Purification and Characterization of a Yeast DNA Polymerase α Complex with Associated Primase, 5′→3′ Exonuclease, and DNA-Dependent ATPase Activities†

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ABSTRACT: We have purified a multimeric form of yeast DNA polymerase α with DNA polymerase, primase, 5′→3′ exonuclease, and single-stranded (ss) DNA-dependent ATPase activities to near-homogeneity. The molecular mass of the complex was 650 kDa with subunits ranging in sizes from 30 to 180 kDa. The α-subunit of the complex could be detected by DNA polymerase α antibody. No cross-reactivity of polypeptides within the complex was observed with antibodies directed against polymerase δ or ε. The multimeric polymerase α could be selectively inhibited by *p-n*-butylphenyl-dGTP (I_{50} of ~0.2 μM), *p-n*-butylanilino-dATP (I_{50} of 1.3 μM), and aphidicolin (I_{50} of 2.5 μg/mL). The complex synthesized RNA primers on various ssDNA templates and rapidly elongated these primers into nascent DNA fragments in the presence of required deoxynucleotides. It had a strong 5′→3′ exonuclease activity. In addition, the complex hydrolyzed both ATP and dATP in a ssDNA-dependent manner. Thus, the multiprotein complex of DNA polymerase α had multiple activities (primase, polymerase, and ATPase) which could act concertedly to synthesize primers and elongate the primers to nascent DNA fragments in the lagging strand of the fork.

Three nuclear DNA polymerases have been identified in eukaryotic systems: polymerase α, polymerase δ, and polymerase ε (Byrnes et al., 1976; Edenburg et al., 1978; Banks et al., 1979; Kornberg, 1980, 1982; Morrison, 1990; Ray et al., 1991), all of which have been implicated, primarily through in vitro DNA replication systems, in some stage of chromosomal DNA replication. Polymerase α with both DNA primase and DNA polymerase activities is involved in the priming and elongation stages of replication in the lagging strand of the replication fork. The mechanistic details of nascent DNA fragment synthesis by DNA polymerase α (pol α)[1] and what other proteins, in addition to primase, participate in this process remain to be elucidated.

DNA polymerase α has been purified from a variety of eukaryotic cells in various laboratories and has been shown to contain four subunits (Banks et al., 1979; Mechali et al., 1980; Gross & Krause, 1981). However, the four-subunit pol α has also been found to be a remarkably slow polymerase with low processivity (Badaracco et al., 1985; Cotterill et al., 1987). These four subunits may likely be a part of a larger multiprotein complex that represents the replicating form of polymerase α. In fact, studies in several laboratories have indicated that DNA polymerase α could be isolated as a large complex accompanied by a number of enzymatic activities such as polymerase, primase, and exonuclease (Hubscher et al., 1982; Baril et al., 1983; Pritchard & DePamphilis, 1983; Pritchard et al., 1983; Takada et al., 1986; Vishwanath et al., 1986; Biswas & Biswas, 1988). The multimeric form of polymerase α is labile, and subunit dissociation appears to be common during purification.

In addition to the polymerases, several other accessory proteins that appear to function as part of the replication machinery have recently been described (Hübscher et al., 1982; Baril et al., 1983; Pritchard & DePamphilis, 1983; Pritchard et al., 1983; Vishwanath et al., 1986; Vishwanath & Baril, 1990; Tsurimoto & Stillman, 1989; Lee et al., 1991; Fien & Stillman, 1992). Vishwanath and Baril (1990) have purified a ssDNA-dependent ATPase from HeLa cells that stimulates polymerase α activity, and several groups (Vishwanath & Baril, 1990; Tsurimoto & Stillman, 1989; Lee et al., 1991; Fien & Stillman, 1992) have identified an ATPase that stimulates DNA polymerase δ from mammalian and yeast cells. These two ssDNA-dependent ATPases may or may not be identical. In SV40 viral DNA replication, the T antigen has been shown to act in the unwinding of the replication origin (Dean et al., 1987; Goetz et al., 1987; Wold et al., 1987). The T-antigen action requires the presence of RP-A in SV40 DNA replication. RP-A has been shown to be a ssDNA binding protein that is involved in DNA replication and recombination (Wold & Kelly, 1987; Heyer & Kolodner, 1989; Heyer et al., 1990; Erdlie et al., 1991).

We identified earlier a high molecular weight form of primase–polymerase complex from *Saccharomyces cerevisiae* which was capable of rapid and processive DNA synthesis on an unprimed single-stranded DNA template (Biswas & Biswas, 1988). In this paper, we describe the purification of a multimeric form of DNA polymerase α to homogeneity. The multimeric form of polymerase α has a strong 5′→3′ exonuclease, ssDNA-dependent ATPase and dATPase, and RP-A-dependent DNA unwinding activity [which is detailed in the following paper (Biswas et al., 1993)]. The multimeric polymerase α appears to be processive (>1 kb) under conditions where ≤1 pmol of deoxynucleotide was incorporated per picomole of template utilizing primers generated in situ.

