Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3993–3997, July 1984
Biochemistry

Mammalian DNA polymerase α holoenzymes with possible functions at the leading and lagging strand of the replication fork

(DEAE-cellulose chromatography/primase/DNA-dependent ATPase/exonuclease/DNA topoisomerase)

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Communicated by Arthur Kornberg, March 12, 1984

ABSTRACT **At an early purification stage, DNA polymerase α holoenzyme from calf thymus can be separated into four different forms by chromatography on DEAE-cellulose. All four enzyme forms (termed A, B, C, and D) are capable of replicating long single-stranded DNA templates, such as parvoviral DNA or primed M13 DNA. Peak A possesses, in addition to the DNA polymerase α, a double-stranded DNA-dependent ATPase, as well as DNA topoisomerase type II, 3′–5′ exonuclease, and RNase H activity. Peaks B, C, and D all contain, together with DNA polymerase α, activities of primase and DNA topoisomerase type II. Furthermore, peak B is enriched in an RNase H, and peaks C and D are enriched in a 3′–5′ exonuclease. DNA methylase (DNA methyltransferase) was preferentially identified in peaks C and D. Velocity sedimentation analyses of the four peaks gave evidence of unexpectedly large forms of DNA polymerase α (>11.3 *s*), indicating that copurification of the above putative replication enzymes is not fortuitous. With moderate and high concentrations of salt, enzyme activities cosedimented with DNA polymerase α. Peak C is more resistant to inhibition by salt and spermidine than the other three enzyme forms. These results suggest the existence of a leading strand replicase (peak A) and several lagging strand replicase forms (peaks B, C, and D). Finally, the salt-resistant C form might represent a functional DNA polymerase α holoenzyme, possibly fitting in a higher-order structure, such as the replisome or even the chromatin.**

DNA replication requires the concerted action of many proteins and enzymes either separately or in a complex (1, 2). These include, for instance, DNA polymerases, DNA polymerase accessory proteins, primase, topoisomerase, helicase, DNA-binding proteins, RNase, and ligase (1, 2). Studies on prokaryotic *in vitro* DNA replication using small bacteriophages or plasmids containing the *oriC* sequence as model replicons, show that >20 polypeptides have to operate coordinately (1–3). *In vitro* adenovirus DNA replication, representing the best studied eukaryotic DNA replication model system so far, again attests to the complex interaction of many proteins (e.g., see ref. 4). The term replisome has been introduced to denote the hypothetical structure working at the prokaryotic replication fork (2). It may consist of a conserved primosome (5), DNA polymerase III holoenzyme (6), helicase, as well as topoisomerase.

Escherichia coli DNA polymerase III holoenzyme is essentially responsible for prokaryotic chromosomal replication (6). Detailed knowledge of this functional multipolypeptide complex has accumulated in the last few years (for review, see ref. 7). In analogy to the prokaryotic DNA polymerase III holoenzyme, we recently described a similar form in eukaryotes (8, 9). Using defined *in vivo*-like templates, we isolated a complex form of DNA polymerase α from freshly harvested calf thymus (8, 9). This enzyme, termed the holoenzyme, is functionally and physicochemically distinct from a homogeneous DNA polymerase α, termed the core enzyme (8, 10), isolated from the same source.

In the course of these studies, we realized that DNA polymerase α holoenzyme extracted from calf thymus and partially purified by phosphocellulose chromatography reproducibly splits into four different DNA polymerase α forms on chromatography on DEAE-cellulose. We provide evidence that at least six additional enzyme activities, whose involvement in DNA replication must be postulated, copurify with one or the other of these four DNA polymerase forms. The results suggest that the eukaryotic DNA polymerase α holoenzyme can be separated into forms that could function at the leading and at the lagging strand.

