum albumin, 15% (v/v) glycerol, 1 mM dithiothreitol, 50 μ g/ml poly(dT) and 2 mM [α - 32 P]ATP[100 μ Ci/assay] for 30 minutes at 30°C. The reactions were terminated by addition of EDTA and SDS to 10 μ M and 0.5% concentrations, respectively, followed by incubation at 35°C for 30 minutes. The product was phenol extracted and precipitated with ethanol. The precipitate was redissolved in 10 mM Tris-HCl, pH 8, 1 mM EDTA and loaded on a 6 ml column of BioGel P-60 in 10 mM Tris-HCl, pH 8, 1 mM EDTA and 20 mM NaCl buffer. The product in the flow-through was concentrated by precipitation with ethanol and divided into three aliquots. One aliquot was digested with T4 polymerase-3',5' exonuclease according to Yamaguchi *et al.* (23). Another aliquot was incubated with 0.35 M NaOH for 16 hours at 35°C. All the products were refractionated by electrophoresis in 22% polyacrylamide gels (0.5 mm x 33 cm x 42 cm) containing 8M urea, 100 mM

Tris-borate (pH 8.3) and 1 mM EDTA. Electrophoresis was carried out at 1500 V for 5 hours using bromophenol blue and xylene cyanol as tracking dyes. Gels were then exposed to Kodak XAR-5 film with a Cronex-plus intensifying screen at -70°C .

Polyacrylamide gel electrophoretic analysis of proteins.

Polyacrylamide gel electrophoretic analysis of proteins under denaturing conditions was performed as previously described (21,32) using 3% stacking gel and 10% polyacrylamide slab gels. The protein staining bands were developed by the silver staining technique of Merril *et al.*, (38).

Other methods.

Unlabeled NTPs were dissolved in 1 ml of 0.5 M ammonium acetate, pH 9.0, and separately chromatographed on a 0.9 X 10 cm column of Affigel-601 in 0.1 M ammonium acetate, pH 8.9. Contaminating dNTPs do not bind and appear in the column flow through. After washing with 10 column volumes of 0.1 M ammonium acetate, pH 8.9, the column was eluted with 0.1 M acetic acid, the eluted NTPs lyophilized and dissolved in glass distilled water. Protein was determined by the procedure of Bradford (39) using crystallized bovine serum albumin as standards or for the purified chromatographic fractions by the absorbance at 280 m μ .

RESULTS

Cofractionation of DNA Primase with DNA Polymerase α_2 .

In a previous report (32) we showed that over 70% of the DNA polymerase α activity and all of the DNA primase activity is eluted by approximately 0.15 M KCl during chromatography of the combined nuclear extract-postmicrosomal supernatant solution from HeLa cells on coupled columns of a DEAE (DE52)-cellulose, native and denatured DNA-cellulose and DEAE-BioGel. This chromatographic fraction of HeLa cell DNA polymerase α was previously designated polymerase α_2 and was shown to contain a Mr 640,000 multiprotein form of the enzyme (31,32).

The DNA primase and DNA polymerase α activities cofractionate during hydrophobic affinity chromatography of pol α_2 on butylagarose (32) and are co-eluted by 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA buffer. It was shown previously that this chromatographic step separates the exonuclease and Ap4A binding activities from the complex and these appear in the 0.8 M $(\text{NH}_4)_2\text{SO}_4$ -TDE buffer column wash and TDE-buffer-1% Triton X-100 column eluate, respectively (32). The DNA primase-polymerase α activities continued to cofractionate during subsequent chromatographic steps

Fig. 1. Flow diagram of the procedure used for the separation of DNA primase and DNA polymerase α activities.

on hexylagarose or phenylSepharose and phosphocellulose. The accessory primer recognition proteins C1, C2, however, are resolved from the multiprotein complex during these chromatographic steps (32).

Dissociation and Purification of Free Primase Activity from the DNA Primase-Polymerase α Complex.

DNA primase could not be resolved from the polymerase α activity using procedures we have previously employed for dissociation of the pol α_2 complex from HeLa cells (31,32). In agreement with the results of Gronastajski *et al.* (21) for DNA primase-polymerase α complex from HeLa cells, we were also unable to resolve the DNA primase-polymerase α activity by glycerol gradient centrifugation in the presence of 2.8 M to 3.5 M urea. We find that both the DNA primase and polymerase α activity from HeLa cells are inactivated under these conditions. We have, however, been able to dissociate the primase and DNA polymerase α activities using the procedure outlined in Figure 1.