MATERIALS AND METHODS

Yeast. S. cerevisiae, wild-type bakers' yeast, was obtained as a gift from the American Yeast Corp., Baltimore, MD, and

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[1] Abbreviations: pol α, DNA polymerase α; pol δ, DNA polymerase δ; pol ε, DNA polymerase ε; BuPhdGTP, *p-n*-butylphenyl analog of dGTP; BuAdATP, *p-n*-butylanilino analog of dATP; ATPase, adenosinetriphosphatase; PMSF, phenylmethanesulfonyl fluoride; NP40, Nonidet P-40; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-trisphosphate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF-C, replication factor C; RP-A, replication protein A.

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was removed from the fermentor at midlog phase, chilled to 4 °C, and harvested by centrifugation. The cell pellet was washed 4 times with cold double-distilled water and once with buffer A + 200 mM NaCl, resuspended in the same buffer at a density of 500 g of cells/L, and stored at −90 °C as thin slabs in 500-mL aliquots until further use.

Nucleotides, Enzymes, and DNA. Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia. These nucleotides were used without further purification. [α-^{32}P]ATP and [α-^{32}P]dATP were obtained from Amersham. [^{3}H]dTTP was from NEN. BuPhdGTP and BuAdATP were obtained as gifts from Dr. George E. Wright (University of Massachusetts Medical School). Aphidicolin was from Sigma Chemical Co. Calf thymus DNA was activated with DNase I according to the procedure of Fansler and Loeb (1974). DNA polymerase I antiserum, raised in rabbit, was a kind gift of Dr. L. M. S. Chang (Uniformed Services Health Sciences University). Antibodies which would recognize either polymerase δ or polymerase ϵ were generated by immunizing mice with conjugated peptides corresponding to the amino acid sequence 671–683 for yeast polymerase δ and 1285–1297 for yeast polymerase ϵ. Each antibody cross-reacted with polypeptides of appropriate size in the yeast extract. Phosphocellulose (P-11) was from Whatman. Q-Sepharose and S-Sepharose were from Pharmacia—LKB. The size exclusion 4000SW HPLC column was from Phenomenex. All chemicals used to prepare buffers and solution were reagent grade and were purchased from Fisher Chemical Co. Protease inhibitors were from Bachem Inc. (Torrance, CA). Poly(ethylenimine)–cellulose TLC plates were from J. T. Baker. All electrophoretic supplies were of molecular biology grade and were purchased from Fisher. Calf thymus DNA polymerase δ and PCNA were generous gifts of Drs. C.-K. Tan and Antero So of the University of Miami.

Buffers. Buffer A was 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.01% (v/v) NP40, 1 mM EDTA, and 2.5 mM DTT. Buffer B was 5 mM Tris-HCl (pH 7.5), 10% glycerol, and 1 mM EDTA. Buffer C was 25 mM Tris-HCl (pH 8.3), 20% (v/v) glycerol, 1 mM EDTA, 0.01% NP40, and 2.5 mM DTT. Buffer D was 25 mM Tris-HCl (pH 7.5), 20% (v/v) glycerol, 0.01% (v/v) NP40, 1 mM EDTA, 150 mM NaCl, and 2.5 mM DTT. Buffer E was 20 mM MES (pH 6.5), 20% glycerol, 1 mM EDTA, 0.01% NP40, and 2.5 mM DTT. Buffer F was 50% glycerol, 125 mM Tris-HCl (pH 7.5), 800 μg/mL BSA, 40 mM DTT, and 40 μg/mL each pepstatin A and leupeptin. TBE buffer was 89 mM Tris–borate and 2.5 mM EDTA. Formamide buffer contained 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF.

Activated Calf Thymus DNA and Primase Assays. These assays were carried out as described in earlier publications (Biswas & Biswas, 1988; Biswas et al., 1987). One unit of activity is defined as the amount of synthesis activity that incorporates 1 nmol of deoxynucleotide per hour at 30 °C into trichloroacetic acid insoluble form.

Denatured DNA Assay. This assay was carried out as described by Vishwanath et al. (1986). Basically, the assay conditions were the same as those for the activated calf thymus DNA assay, except that heat-denatured DNA was utilized as the template.

Coupled Primase–Polymerase and Primed DNA Assays. These assays were carried out as described earlier (Biswas & Biswas, 1988). One unit of activity was defined as the amount of synthesis activity that incorporates 1 nmol of deoxynucleotide per hour at 30 °C into trichloroacetic acid insoluble form.