MATERIALS AND METHODS

Chemicals. Unlabeled NTPs, dNTPs, and araCTP were purchased from P-L Biochemicals. [^{3}H]dTTP, [8-^{3}H]ATP, [γ-^{32}P]ATP, poly[8-^{3}H]A, and *S*-adenosyl-L-[*methyl*-^{3}H]methionine were from Amersham; pepstatin, novobiocin, and spermidine were from Sigma. Whatman was the supplier of DEAE-cellulose, and aphidicolin was provided by S. Spadari (Pavia, Italy).

Production, Isolation, and Preparation of Nucleic Acids. Single-stranded (ss) DNA from minute virus of mice, a parvovirus, and phage M13 (mp8) were isolated as described (8). Activation and denaturation of calf thymus DNA and hybridization of poly(dA) to poly(dT)$_{12-18}$ were done as described (8). Plasmid pβG forms I and II were gifts from E. Di Capua (Zürich). Hemimethylated *Micrococcus luteus* DNA and supercoiled pAT153 DNA (both prepared according to ref. 11) were provided by S. Spadari (Pavia, Italy). Poly(dT) and poly(dA)·oligo(dT) were purchased from P-L Biochemicals and Boehringer Mannheim, respectively. The hook polymer, poly(dA)$_{100}$-oligo(dT)$_{25}$, was prepared as outlined by Fisher *et al.* (12). Primed M13 DNA was prepared as follows: the pentadecamer oligonucleotide T-C-C-C-A-G-T-C-A-C-G-A-C-G-T (New England Biolabs) was hybridized to M13 ss DNA (mp8) in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/30 mM NaCl, at a ratio of 1:400 (wt/wt). After incubation at 55°C for 5 min, the primed M13 DNA was cooled to room temperature and stored at 4°C until use.

***In Vitro* Assays of DNA Synthesis.** The reaction mixtures (25 μl) included the following components; (*i*) for activated and heat-denatured DNA: 20 mM potassium phosphate, pH 7.2/0.1 mM EDTA/4 mM dithiothreitol/bovine serum albumin (0.25 mg/ml)/10 mM $MgCl_2$/dATP, dGTP, and dCTP each at 48 μM/18 μM [^{3}H]dTTP (100–200 cpm/pmol)/16.5 nmol of activated (or heat-denatured) DNA as nucleotides and DNA polymerase to be assayed; incubation was at 37°C for 10 min; (*ii*) for poly(dA)·oligo(dT)$_{12-18}$ (different base ratios) and hook poly(dA)$_{100}$-oligo(dT)$_{25}$: 20 mM Tris·HCl, pH

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Abbreviations: ss, single stranded; ds, double stranded.

7.5/4% (wt/vol) sucrose/8 mM dithiothreitol/bovine serum albumin (80 μg/ml)/1 mM $MnCl_2$/18 μM [^{3}H]dTTP (100–200 cpm/pmol)/225 pmol of homopolymer (as nucleotides) and enzymes to be assayed; incubation was at 37°C for 60 min; (*iii*) for elongation of parvoviral ss DNA and primed M13 DNA: 20 mM Tris·HCl, pH 7.5/4% (wt/vol) sucrose/8 mM dithiothreitol/bovine serum albumin (80 μg/ml)/10 mM $MgCl_2$/5 mM ATP/10 mM KCl (omitted in M13 assay)/ dATP, dGTP, and dCTP each at 48 μM/18 μM [^{3}H]dTTP (100–200 cpm/pmol)/200 pmol (total nucleotides) of ss parvoviral or primed M13 DNA and enzyme to be assayed; incubation was at 37°C for 1 hr. When double-stranded (ds) pβG form I or II was used for assay, the reaction mixtures contained, in addition to the ingredients of the elongation assays, UTP, GTP, and CTP each at 100 μM. The reactions were stopped, the DNA was precipitated, the precipitate was collected on a Whatman GF/C filter and dried, and the radioactivity was determined as described (13). One unit of activity is defined as the incorporation of 1 nmol of dNTP into acid-insoluble DNA in 60 min at 37°C.