The DEAE-BioGel 0.15 M KCl eluate (Fraction II in Table I) from the coupled column procedure (32) for purification of pol α_2 from the

TABLE I. Resolution of free DNA primase and its purification

Fraction	Protein (mg)	DNA Primase			Polymerase α		
		Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
I Crude Extract	946	7195	7.61	100	10462	11.1	100
II DNA Polymerase α_2	285	6238	21.9	87	5751	20.0	55
III Phenyl-Sepharose (TDE/0.5% Triton X-100)	114	5637	49.4	78	1949.2	17.1	34
IV Hexylagarose							
A. Flow thru	2.2	1442	654.0	20	-	-	-
B. TDE/0.05M KCl	3.2	2550	800.0	35	-	-	-
C. TDE/0.1% TDC	13.9	124	9.0	1.7	891.0	64.0	8.5
V Phosphocellulose	0.88	1108	1259.0	15.4	-	-	-
VI Blue Agarose	0.50	731	1461.0	10.1	-	-	-
VII Glycerol gradient	0.16	311	1970.0	4.3	-	-	-

The purification was from 25 g wet weight of HeLa cells. The crude extract represents the combined 0.15M KCl nuclear extract plus the postmicrosomal supernatant solution obtained as described in Materials and Methods. DNA primase activity was assayed by the coupled assay procedure with *E. coli* DNA polymerase I (Klenow fragment) and DNA polymerase α with activated DNA by the procedures described in Materials and Methods.

combined nuclear extract postmicrosomal supernatant from HeLa cells (Fraction I in Table I) served as the starting fraction for the resolution and purification of primase activity. As shown previously (32) all of the primase activity in the crude extract from HeLa cells cofractionates with the pol α_2 complex. Hydrophobic affinity chromatography of pol α_2 on a phenylSepharose column equilibrated with TDE-buffer, 0.8 M $(\text{NH}_4)_2\text{SO}_4$ separated about 60% of the protein in this fraction that appeared in the TDE-buffer, 0.8 M $(\text{NH}_4)_2\text{SO}_4$ column wash (Fig. 2A). Following elution of the column by a discontinuous gradient containing 10 column volumes each of TDE-buffer and TDE-buffer containing 0.5% or 1.5% Triton X-100, the DNA primase and polymerase α activities were coeluted by 0.5% Triton X-100 (Fraction III, Table I). The recovery of primase and DNA polymerase α activity were 90% and 34%, respectively. This fraction was dialyzed overnight against TDE-buffer, 0.8 M $(\text{NH}_4)_2\text{SO}_4$ and subjected to a second hydrophobic chromatographic step on a hexylagarose column that was equilibrated with TDE-buffer, 0.8 M $(\text{NH}_4)_2\text{SO}_4$. After washing with 4 column volumes of equilibration buffer the column was eluted by 8 volumes of TDE-buffer, 0.05 M KCl. As shown in Fig. 2B and Table I about 26% of the primase activity loaded onto the column was recovered in the hexylagarose column flow-through and 45% was eluted by TDE-buffer, 0.05 M KCl. There was no detectable DNA polymerase α activity in either of these chromatographic fractions. DNA primase and DNA polymerase α activities were

Fig. 2. Dissociation of free DNA primase from the DNA primase-polymerase α complex. **A** HeLa cell pol α_2 (Fraction II, Table I) was dialysed against TDE-buffer/ $0.8\text{M}(\text{NH}_4)_2\text{SO}_4$ and loaded onto a 12 ml column of phenylSepharose that was equilibrated with the dialysis buffer. The column was washed with four column volumes of dialysis buffer and then eluted with eight column volumes each of TDE/ 0.05M KCl, TDE/ 0.5% Triton X-100 and TDE/ 1.5% Triton X-100. Four ml fractions were collected at a flow rate of 1 ml/5 min. Aliquots of the fractions were assayed for DNA pol α (o---o) and primase (●---●) as described in Materials and Methods. **B** Fractions from phenylSepharose containing pol α and primase activities were pooled (Fraction III), dialysed against TDE-buffer/ $0.8\text{M}(\text{NH}_4)_2\text{SO}_4$ and loaded onto a 12 ml column of hexylagarose equilibrated with the dialysis buffer. The column was washed with four column volumes of dialysis buffer, and eluted with eight