5′→3′ and 3′→5′ Exonuclease Assays. 5′→3′ exonuclease assay was carried out using 5′-^{32}P-labeled activated calf thymus DNA as template. Reactions were set up on ice, contained 5 μL of buffer F, 5 mM $MgCl_2$, 300 pmol of 5′ ^{32}P-labeled activated calf thymus DNA, and the indicated amount of protein in a final volume of 25 μL, and were incubated at 37 °C for 30 min. One unit of activity is defined as that amount which liberates 1 nmol of dNTP per hour at 37 °C. 3′→5′ exonuclease activity was measured using 3′ ^{3}H-labeled activated calf thymus DNA and was carried out as described by Bauer et al. (1988).

Immunological Analyses. Western transfer was performed using a Bio-Rad Trans-blot apparatus according to the manufacturer's instructions. Cross-reactivity of antibody to protein was detected by utilizing alkaline phosphatase conjugated second antibody with NBT and BCIP as its substrates. Slot blot analysis was performed using a Gibco-BRL slot blot apparatus.

Primer Analysis. Synthesis of primers on poly(dT) template was carried out essentially as described (Biswas & Biswas, 1988). The reaction conditions were essentially the same as described for a standard primase assay except that *Escherichia coli* DNA polymerase I and all deoxynucleotides were omitted. Two hundred fifty picomoles of poly(dT) template and 2 nmol, 10 μCi, of [α-^{32}P]ATP were added. The reactions were incubated at 30 °C for 15 min and terminated by adding 2 μL of 500 mM EDTA followed by chilling on ice. The mixtures were treated with calf alkaline phosphatase at 37 °C for 30 min. The primers were then extracted with phenol/CHCl, and CHCl, and ethanol-precipitated using 10 μg of yeast tRNA as a carrier. The pellets were resuspended in 5 μL of formamide buffer and heated at 100 °C for 3 min followed by chilling on ice. The samples were loaded on a preelectrophoresed 20% polyacrylamide/7 M urea gel. Electrophoresis was carried out in TBE at 40 W, 50 °C, for 3 h. Following electrophoresis, the gels were dried and autoradiographed using Fuji RX-G film.

Analysis of the Products of DNA Synthesis. Reactions were carried out with 5 μCi of [α-^{32}P]dATP, 1000 pmol (as nucleotide) of M13mp18 ssDNA, and 2.5 mM each of the four rNTPs with a predetermined amount of polymerase α complex so that the total incorporation of dNTP will be less than one nucleotide per template. The reactions were terminated by the addition of 2 μL of 500 mM EDTA and heating at 75 °C for 3 min followed by chilling on ice. Four microliters of each reaction mixture was added to 2 μL of formamide buffer, heated at 90 °C for 1 min, and loaded on a 6% polyacrylamide/7 M urea, gel. Electrophoresis was carried out for 6 h in 1× TBE. Following electrophoresis, the gel was dried and autoradiographed using Fuji RX film.

ATPase Assay. The ATPase assays were carried out essentially as previously described (Biswas et al., 1986).

RESULTS

Purification of a Multiprotein Complex of Yeast DNA Primase–Polymerase Complex. All steps in the purification were carried out at 0–4 °C, and all buffers used in the procedure contained the following protease inhibitors: 1 μg/mL each of leupeptin, pepstatin A, antipain, chymostatin; 0.1 mM each of benzamidine hydrochloride and $NaHSO_3$; and 2.5 μg/mL each of TPCK and TLCK unless otherwise indicated. The yeast extract (fraction I) was prepared from wild-type yeast as described earlier (Biswas et al., 1987) in the presence of