Other Enzyme Assays. (*i*) *Primase.* The reaction was performed as described by Conway and Lehman by using 0.5 unit of DNA polymerase I per assay (14). One unit of primase activity is the amount that catalyzes the incorporation of 1 nmol of dATP (with M13 ss DNA of dNTPs) into acid-insoluble material in 60 min at 37°C.

(*ii*) *DNA-dependent ATPase.* This assay was carried out exactly as described by Shlomai and Kornberg (15) by using denatured calf thymus DNA, φX174 ss DNA or ds supercoiled pAT153 DNA at 6600, 400, and 600 pmol as nucleotides, respectively. One unit of DNA-dependent ATPase is defined as the cleavage of 1 nmol of ATP per 30 min at 37°C.

(*iii*) *3′–5′ exonuclease.* The poly(dA-dT) substrate was labeled at the 3′ terminus with [^{3}H]dTTP, and the 3′–5′ exonuclease activity was determined according to Byrnes *et al.* (16). One unit of 3′–5′ exonuclease was determined as the conversion of 1 nmol of terminally labeled poly(dA-dT) [^{3}H]dTMP into acid-soluble nucleotides in 60 min at 37°C.

(*iv*) *RNase H.* The assay for RNase H and preparation of a RNA·DNA hybrid was as described by Berkower *et al.* (17). One unit of RNase H activity was the amount of enzyme producing 1 nmol of acid-soluble material in 30 min at 37°C. The control for RNase contamination was as described for RNase H except the RNA·DNA hybrid was replaced by 2.5 pmol of [^{3}H]poly(A) (15,000 cpm/pmol).

(*v*) *DNA ligase.* Ligation was measured according to Zimmermann and Levin (18). One unit of ligase activity is the amount that converts 1 pmol of ^{32}P into a phosphatase-resistant form in 30 min at 37°C.

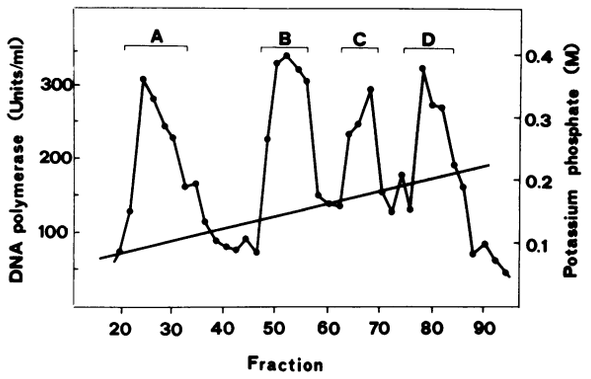

FIG. 1. Resolution of four DNA polymerase forms on DEAE-cellulose chromatography. The DNA polymerase α holoenzyme was purified from 3000 g of freshly harvested calf thymus up to fraction III, exactly as described (8). Fraction III (2400 ml; 408,000 units; eluate from the phosphocellulose column) was precipitated with an equal volume of saturated neutralized ammonium sulfate. The solution was stirred for 30 min and centrifuged at 20,000 × *g* for 30 min. The resulting pellet was dissolved in 100 ml of buffer A [30 mM potassium phosphate, pH 6.8/10 mM 2-mercaptoethanol/10 mM sodium bisulfite/1 μM pepstatin/10% (vol/vol) glycerol/20% (wt/vol) glucose], and was dialyzed against this buffer until the conductivity in the retentate reached that of the pure buffer. The retentate was then adsorbed onto a 350-ml DEAE-cellulose column (9 × 8 cm) equilibrated with buffer A. A 2000-ml linear gradient of 30–250 mM potassium phosphate (pH 6.8) in buffer A was applied. Fractions of 17 ml were collected, the conductivity was measured, and DNA polymerase activity was determined using activated DNA as template. The combined activity of peaks A, B, C, and D recovered from the DEAE-cellulose column calculated relative to the applied activity was 41% (peak A, 11%; peak B, 7%; peak C, 15%; and peak D, 8%). The specific activity of peaks A, B, C, and D increased on this column 8-, 56-, 23-, and 27-fold, respectively. Fractions were pooled as indicated, divided into small aliquots, and shock-frozen in liquid nitrogen. Individual aliquots were thawed only once and used for a single experiment.