column volumes each of TDE/0.05 M KCl, TDE/1.5% Triton X-100 and TDE/0.1% taurodeoxycholate. Aliquots of the column fractions were assayed for DNA pol α and primase activities as described in Materials and Methods. The eluates (Fractions IVa and IVb) containing DNA primase activity were pooled and subjected to chromatography on phosphocellulose and Remazol blue agarose (Fractions V and VI respectively). Fraction VI was precipitated with 80% saturation of $(\text{NH}_4)_2\text{SO}_4$, the precipitate resuspended in 20 mM K_2PO_4 buffer, pH 7.6/1mM DTT and loaded onto a 10-30% glycerol gradient containing 50 mM K_2PO_4 , pH 7.6/200 mM $(\text{NH}_4)_2\text{SO}_4$ /1 mM DTT/1 mM EDTA and centrifuged in a Spinco SW 41 rotor for 39 hours at 180,000 x g. Fractions (15 drops) were collected from the bottom of the tube and aliquots assayed for primase activity as described in Materials and Methods.

not recovered by further elution of the column by increasing Triton X-100 concentrations up to 1.5% in TDE buffer. However, about 46% of the polymerase α and 2% of the DNA primase activity that were applied to the column were co-eluted by 10 column volumes of TDE-buffer containing 0.1% TDC (Table I and Fig. 2B). The DNA polymerase and primase activity were not inactivated by 0.1% taurodeoxycholate and the activities were fully recovered following dialysis against TD-buffer, 0.15 M KCl (data not shown).

The fractions in the column flow-through and TDE-buffer, 0.05 M KCl eluate from hexylagarose (Table I, Fractions IV A-B) containing DNA primase activity were pooled and dialyzed overnight against 4 liters of 0.02 M KH_2PO_4 - K_2HPO_4 (K_2PO_4) pH 6.8, 1 mM DTT, 1 mM EDTA and 10% glycerol. The dialyzed fraction was loaded onto a phosphocellulose column (1 X 2 cm) that was equilibrated with the dialysis buffer. After washing with 10 column volumes of the equilibration buffer the column was eluted by a discontinuous gradient of increasing K_2PO_4 concentration from 0.02 M to 0.5 M K_2PO_4 and increasing pH from 6.8 to 7.5 in buffers containing 1 mM EDTA, 1 mM DTT and 10% glycerol. DNA primase activity was eluted by 10 column volumes of 0.25 M K_2PO_4 , pH 7.2, 1 mM EDTA, 1 mM DTT and 10% glycerol (Table I, Fraction V). The fractions containing primase were pooled, dialyzed overnight against 4 liters of TDE buffer containing 10% glycerol and loaded onto a 1 X 2 cm column of Remazol blue agarose that had been equilibrated with the dialysis buffer. After washing with 10 column volumes of the equilibration buffer the column was eluted by a discontinuous gradient of increasing KCl concentration from 0 to 2 M in TDE buffer containing 10% glycerol. DNA primase activity eluted at 1 M KCl (Table I, Fraction VI). These active fractions were pooled, precipitated with 80% saturation of ammonium sulfate, centrifuged and the precipitate was resuspended in 20 mM K_2PO_4 buffer, pH 7.6, containing 1 mM DTT by the procedure of Kaguni *et al.* (18). The suspended fraction was loaded onto a 10 to 30% glycerol

Fig. 3. SDS polyacrylamide gel electrophoretic analysis of the purified DNA primase. 4 μ g of the glycerol gradient fraction of primase (Fraction VII, Table I) was analyzed by SDS polyacrylamide slab gel electrophoresis using a 3% stacking gel and 10% separating gel as described (31). Protein bands were visualized by silver staining. Lane A, the purified primase fraction; and Lane B, molecular weight markers which were: β galactosidase (130 kDa), phosphorylase B (94 kDa), bovine serum albumin (68 kDa) and ovalbumin (43 kDa).

gradient containing 50 mM K_2PO_4 , pH 7.6, 200 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM DTT and 1 mM EDTA prepared in polyallomer tubes for the SW41 (Spinco) rotor. After centrifugation for 39 hrs at $180,000 \times g$ fractions (15 drop) were collected by puncturing the bottom of the tube and assayed. DNA primase activity sedimented as a single peak with an $S_{20,W}$ of 5 (Fig. 2C). The gradient fractions containing DNA primase activity (Table I, Fraction VII) were pooled, dialyzed against TDE buffer containing 50% glycerol and stored in aliquots at -80°C .