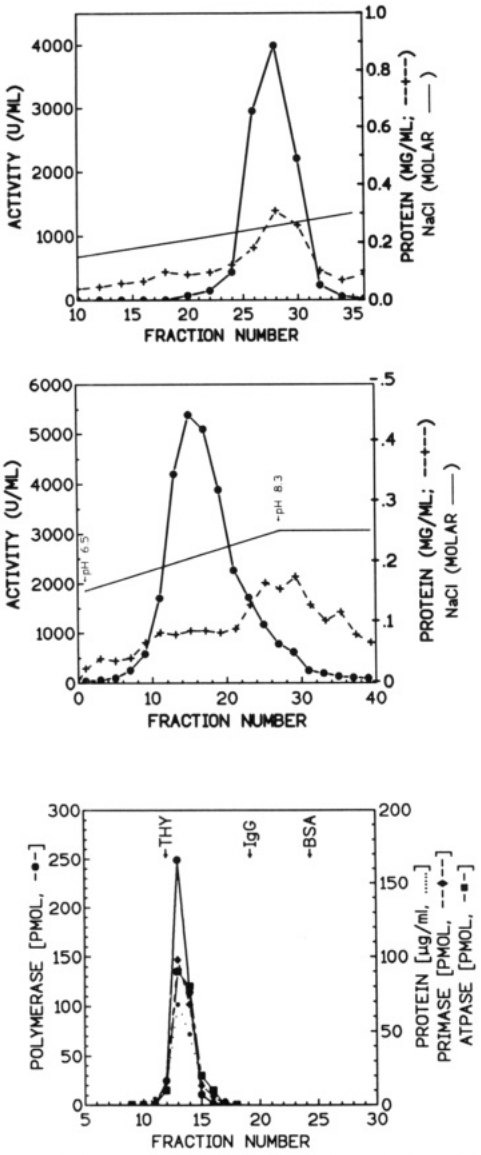

FIGURE 1: Chromatographic fractionation of the polymerase α complex. Details of the purification procedures have been presented under Results. (Top) Q-Sepharose; (middle) S-Sepharose chromatography; (bottom) size-exclusion high-performance liquid chromatography. Aliquots of the collected fractions were assayed for the following activities: polymerase, primase, ATPase, and protein. The assays were carried out as described under Materials and Methods, and 5 μL of the indicated fractions was tested in each case. Size markers were as follows: thyroglobulin (THY); immunoglobulin G (IgG); bovine serum albumin (BSA).

5 mM spermidine hydrochloride, 1 M NaCl, and 0.1 mM PMSF. The S-100 supernatant was dialyzed for 3 h against buffer A + 10% ultrapure sucrose and diluted to the conductivity of buffer A + 50 mM NaCl by adding buffer B. The dialyzed extract was then chromatographed on phosphocellulose (fraction II) as described earlier (Biswas et al., 1987). The fractionation was carried out in buffer A with a gradient of buffer A+200 to buffer A+600. The active fractions were pooled and dialyzed 2 h against buffer C + 10% sucrose. The dialysate was adjusted to pH (8.3) and the conductivity of buffer C + 150 mM NaCl and loaded on a 15-mL (2.5 cm × 3 cm) Q-Sepharose column equilibrated with the same buffer. The column was washed with 100 mL of buffer C + 150 mM NaCl followed by 100 mL of buffer E + 150 mM NaCl. The polymerase α complex was eluted with a 100-mL gradient of buffer E + 150 mM NaCl to buffer E + 300 mM NaCl. The active fractions were pooled (fraction III) and diluted with 5 mM MOPS (pH 6.5), 20% glycerol, 1 mM EDTA, and 2.5 mM DTT to the conductivity of buffer E + 150 mM NaCl. The diluted pool was loaded on a 2-mL (0.7 cm × 5.2 cm) S-Sepharose column equilibrated with buffer E + 150 mM NaCl. The column was washed with 50 mL of buffer E + 150 mM NaCl, and the polymerase α complex was eluted with a 50-mL gradient of buffer E + 150 mM NaCl to buffer C + 250 mM NaCl. The active fractions were pooled (fraction IV) and concentrated by ultrafiltration using an Amicon YM100 membrane to a concentration of 0.5–1 mg/mL. The activity and protein profiles of Q-Sepharose and S-Sepharose columns are shown in Figure 1 (top and middle panels).

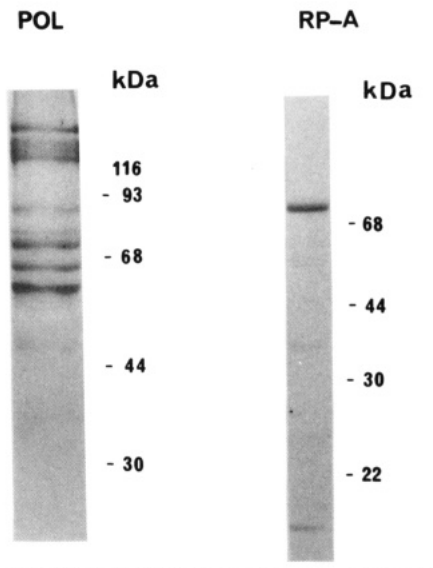

FIGURE 2: SDS–PAGE (10%) of polymerase α complex (7.5 μg) fraction VI (lane 1) and purified RP-A (2 μg) (lane 2). Molecular mass standards were as follows: β-galactosidase (116 kDa); phosphorylase B (93 kDa); BSA (68 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (22.4 kDa); lysozyme (14.4 kDa). The gel was stauned with Coomassie blue R-250.

The polymerase α complex was further purified by size-exclusion HPLC in a Phenomenex S4000 SW column, and the running buffer was buffer D + 150 mM NaCl. The active fractions from HPLC were concentrated and refractionated by HPLC. Profiles of various activities of the polymerase complex in HPLC fractions are shown in Figure 1 (bottom panel). The second HPLC did not improve the specific activities. The highly purified complex is stable for at least 6 months at –80 °C. The complex in the unpurified state was very sensitive to the freeze–thaw cycle. We also avoided too much dilution of the polymerase at various stages of purification. Addition of sucrose in the dialysis buffers diminished osmotic dilution of the samples. All of the activities were quite sensitive to dilution (data not shown).