(*vi*) *DNA methylase.* The final volume of 100 μl included 50 mM Tris·HCl, pH 7.5/10 mM EDTA/1 mM dithiothreitol/25 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (8000 cpm/pmol)/7 μg of hemimethylated *M. luteus* DNA and enzyme to be assayed; incubation was for 5 hr at 37°C, and the acid-insoluble radioactivity was determined as described (13). One unit of methylase activity is defined as 1 pmol of [^{3}H]methyl group incorporated into acid-insoluble material in 60 min at 37°C.

(*vii*) *DNA topoisomerase.* The final volume of 30 μl contained 10 mM Tris·HCl, pH 7.9/0.2 mM EDTA/0.5 mM di-

Table 1. Characteristics of the four different DNA polymerase forms

Property	DNA polymerase peak A	B	C	D	Purified DNA polymerase α holoenzyme*
DNA polymerase β†	<0.1	<0.1	<0.1	<0.1	<0.1
DNA polymerase γ†	<0.1	<0.1	<0.1	<0.1	<0.1
Terminal deoxynucleotidyltransferase†	<0.1	<0.1	<0.1	<0.1	<0.1
Aphidicolin (80 μg/ml), % inhibition‡	89	93	95	97	98
d_2TTP (d_2TTP/dTTP, 25:1), % inhibition‡§	5	9	12	17	6
N-Ethylmaleimide (0.5 mM), % inhibition‡	96	97	98	95	97
Salt (mOsm) resulting in 50% inhibition‡¶	125	85	190	100	90
Heat (°C) resulting in 50% inhibition‡	49	58	60	56	54
Spermidine (mM) resulting in 50% inhibition‡	1	1.7	2.3	1	1.2

*DNA polymerase α holoenzyme was isolated as described (8, 9).
†DNA polymerase β, -γ, and terminal deoxynucleotidyltransferase are given as the percentage of α activity.
‡DNA polymerase activity was determined using activated DNA and 48 μM dCTP.
§d_2TTP, dideoxythymidine triphosphate.
¶The ionic strength derives from 20 mM potassium phosphate and from variable concentrations of KCl.

Table 2. Template specificity of the four DNA polymerase α forms and purified DNA polymerase α holoenzyme

Template	Nucleotides per assay, pmol	DNA synthesis, pmol*				
		DNA polymerase α peak				Purified DNA polymerase α holoenzyme
		A	B	C	D	
Activated DNA	16,500	207	180	184	214	123
Heat-denatured DNA	16,500	96	84	78	91	112
Poly(dA)·oligo(dT)$_{12-18}$						
Base ratio, 1:1	225	10	11	16	23	24
Base ratio, 3:1	225	6	19	29	33	31
Base ratio, 10:1	225	9	15	32	38	51
Poly(dA)$_{100}$–oligo(dT)$_{25}$ (hook)	225	8	52	122	170	109
ss parvoviral DNA	200	19	46	27	23	47
Singly primed M13 DNA	200	24	32	29	25	67
Supercoiled pβG DNA (form I)	200	<0.5	<0.5	<0.5	<0.5	<0.5
Relaxed pβG DNA (form II)	200	<0.5	<0.5	<0.5	<0.5	<0.5

*DNA synthesis was determined by measuring dNTP incorporation during 60 min, using saturating amounts of enzyme. For each template, saturation was corroborated separately.

thiothreitol/1 mM ATP/10 mM $MgCl_2$/0.4 μg of supercoiled pAT153DNA and enzyme to be assayed; after incubation at 37°C for 30 min, 1% (wt/vol) $NaDodSO_4$, 10% (vol/vol) glycerol, and bromphenol blue (0.25 mg/ml) were added. After 5 min, the samples were loaded onto a 1% agarose gel (16 × 13 cm) containing 0.09 M Tris borate and 20 mM EDTA; electrophoresis was carried out at 30 V for 15 hr; the gel was stained for 15 min in gel buffer containing ethidium bromide (1 μg/ml) and was then photographed. One unit of topoisomerase activity is defined as the amount of enzyme necessary to relax 1 nmol of supercoiled DNA in 30 min at 37°C.