At this stage in the purification, DNA primase activity has been purified about 260 fold with 4% recovery of activity (Table I). There is no

Table 2. Requirements for DNA primase activity.

Addition and Template	dNMP incorporated (pmol/30 min)
Complete: M13 ssDNA	9.4
minus: template	<0.1
enzyme	<0.1
Mg ²⁺	0.1
Mg ²⁺ plus Mn ²⁺ (0.2 mM)	1.0
plus: ATP, GTP only	9.1
GTP only	5.5
ATP only	6.4
Complete: poly(dT)	7.2
minus: ATP	<0.1
minus ATP plus AMP-PCP	<0.1
minus ATP plus ADP	<0.1
plus: 0.5 mM ATP	1.1
1 mM	2.6
2 mM	6.5
4 mM	7.1
Complete: poly(dC)	31.7
minus: GTP	<0.1
plus: 0.1 mM GTP	5.3
0.2 mM	13.2
0.5 mM	33.1
1.0 mM	39.0

The reaction was carried out as described in Materials and Methods for the coupled DNA primase polymerase α assay with the omission and addition of components as indicated and using 0.5 units of the DNA primase-polymerase α preparation (Fraction III). The reaction was measured by the amount of labeled dNMP incorporated per 30 min which for assays with M13 DNA was [³H]dTTP (600 opm/pmol), for poly(dT) it was [³H]dATP (500 opm/pmol) and for poly(dC) it was [³H]dGTP (220 opm/pmol).

significant DNA polymerase α activity with activated or denatured DNA templates, DNase or DNA-dependent ATPase activities in the purified DNA primase fraction. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of the purified DNA primase showed it to be over 90% pure with the major silver staining band of M_r 70,000 and minor bands of higher and lower molecular weight material (Fig. 3). As we previously reported, a M_r 70,000 protein band is present in SDS-polyacrylamide gel electrophoretic analysis of pol α_2 that was purified to homogeneity (32). The M_r 70,000 protein band is also present in SDS-polyacrylamide gel electrophoretic analysis of fractions at each step during the purification of DNA primase activity that is resolved from DNA polymerase α_2 (data not shown). This polypeptide

Fig. 4. The effects of inhibitors on DNA primase and DNA polymerase α activities. Upper Left Panel. The effect of neutralizing monoclonal SJK-132-20 antibody produced to human DNA polymerase α . Aliquots of the DNA primase-polymerase α complex (Fraction III in Table I) containing 0.08 unit primase and 0.5 unit of polymerase α respectively were preincubated with the designated amounts of the purified IgG protein in the presence of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 5 mg/ml bovine serum albumin at 4°C for 1 hour. Following the preincubation the samples were assayed for DNA polymerase α ●-● activity with activated DNA and DNA primase ▲-▲ activity with poly(dT) as template by the procedures described in Materials and Methods.

Upper Right Panel. Effect of BuPdGTP on free DNA primase and DNA polymerase α activities. Aliquots of the glycerol gradient fraction (Fraction VII in Table I) containing 0.08 unit of free primase and the purified DNA polymerase α catalytic subunit (32) containing 0.5 unit of polymerase α were incubated with the designated amounts of BuPdGTP in the assay for DNA primase activity ▲-▲ with poly(dT) template and polymerase α activity ●-● with poly(dT)-oligo(A)₁₀₋₂₀ template-primer by the procedures described in Materials and Methods.