Polypeptide Structure and Associated Enzymatic Activities. The HPLC size-exclusion chromatography indicated that the polymerase α complex was close in size to the marker thyroglobulin (679 kDa, 19 S) in elution profile. The computed molecular mass of the complex is 650 kDa. The SDS–PAGE gel of the purified polymerase α complex is shown in Figure 2. The complex had 180-, (140-), 78-, 58-, and 48-kDa known subunits of polymerase α, and in addition, there were several other polypeptides present in the complex: 90-, 68-, 59-, 34-, and 30-kDa subunits. The doublet observed at 90 kDa was perhaps due to phosphorylation. It corresponded to ATP binding [see Biswas et al. (1993)] and perhaps ATPase and/or helicase polypeptide. The functions of other polypeptides

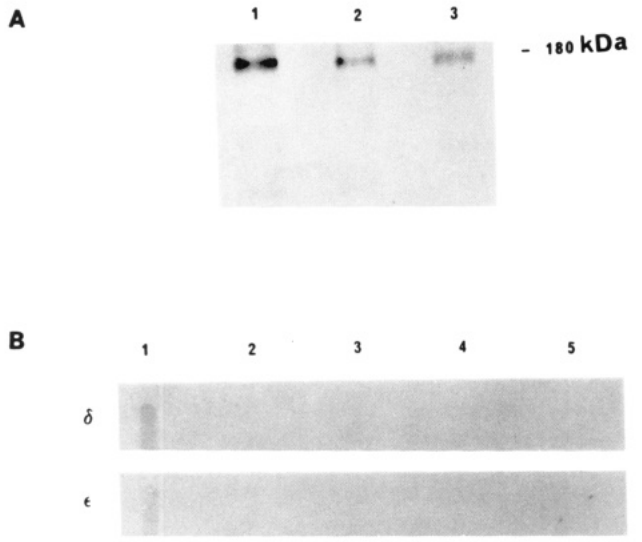

FIGURE 3: (A) Western analysis of the polymerase α complex. Proteins were separated by 5–15% SDS–PAGE prior to transfer onto nitrocellulose and immunodetection. Lane 1, immunoaffinity-purified polymerase α (1 unit); lane 2, polymerase α complex, fraction IV (1 unit); lane 3, polymerase α complex, fraction VI (1 unit). (B) Immunological analysis of polymerase fractions and the blot probed with pol δ (δ) and pol ϵ (ϵ) antibodies. Lanes were as follows: 1, fraction II (1 μg); 2, fraction III (600 ng); 3, fraction IV (360 ng); 4, fraction V (200 ng); 5, fraction VI (120 ng). Details of the electrophoresis and immunodetection are given under Materials and Methods.