Other Methods. The determination of DNA polymerase β, -γ, and terminal deoxynucleotidyltransferase was as described (8). Protein determination was according to Bradford (19), using bovine serum albumin as the standard.

RESULTS

At an Early Purification Stage DNA Polymerase α Holoenzyme Can Be Separated into Four Different DNA Polymerase α Forms by Chromatography on DEAE Cellulose. We have shown that a calf thymus DNA polymerase α can be purified by using as probes *in vivo*-like DNA templates such as ss parvoviral DNA or primed ss M13 DNA (8). The purification procedure has been extended to include an additional chromatography on Bio-Rex 70 and a second phosphocellulose step, and this results in a DNA polymerase α holoenzyme containing at least 11 polypeptides (9). In the course of these studies, it was observed that early purification stages of DNA polymerase α holoenzyme were split into four different DNA polymerase forms when subjected to DEAE-cellulose chromatography (Fig. 1). This elution pattern is reproducible provided that (*i*) the extremely fresh harvested calf thymus is used as an enzyme source, (*ii*) the protease inhibitors sodium bisulfite and pepstatin are included (see legend to Fig. 1), and (*iii*) elution is carried out with a shallow potassium phosphate gradient. The four peaks are designated A, B, C, and D, respectively, indicating elution order from the DEAE-cellulose column.

All Four DNA Polymerase Forms Have Properties Characteristic of DNA Polymerase α. None of these four DNA polymerase forms contained DNA polymerase β, -γ, or terminal deoxynucleotidyl transferase (Table 1). All forms were ex-

Table 3. Copurification of six enzymatic activities with different DNA polymerase α forms

Enzyme	Activity, units/ml				
	DNA polymerase α peak				Purified DNA polymerase α holoenzyme
	A	B	C	D	
DNA polymerase α	109	152	160	123	20
Primase					
Poly(dT) assay	<0.1	28	49	25	5.2
ss M13 assay	<0.1	50	47	12	4.7
DNA-dependent ATPase					
Without DNA	58	<1	1	14	<0.1
Activated DNA	112	41	26	28	<0.1
ss φX174 DNA	103	33	20	32	<0.1
ds supercoiled pAT153 DNA	217	52	37	48	<0.1
DNA topoisomerase II	567	376	650	492	<0.1
3′–5′ exonuclease	11	3	76	152	18
RNase H	11	12	3.9	1.8	<0.1
DNA methylase	5	5.4	12	11	<0.1
DNA ligase	<0.1	<0.1	<0.1	<0.1	<0.1

All assays were carried out using a pure replication enzyme from another source as a positive control. These were as follows: for primase, purified DNA polymerase α core enzyme containing primase (see ref. 20); for DNA-dependent ATPase, *E. coli* rep protein, gift of N. Arai (Palo Alto, CA); for topoisomerase type II, *E. coli* gyrase (relaxation of negatively supercoiled DNA without ATP), gift of R. Low (St. Louis, MO); for 3′–5′ exonuclease, *E. coli* DNA polymerase I (Boehringer Mannheim); for RNase H, *E. coli* DNA polymerase I; for DNA methylase, *Hpa* II methylase (New England Biolabs); for DNA ligase, T4 DNA ligase (Boehringer Mannheim).