Lower Left Panel. Effect of aphidicolin on DNA primase and DNA polymerase α activities. Aliquots (0.5 unit) of the purified DNA polymerase α catalytic subunit (32) were incubated with the designated amounts of aphidicolin in the polymerase α assay with activated DNA. DNA primase-polymerase α complex (0.4 unit of primase, Fraction III in Table I) and purified free primase (0.08 unit of Fraction VII in Table I) were incubated with the designated amounts of aphidicolin in the assays with single-stranded, circular M13 DNA as template. Primase activity of the DNA primase-polymerase α complex was assayed by the coupled assay with polymerase α and free primase was assayed by the coupled assay with *E. coli* polymerase I

(Klenow fragment) as described in Materials and Methods.

The 100% values represent for: DNA polymerase α - 544 pmoles of dNMP incorporated; free DNA primase - 77 pmoles NMP incorporated; and for primase of the DNA primase-polymerase α complex - 388 pmoles of dNMP incorporated.

Lower Right Panel. Effect of KCl concentration on DNA primase activity. Aliquots (0.08 unit) of the purified, free DNA primase (Fraction VII) or 0.5 units of polymerase α were assayed in the presence of the designated concentrations of KCl with poly(dT) as template described in Materials and Methods.

The 100% values in all cases represent: for DNA polymerase α - 550 pmoles dNMP incorporated; free primase - 77 pmoles NMP incorporated; and for primase of the DNA primase-polymerase α complex - 390 pmoles dNMP incorporated.

does not correspond to any of the polypeptides responsible for catalytic or modulating activities that have previously been resolved from the DNA polymerase α_2 complex (31,32,36).

Requirements for DNA Primase Activity.

The DNA primase activity that we have purified has similar properties as primase isolated by others from a variety of eukaryotic cells (14,26). The enzyme will synthesize oligoribonucleotide primers on natural single-stranded DNA templates and on homopolydeoxynucleotide polymers such as poly(dT) and poly(dC) (Table II). In agreement with the observations by others (17,21,23), the purified primase has highest catalytic activity with poly(dC) as a template. There is a requirement for Mg^{2+} in the reaction although Mn^{2+} is about 1/10th as effective at 0.2 mM concentration. In the reaction with natural single-stranded DNA templates about 50% of the activity is retained in the presence of only ATP or GTP. The ATP concentration required for half-maximal synthesis is high, and with poly(dT) as template the K_m for ATP is about 1 mM. This value is approximately the same for free primase and for the DNA primase-polymerase α complex. RNA primer synthesis does not occur in the presence of ATP analogs such as AMP-PCP and ATP γ S (Table II and data not shown). In agreement with the results reported by Gronostajski *et al.* (21) the concentration of GTP required for half-maximal rate of synthesis for the HeLa cell DNA primase is lower than the ATP requirement. With poly(dC) as the template the K_m for GTP is 0.1 mM (Table II).

Influence of Inhibitors on DNA Primase Activity.

The purified DNA primase that is resolved from HeLa cell DNA polymerase α is not inhibited by murine monoclonal antibody SJK 132-20 that was produced against human DNA polymerase α (19). In contrast, the activity of HeLa cell DNA polymerase α catalytic subunit, however, is inhibited over 90% in the presence of 0.6 μ g of the antibody protein (Fig. 4). The purified primase

activity is also not inhibited by neutralizing polyclonal antibody produced against the purified Mr 183,000 catalytic subunit of HeLa cell DNA polymerase (32) or by neutralizing monoclonal antibody directed against chicken oviduct RNA polymerase II that cross-reacts with HeLa cell RNA polymerase II (40, data not shown). Further evidence that the DNA primase and polymerase α catalytic sites are distinct was obtained by the use of specific inhibitors of DNA polymerase α activity and DNA synthesis. BuPdGTP (41) is a specific, potent inhibitor of DNA polymerase α activity. Likewise, aphidicolin has been shown to specifically inhibit DNA synthesis and DNA polymerase α activity in eukaryotic cells (42). As shown in Figure 4, the purified free primase activity was resistant to BuPdGTP, whereas, the activity of the free polymerase α catalytic subunit was inhibited about 50% at 100 nM concentration of this analogue. The activity of the free polymerase α catalytic subunit or the polymerase α -primase complex measured by the coupled assay procedure were inhibited over 90% in the presence of 100 μ g/ml of aphidicolin. However, the purified free primase activity measured by a coupled assay with *E. coli* pol I (Klenow fragment) was relatively resistant to aphidicolin.