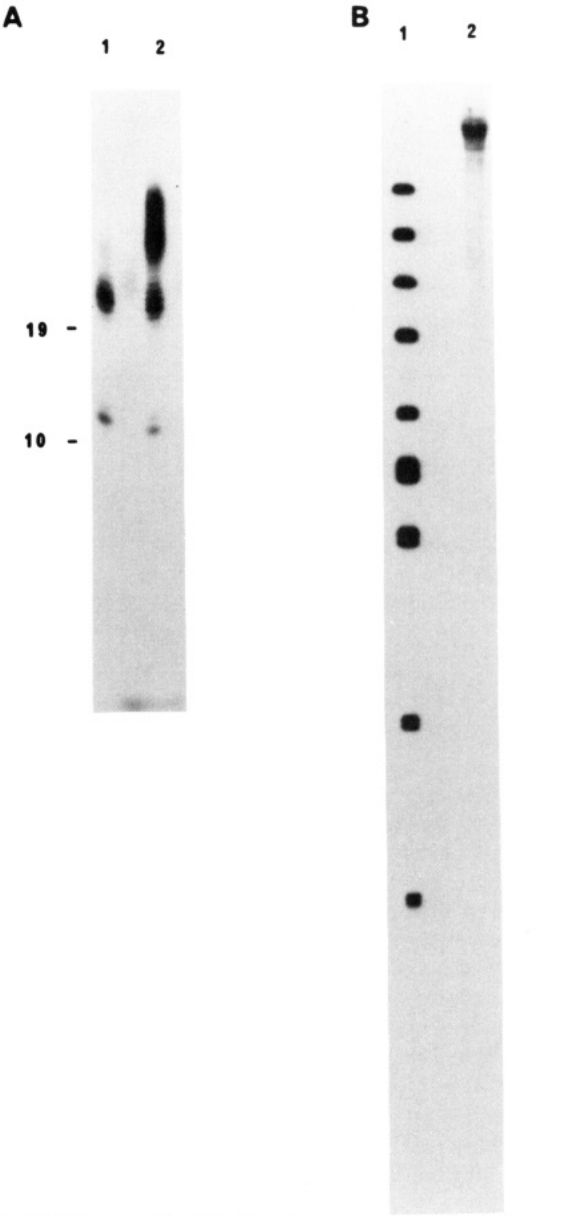

FIGURE 4: (A) Primer synthesis by the polymerase α complex. Primers synthesized by 320 ng of polymerase α complex for 5 and 15 min at 30 °C on 250 pmol of poly(dT) template. (B) Analysis of products of coupled DNA synthesis by the polymerase α complex (fraction V) using 1000 pmol of unprimed M13mp18 ssDNA. The reaction conditions were such that <1 pmol of dNTP was incorporated per minute. Size markers were the 5′ ^{32}P-labeled *Hin*cII digest of ϕX174 DNA.

that were present remain unknown. Two of the polypeptides are likely similar to C1 and C2 proteins as described earlier (Baril et al., 1983; Pritchard & DePamphilis, 1983; Pritchard et al., 1983; Vishwanath et al., 1986; Vishwanath & Baril, 1990). Protease degradation is a significant problem in *Saccharomyces cerevisiae*. Although we have attempted to prevent proteolysis by using a large number of protease inhibitors, proteolysis could not be ruled out in the origination of one or more of these polypeptides.

We have analyzed the polymerase activity of the complex using a number of DNA templates: activated DNA, denatured DNA, M13mp18 single-stranded DNA, and singly primed M13mp18 ssDNA. Activities of the polymerase on these templates were highly comparable except the polymerase appeared to be more active when denatured DNA was used as a template. The polymerase, primase, ATPase, and a 5′→3′ exonuclease activities comigrated in the highly purified complex (Figure 1, bottom). The 5′→3′ exonuclease comigrates with the polymerase activity. The size of this complex is large (650 kDa), and it migrates as a single entity on HPLC; as a result, it appeared that these individual polypeptides and activities are components of this complex.

The purified complex was also analyzed using various polymerase inhibitors that can effectively distinguish yeast DNA polymerases α, δ, and ϵ. The results of the inhibitor analysis are given in Table II. The I_{50} values for BuPhdGTP, BuPhdATP, and aphidicolin were 0.2 μM, 1.3 μM, and 2.5 μg/mL, respectively. The data indicated that the polymerase component of the complex was most likely the DNA polymerase α (Bauer et al., 1988).

Immunological Analysis of the Polymerase α Complex with Antibodies against Yeast Polymerase α, δ, and ϵ. The highly purified polymerase α complex contained an accompanying primase activity that is a well-known indicator of the presence of pol α; however, two other eukaryotic nuclear DNA polymerases have been described and have been implicated in certain stages of chromosomal DNA replication. Therefore, we have analyzed the purified polymerase complex for the presence of pol α, pol δ, and pol ϵ. We have prepared polyclonal mouse antibodies against synthetic 13-mer oligopeptides derived from the primary sequences pol δ and pol ϵ (Boulet et al., 1989; Morrison et al., 1990). The most antigenic oligopeptide sequence was determined for each polymerase by analyzing its protein sequence with the "Antigen" program of PCGENE sequence analysis software (Intelligenetics Inc.). Western blot analysis of polymerase α complex from various stages of purification confirmed the presence of the 180-kDa subunit of polymerase α in the complex (Figure 3). A slot blot analysis of the polymerase at various stages of purification and probing with pol δ or pol ϵ demonstrated the presence of these polymerases in the phosphocellulose pool (fraction II). However, neither pol δ nor pol ϵ was detectable after this stage (Figure 3).

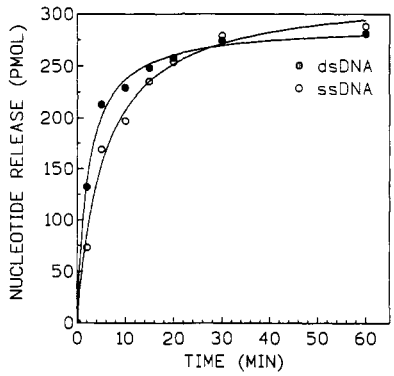

FIGURE 5: 5′→3′ exonuclease activity of the polymerase α complex on native and denatured substrates. The assay was carried out as described under Materials and Methods using 320 ng of polymerase α complex (fraction V) and 200 pmol of native or heat-denatured DNA.

Synthesis of Oligonucleotide Primers and Nascent DNA Fragments. Previously it has been shown that primase in the primase–polymerase α complex synthesizes multimeric mixed oligoribo–deoxynucleotide primers on single-stranded DNA templates (Singh & Dumas, 1985; Biswas & Biswas, 1988). The multimeric nature of the primers is in general most prominent when poly(dT) is used as template. The primers synthesized by purified pol α complex on poly(dT) template are shown in Figure 4A. At both 5- and 15-min time points, primers of various sizes were formed. However, larger (>35 bp) primers were observed only at 15 min. The primer labeling could be chased effectively with unlabeled ATP or dATP.