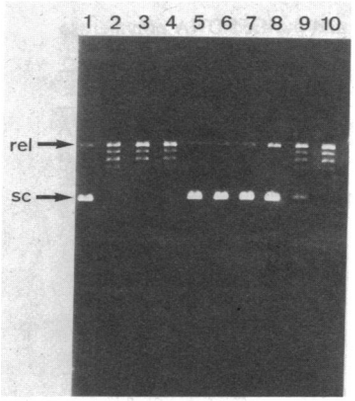

FIG. 2. Characterization of DNA topoisomerase type II copurifying with the C form of DNA polymerase α. DNA topoisomerase determination and electrophoresis were carried out as described in *Materials and Methods*. Lanes: 1, no enzyme; 2, 0.32 unit peak C DNA polymerase α; 3, same as lane 2, but with 0.64 unit; 4, same as lane 2, but with 0.96 unit; 5–10, each lane contained 0.64 unit of peak C DNA polymerase α with the following modifications: lane 5, addition of novobiocin (250 μg/ml); lane 6, addition of novobiocin (250 μg/ml) and ATP omitted; lane 7, ATP omitted; lane 8, ATP and $MgCl_2$ omitted; lane 9, incubation time, 10 min; lane 10, incubation time, 20 min. sc, Supercoiled; rel, relaxed.

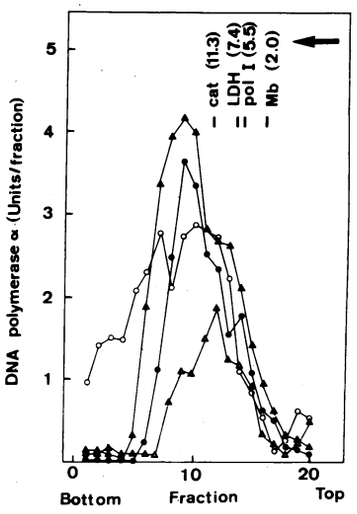

FIG. 3. Velocity sedimentation of the four different DNA polymerase α forms. Preformed 4.8-ml linear 10%–30% (vol/vol) glycerol gradients in 100 mM potassium phosphate, pH 6.8/5 mM dithiothreitol/0.1 mM EDTA/10% (wt/vol) glucose, were prepared in Beckman SW 50.1 tubes. Twenty-seven units each of the four DNA polymerase α peaks were loaded onto individual gradients. Centrifugation was with an SW 50.1 rotor at 40,000 rpm at 4°C for 14 hr. Gradient fractions (8 drops) were collected from the bottom. The sedimentation standards were run in a separate tube and detected as follows: myoglobin (Mb) at A_{430}; DNA polymerase I (pol I) as described in *Materials and Methods* for DNA polymerase α; L-lactate dehydrogenase (LDH) and catalase (cat) as described in the Worthington manual. Arrow indicates direction of sedimentation. ●, Peak A; ○, peak B; ▲, peak C; △, peak D.

tremely sensitive to aphidicolin and *N*-ethylmaleimide but were insensitive to dideoxythymidine triphosphate. Low concentrations of monovalent cations inhibited peaks A, B, and D as well as purified DNA polymerase α holoenzyme. In contrast, higher than physiological salt concentrations were necessary to inhibit form C.

Template Specificity of the Four DNA Polymerase α Forms and the Purified DNA Polymerase α Holoenzyme. The ability of the four DNA polymerase α forms to replicate artificial and natural DNA templates was compared to the purified holoenzyme (Table 2). The holoenzyme, isolated by using ss parvovirus DNA as the template, was active on all kinds of DNA with long single strands. All the four peaks contained activities capable of replicating long ss DNA, such as primed M13 DNA and ss parvoviral DNA. No replication was measured on ds circular forms I and II DNA by any of these different DNA polymerase α peaks or by purified holoenzyme.

Copurification of Six Enzyme Activities with the Different DNA Polymerase α Forms. The four DNA polymerase α forms possibly could represent different functional entities separated on the DEAE-cellulose column (see also *Discussion*). We reasoned that in this case we should be able to identify in the different DNA polymerase α forms other enzymes known to participate in replication. Thus, the four fractions were tested for primase, DNA-dependent ATPase, topoisomerase type II, 3′–5′ exonuclease, RNase H, DNA methylase, and DNA ligase (Table 3). All of these, except DNA ligase, were detected and shown to be preferentially associated with one or the other polymerase form.