The purified, free DNA primase activity is much more sensitive to salt concentrations and is more labile than the free DNA polymerase α catalytic subunit. The primase activity is inhibited more than 90% in the presence of 50 mM KCl whereas the DNA polymerase α activity requires about 100 mM KCl concentration for this amount of inhibition (Fig. 4). The purified free DNA primase activity is more labile to storage at 4°C or 80°C than the DNA polymerase α catalytic subunit under the same conditions (i.e. in 50 mM Tris, pH 7.5, 1 mM DTT, 0.15 M KCl and 10% glycerol). This is similar to what has been observed for free primase from yeast (24, 26). More than 50% of the primase activity is lost in 12 hrs at 4°C and in 24 hrs when stored at -80°C. The free DNA polymerase α activity is stable at 4°C for over a week and for at least 6 months with storage at -80°C under these conditions. We do find, however, that both the DNA primase and

Fig. 5 Polyacrylamide gel electrophoresis of RNA primers. HeLa cell DNA primase (lanes A-C) or DNA primase-polymerase α (lanes D and E) was incubated with poly(dT) and [α -³²P]ATP and the product was isolated. Aliquots of the product were either left untreated (lanes A and D), digested with T₄ DNA polymerase-3',5' exonuclease (lanes B and E) or digested with 0.35 M NaOH (lane C) and the products were fractionated by electrophoresis in a 22% polyacrylamide gel containing 8M urea as described under Materials and Methods. The values on the vertical axis indicate the length in nucleotides of the markers run in parallel on the same gel.

polymerase α activity in the DNA polymerase α_2 complex are stable at 40°C for at least 6 weeks when stored under these same conditions (32).

Analysis of RNA Primers Formed in Primase Reactions.

The DNA primase-polymerase α complex from a variety of eukaryotic cells was shown to synthesize in vitro RNA primers of 10 to 15 nucleotides in length on single-stranded DNA templates (14,17,19,20,23,24). In this study we analyzed the RNA product synthesized in vitro by the purified DNA primase-polymerase α complex and free DNA primase on poly(dT) template. The purified DNA primase-polymerase α complex and free DNA primase that was resolved from it both synthesized RNA primers of 10 to 12 nucleotides in length and multiples, thereof, on poly(dT) template in the presence of [α -³²P]ATP (Fig. 5 lanes A and D). Digestion with T4 DNA polymerase associated 3'→5' exonuclease did not decrease the length of the RNA primers indicating that the incorporation is into RNA (Fig. 5 lanes B and E). This is also supported by the fact that the product is sensitive to alkaline hydrolysis (Fig. 5 lane C). Synthesis of multiples of RNA primer units of 10 to 12 nucleotides on poly(dT) templates has also been observed with purified yeast DNA primase-polymerase I complex (25) and DNA primase-polymerase α complex from Drosophila embryos (14). The RNA primers synthesized by the purified DNA primase-polymerase α and free DNA primase on single-stranded ϕ X 174 DNA template were also 10 to 12 nucleotides in length (data not shown). Thus, it appears that the association of primase in a complex with DNA polymerase α does not affect the length of RNA primers synthesized in vitro on single-stranded DNA templates.

DISCUSSION

DNA primase activity has now been shown to be in tight association with major or minor species of DNA polymerase α , or the alpha-like polymerase from a wide range of phylogenetically unrelated eukaryotic cells (14-26). It has not been possible in most instances to separate the DNA primase from the polymerase α activity of the complex; the notable exceptions being, in one case, the reported separation of DNA primase from the complex purified from mouse myeloma cells (10), and the separation of primase from yeast DNA polymerase I (24,26,43). The separation of primase from DNA polymerase α of the complex purified from Drosophila embryo extracts required glycerol gradient centrifugation in the presence of urea (29). Also, the separation of DNA primase from a DNA polymerase α complex in mouse (44) and HeLa cells (45) required the use of high concentrations of ethylene glycol in the

elution buffers during DEAE-cellulose and immunoaffinity chromatography, respectively. These conditions are known to disrupt hydrophobic associations between proteins. In this report we describe a procedure for the resolution of DNA primase from a multiprotein complex of DNA polymerase α from human cells that results in a reasonable yield of DNA primase that is free from polymerase α activity. This has enabled us to characterize for the first time the properties of free DNA primase from human cells.