We have also examined the synthesis of RNA–DNA hybrids or "nascent DNA fragments" by the pol α complex on M13mp18 ssDNA template. Under conditions where less than one deoxynucleotide was incorporated per molecule of the template DNA, the products observed were >1000 bp in length, as shown in Figure 4B. Consequently, the primase/polymerase activities of the complex in the presence of the other accessory subunits of the complex were capable of synthesizing primers and rapid extension of the primers into long nascent DNA fragments.

Exonuclease Activities Associated with the Polymerase α Complex. We have explored both 5′→3′ and 3′→5′ proofreading exonuclease activities associated with the purified complex (Skarnes et al., 1986). The 3′→5′ exonuclease activity of the purified complex was too low to measure accurately on either native or denatured DNA templates in our assay system. In the early stages of the purification (fractions I, II, and III), 3′→5′ exonuclease was easily detectable. On further purification (fractions IV and V), the 3′→5′ exonuclease activity disappeared drastically. Therefore, these observations led us to conclude that the 3′→5′ exonuclease activity detected in the early stages of purification was due to a nuclease impurity. However, the complex had a very strong 5′→3′ exonuclease activity on both native and denatured DNA templates (Figure 5). The 5′→3′ exonuclease activity copurified with the polymerase α complex through five steps of chromatography, and it comigrated with the other pol α complex associated activities (data not shown). Thus, the 5′→3′ exonuclease activity appeared to be an integral part of the polymerase complex. The exonuclease activity was comparable on both denatured and native DNA. The 5′→3′ exonuclease activity had a fairly broad [Mg^{2+}] maximum between 2 and 15 mM whereas the [Mg^{2+}] maximum for polymerase activity was 10 mM (Figure 6A). These activities also differed in the relative sensitivity to KCl concentration. The peak exonuclease

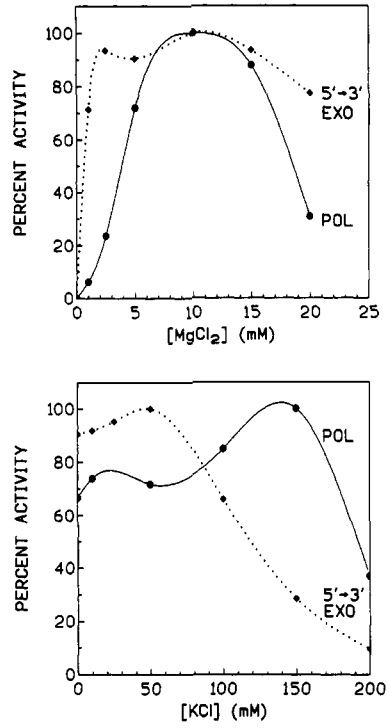

FIGURE 6: Influence of [Mg^{2+}] (A) and [KCl] (B) on the polymerase and 5′→3′ exonuclease activities. These assays were carried out as described under Materials and Methods using 320 ng of pol α complex (fraction V).

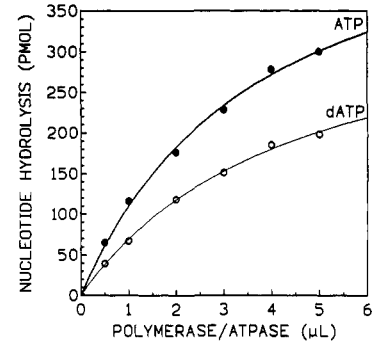

FIGURE 7: Titration of ATPase and dATPase activities of purified polymerase α complex. The assays were carried out in the presence of 200 pmol of M13mp18 ssDNA and polymerase α complex (0.5 unit/μL). The details of this assay have been described under Materials and Methods.

activity was observed at 50 mM KCl, and the activity decreased exponentially with increasing KCl concentration (Figure 6B). The peak polymerase activity was observed at 150 mM KCl, and the activity decreased rapidly with further increase in the KCl concentration.

ssDNA-Dependent ATPase Activity. The polymerase α complex displayed a strong DNA-dependent ATPase (2669 units/mg, Table I) and dATPase activity (Figure 7), and this activity comigrated with polymerase/primase activities in HPLC analysis of the purified complex (Figure 1, bottom panel). The complex had a weak basal ATPase or dATPase activity, but ssDNA stimulated the activity significantly. The ATPase activity was not inhibited by other ribo- and deoxynucleotides, AMPPNP, BuPhdGTP, aphidicolin, or α-amanitin.