For instance, DNA-dependent ATPase was predominantly found in peak A with a ds supercoiled DNA acting as the preferred effector for ATP hydrolysis. In addition, this peak included activities of topoisomerase type II, 3′–5′ exonuclease, and RNase H. Peaks B, C, and D all contained primase,

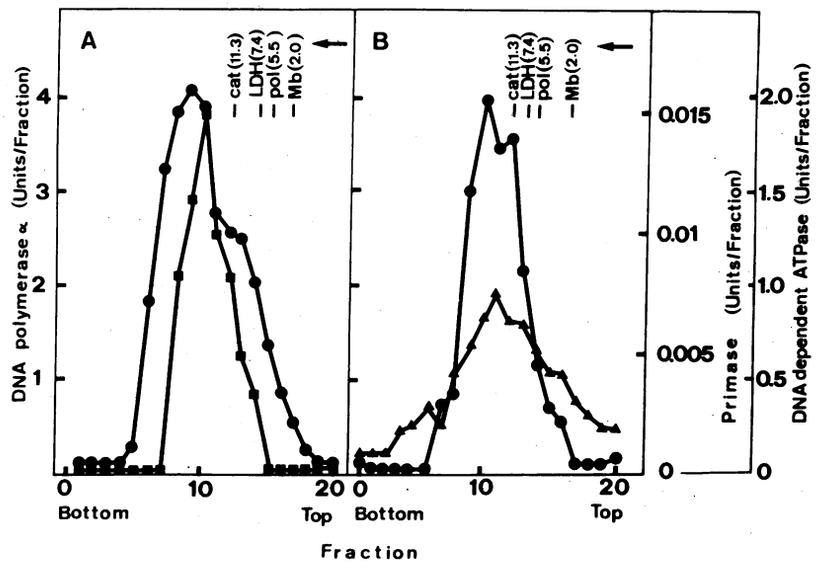

FIG. 4. Cosedimentation of two enzyme activities with DNA polymerase α under moderate salt concentration (*A*) or high salt concentration (*B*). *A* documents the DNA polymerase α (●) peak C shown in Fig. 3 as well as DNA primase (■). DNA primase was determined with M13 ss DNA and DNA polymerase I in the assay mixture. (*B*) A preformed 4.8-ml linear 10%–30% (vol/vol) glycerol gradient in 50 mM potassium phosphate, pH 6.8/5 mM dithiothreitol/0.1 mM EDTA/10% (wt/vol) glucose/200 mM ammonium sulfate was prepared in a Beckman SW 50.1 tube. Twenty-seven units of peak A DNA polymerase α were loaded, centrifuged, and the fraction was collected as described in the legend to Fig. 3. DNA polymerase α (●) and DNA-dependent ATPase with ds supercoiled pAT153 DNA as the effector for ATP hydrolysis (▲) were determined as described in *Materials and Methods*. Markers are as in Fig. 3.

which, on the other hand, was totally absent from peak A. A novobiocin-dependent topoisomerase type II was present in all four peaks, as shown in Fig. 2 for peak C. A 3′–5′ exonuclease was present, as mentioned, in peak A, but preferentially in peaks C and D. A RNase H was found, in addition to peak A, predominantly in peak B. The main DNA methylase activities were identified in peaks C and D. On the other hand, the purified DNA polymerase α holoenzyme only contained primase and 3′–5′ exonuclease as additional activities.