The primase activity of the DNA primase-polymerase α complex could not be separated from polymerase α by our usual procedure for dissociating the activities associated with pol α_2 (31,32). We did find, however, that if pol α_2 was first chromatographed on a phenylSepharose column, then this permitted the separation of DNA primase from polymerase α activity at a subsequent chromatographic step on hexylagarose. We cannot explain at this time why chromatography on phenylSepharose does not separate the polymerase α -primase activities that are separated at the next step in purification on hexylagarose. However, about 60% of the protein in the pol α_2 fraction appears in the flow-through fraction during chromatography on phenylSepharose while the primase-polymerase α activities bind to this matrix. It is possible that protein(s) removed during this chromatographic step and during the subsequent chromatography on hexylagarose may stabilize the interaction between the DNA primase and polymerase α .

The purified free DNA primase activity is much more labile than when complexed with DNA polymerase α . This is in agreement with the findings for yeast DNA primase (43). We find that the purified free DNA primase is much more labile at 4°C or -80°C than when complexed with polymerase α and is more sensitive to salt concentrations. The labile nature of the free DNA primase presented problems to its purification and characterization and probably contributes an underestimation of the actual purification of the enzyme activity. In general, the catalytic properties of the free primase are similar to the enzyme when complexed with DNA polymerase α .

The predominant protein band in SDS-polyacrylamide gel electrophoretic analysis of our purified, free primase is 70 kDa. The 70 kDa protein copurifies during the course of purification of the free primase activity including glycerol gradient centrifugation in which free primase activity has an $S_{20,w}$ value of 5. This is similar to an $S_{20,w}$ value of 5.8 that has been reported for free primase activity from other cell systems (44-46). Due to the labile nature of free primase activity, we have been unable to recover activity following non-denaturing gel electrophoresis in order to

perform gel transfer analysis. Thus, the subunit structure of the purified free primase could not be defined in this study.

In summary, the outcome of this study shows for the first time the separation of DNA primase from the major DNA polymerase α activity in human cells and characterization of its properties. Our results support the published evidence that the primase and polymerase α activities reside on separate polypeptides (9,17,43). Moreover, the results show that DNA primase and polymerase α are in tight association by hydrophobic interactions. It provided a simple, mild procedure to resolve free DNA primase from the DNA primase-polymerase α complex with a reasonable yield. Studies are now in progress to define the subunit structure of the purified, free DNA primase of HeLa cells.

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REFERENCES

1. Kornberg, A. (1980) DNA Replication, W.H. Freeman and Company, San Francisco.
2. DePamphilis, M.L. and Wassarman, P.M. (1980) Ann. Rev. Biochem., 49, 627-666.
3. Eliasson, R. and Reichard, P. (1978) Nature, 272, 184-185.
4. Benz, E.W., Reinberg, D., Vicuna, R. and Hurwitz, J. (1980) J. Biol. Chem. 255, 1096-1106.
5. Rowen, L. and Kornberg, A. (1978) J. Biol. Chem. 253, 758-764.
6. Romano, L.J. and Richardson, C.C. (1979) J. Biol. Chem. 254, 10476-10482.
7. Liu, C.C. and Alberts, B.M. (1981) J. Biol. Chem. 256, 2813-2820.
8. Nossal, N.G. (1980) J. Biol. Chem. 255, 2176-2182.
9. Kaufmann, G. and Falk, H.H. (1982) Nucleic Acids Res. 10, 2309-2321.
10. Tseng, B.Y. and Ahlem, C.N. (1982) J. Biol. Chem. 257, 7280-7283.
11. Yoda, K. and Okazaki, T. (1983) Nucleic acids Res. 11, 3433-3450.
12. Mechali, M., and Harland, R.M. (1982) Cell 30, 93-101.
13. Rieder, H.D., Konig, H., Stahl, H. and Knippers, R. (1982) Nucleic Acids Res. 10, 5621-5635.
14. Conaway, R.C. and Lehman, I.R. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2523-2527.

15. Yagura, T., Kozu, T. and Seno, T. (1982) *J. Biochem. (Tokyo)* 91, 607-618.
16. Shioda, M., Nelson, E.M., Bayne, M.L. and Benbow, R.M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 7209-7213.
17. Tseng, B.Y. and Ahlem, C.N. (1983) *J. Biol. Chem.* 258, 9845-9849.
18. Hubscher, U. (1983) *EMBO J.* 2, 133-136.
19. Wang, T.S-F., Hu, S-Z. and Korn, D. (1984) *J. Biol. Chem.* 259, 1854-1865.
20. Chang, L.M.S., Rafter, E., Augl, C. and Bollum, F.J. (1984) *J. Biol. Chem.* 259, 14679-14687.
21. Gronostajski, R.M., Field, J. and Hurwitz, J. (1984) *J. Biol. Chem.* 259, 9479-9486.
22. Grosse, F. and Krauss, G. (1985) *J. Biol. Chem.* 260, 1881-1888.
23. Yamaguchi, M., Hendrickson, E.A. and DePamphilis, M.L. (1985) *J. Biol. Chem.* 260, 6254-6263.
24. Plevani, P., Badaracco, G., Augl, C. and Chang, L.M.S. (1984) *J. Biol. Chem.* 259, 7532-7539.
25. Singh, H. and Dumas, L.B. (1984) *J. Biol. Chem.* 259, 7936-7940.
26. Wilson, F. and Sugino, A. (1985) *J. Biol. Chem.* 260, 8173-8181.
27. Wong, T.W. and Clayton, D.A. (1985) *J. Biol. Chem.* 260, 11530-11535.
28. Ledwith, B.J., Manam, S. and Van Tuyle, G.C. (1986) *J. Biol. Chem.* 261, 6571-6577.
29. Kaguni, L.S., Rossignol, J-M., Conaway, R.C. and Lehman, I.R. (1983) *Proc. Nat. Acad. Sci. U.S.A.* 80, 2221-2225.
30. Ashani, Y. and Catravas, G.N. (1980) *Anal. Biochem.* 109, 55-62.
31. Lamothe, P., Baril, B., Chi, A., Lee, L. and Baril, E. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 4723-4727.
32. Vishwanatha, J.K., Coughlin, S.A., Wesolowski-Owen, M. and Baril, E. (1986) *J. Biol. Chem.* 261, 6619-6628.
33. Baril, E., Mitchener, J., Lee, L. and Baril, B. (1977) *Nucleic Acids Res.* 4, 2641-2653.
34. Chiu, R.W. and Baril, E.F. (1975) *J. Biol. Chem.* 250, 7951-7957.
35. Novak, B., and Baril, E.F. (1978) *Nucleic Acids Res.* 5, 221-239.
36. Skarnes, W., Bonin, P. and Baril, E. (1986) *J. Biol. Chem.* 261, 6629-6636.
37. Pritchard, C.G., Weaver, D.T., Baril, E.F. and De Pamphilis, M.L. (1983) *J. Biol. Chem.* 258, 9810-9819.
38. Merrill, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) *Science* 211, 1437-1438.
39. Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.
40. Tsai, S.Y., Dicker, P., Fang, P., Tsai, M.-J. and O'Malley, B.W. (1984) *J. Biol. Chem.* 259, 11587-11593.
41. Khan, N., Wright, G., Dudycz, L. and Brown, N. (1984) *Nucleic Acids Res.* 12, 3695-3706.
42. Ohashi, M., Taguchi, T. and Ikegami, S. (1978) *Biochem. Biophys. Res. Commun.* 82, 1084-1090.
43. Plevani, P., Fiolani, M., Valsasnini, P., Badaracco, G., Cheriathundam, E. and Chang, L.M.S. (1985) *J. Biol. Chem.*, 260, 7102-7107.
44. Suzuki, M., Enomoto, T., Hanaoka, F. and Yamada, M. (1985) *J. Biochem. (Tokyo)* 98, 581-584.
45. Murakami, Y., Wobbe, C.R., Weissbach, L., Dean, F.B. and Hurwitz, J. (1986) *Proc. Natl. Acad. Sci. USA.* 83, 2869-2873.
46. Masaki, S., Tanabe, K. and Yoshida, S. (1984) *Nucleic Acids. Res.* 12, 4455-4467.