The four DNA polymerase α forms were sedimented in glycerol gradients near physiological salt conditions (250 mOsm). Fig. 3 shows that all four forms had *s* values larger than the largest marker used (catalase, 11.3 *s*). In addition, all four DNA polymerases displayed an activity in the region around 9–10 *s*, where the large form of a purified calf thymus DNA polymerase α normally sediments (21). With forms A, B, and C, this activity appeared as a shoulder on the trailing slope of the peak, whereas with form D, it coincided with the peak center. The putative replication enzymes cosedimented under moderate salt conditions with all of the four DNA polymerase α, as shown in Fig. 4*A* for primase and form C DNA polymerase α. To exclude unspecific aggregation of DNA polymerase α under these salt conditions, identical gradients were prepared in high salt concentration (625 mOsm). As documented in Fig. 4*B*, even under these high salt concentrations, DNA-dependent ATPase cosedimented with form A DNA polymerase α.

Other Properties of the Four DNA Polymerase α Forms. All four forms protected ss DNA from digestion with nuclease S1. Peak C was most resistant to inhibition by spermidine and salt or to inactivation by heat (Table 1). All enzyme forms were similarly inhibited by araCTP (120 μM). Finally, novobiocin (500 μM) inhibited all forms of DNA polymerase α with a preferential effect on peak B and the purified holoenzyme (data not shown).

DISCUSSION

At an early stage of purification, DNA polymerase α holoenzyme from calf thymus (8, 9) can be separated by chromatography on DEAE-cellulose into four forms, designated A, B, C, and D. All four forms clearly possess DNA polymerase α holoenzyme activity (8); that is, they are able to replicate artificial and natural ss DNA templates (Table 2). Six different enzyme activities that are involved in DNA replication copurify with the DNA polymerase α at this early stage of purification and distribute differentially between the four forms (Table 3). Based on the results, we present the following hypotheses. Form A is a candidate for the leading-strand replicase, because, in addition to DNA polymerase α activity, it possesses a DNA-dependent ATPase acting preferentially on ds supercoiled DNA, topoisomerase type II, RNase H, and to a lesser extent 3′–5′ exonuclease. Peaks B, C, and D might represent different forms of lagging strand DNA polymerase α, because they all contain DNA primase. Furthermore, we identified in peak B a RNase H. Peaks B, C, and D were associated with additional enzymes—namely, a DNA topoisomerase type II in peaks B, C, and D; a DNA methylase in peaks C and D; and a 3′–5′ exonuclease particularly abundant in peaks C and D. The purified holoenzyme, on the other hand, contained as the only additional activities the primase and the 3′–5′ exonuclease. Form C differs from the other two primase-containing species by its higher stability to interference by monovalent cations, spermidine, and heat. ATP-activated *E. coli* DNA polymerase III holoenzyme is insensitive to monovalent cations at concentrations (150 mM) that completely inhibit both nonactivated holoenzyme and incomplete forms of holoenzyme (22). Similarly, form C could represent such a form of holoenzyme with increased resistance to salt and spermidine.

The fact that all four forms sedimented as high molecular weight DNA polymerase complexes (>11.3 *s*) suggests that copurification of all these additional replication enzymes is not fortuitous (Fig. 3). The replication enzymes cosedimented under moderate salt conditions (250 mOsm) with the DNA polymerase α forms, as exemplified for DNA primase in Fig. 4*A*, and even under conditions of high salt concentration (625 mOsm) DNA dependent-ATPase cosedimented with form A DNA polymerase α (Fig. 4*B*).

In summary, we have presented evidence for the existence of different functional forms of DNA polymerase α. We suggest that these represent a leading strand DNA polymerase α (form A) and several forms of lagging strand DNA polymerase α (forms B, C, and D). Among the latter, peak C is more resistant to inhibition by salt and spermidine or to inactivation by heat than the other enzyme forms. This is suggestive of a functional DNA polymerase α holoenzyme possibly fitting in a higher-order structure, such as the replisome or even the chromatin. The functional distinction elaborated in the present work provides a basis for the isolation, dissection, and reconstitution of DNA replication enzymes.

We thank C. C. Kuenzle for critical reading of the manuscript. G. Morgenegg for stimulating discussions, and H. P. Stalder for expert technical assistance. This work was supported by Swiss National Science Foundation Grant 3.006-0.81 and by the Kanton of Zürich.

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