DISCUSSION

DNA polymerase α has been proposed to be the enzyme whose major role is in the replication of the lagging strand of

Table I: Purification Table

fraction	polymerase		primase		5′→3′ exo		ATPase		protein
	units	units/mg	units	units/mg	units	units/mg	units	units/mg	(mg)
(I) extract	7200	2.4	ND	ND	ND	ND	ND	ND	3000
(II) phosphocellulose	11760	147	1760	22	ND	ND	ND	ND	80
(III) Q-Sepharose	6120	450	1414	104	13641	1003	11292	830	14
(IV) S-Sepharose	5600	2812	1205	603	5040	2520	2880	1440	2
(V) HPLC I	1930	6300	430	1403	1375	4583	803	2669	0.3

Table II: Influence of Various Nucleotide Analogues on Replication by the Polymerase α Complex

inhibitor	I_{50}	inhibitor	I_{50}
BuPhdATP	1.29 μM	Mg^{2+}	18.5 mM
BuPhdGTP	0.2 μM	KCl	190 mM
aphidicolin	2.5 μg/mL		

the replication fork. Earlier (Biswas & Biswas, 1988) we identified a high molecular weight form of DNA polymerase α in yeast that is capable of replicating long single-stranded DNA templates through the concerted action of the primase–polymerase components of the complex and required ATP for efficient DNA synthesis. Vishwanath et al. (1986) and Hübscher et al. (1982) purified similar complexes of mammalian DNA polymerase α from HeLa cells and calf thymus, respectively. We have purified a large complex of yeast DNA polymerase α which possesses a number of activities in addition to the primase and polymerase activities. The complex has been purified approximately 3000-fold to homogeneity (Table I and Figure 1). We have used pH and NaCl gradients in conjunction in order to maximize the recovery and purification of the polymerase in Q-Sepharose and S-Sepharose chromatography. The final step in the purification is the size-exclusion HPLC using a Phenomenex S4000 column which resolves large proteins. This step removed a number of lower molecular weight impurities and allowed determination of the size of the complex.

Four separate activities (polymerase, primase, 5′→3′ exonuclease, and ssDNA-dependent ATPase) copurified through the chromatographic steps, each of which was optimized for high-resolution separation of proteins. The complex was ~650 kDa which was large enough to accommodate all of the polypeptides observed in SDS–PAGE (Figure 2). Inhibition of the polymerase activity by several eukaryotic polymerase inhibitors (Table II) suggests that the polymerase was most likely polymerase α. Immunological analysis using antibodies against yeast polymerases α, δ, and ϵ (Figure 3) supported that the polymerase component of the complex was pol α and not pol δ or ϵ. The polymerase was not stimulated by PCNA (Tan et al., 1986) nor can it stimulate pol δ (data not shown). As would be anticipated, the primase activity copurified with the polymerase activity of the complex. The primase activity synthesized multimeric primers on poly(dT) template (Figure 4A). Combined primase and polymerase activities rapidly synthesized RNA-primed DNA on M13mp18 ssDNA (Figure 4B). Under conditions where less than one deoxynucleotide incorporated per template, the synthesized DNA was larger than 1 kb. Thus, the complex appeared to be capable of synthesizing DNA in a processive manner. Further studies are required for a more detailed analysis of the mechanism of action of the polymerase.

The complex displayed a strong 5′→3′ exonuclease activity on both denatured and native DNA templates. In contrast, the 3′→5′ exonuclease activity was too low to detect in purified polymerase α complex. Skarnes et al. (1986) describe the isolation of a 69-kDa exonuclease from a multisubunit pol α complex of HeLa cells. However, in that instance, both 5′→3′ and 3′→5′ activities reside in the same polypeptide. It did not appear to be the case with the yeast polymerase α complex.

In addition to the exonuclease activity, the complex had a unique ssDNA-dependent ATPase activity. The ATPase also hydrolyzed dATP quite efficiently in a ssDNA-dependent manner. The activity appeared to be distinct from the polymerase and primase activities of the complex. Several groups have reported the purification of DNA-dependent ATPases from a number of eukaryotic systems. Vishwanath and Baril (1990) reported the purification of a DNA-dependent ATPase (ATPase1) from HeLa cells that stimulated DNA polymerase α. In addition, Baril et al. (1988) reported a 16S multienzyme polymerase α complex, and Malkas et al. (1990) reported a 21S complex, both of which have similar a ssDNA-dependent ATPase activity. Tsurimoto and Stillman (1989) purified a DNA-dependent ATPase (RF-C) that stimulates DNA polymerase δ in SV40 DNA replication in vitro. Hoeckensmith et al. (1986) have discovered a 140-kDa ATPase from calf thymus that is stimulated especially by primer-template junctions similar to that reported for RF-C. In the case of RF-C, it would be reasonable to expect it to associate more efficiently to DNA polymerase δ. At the present time, we cannot directly correlate the pol α associated ATPase with any of the known DNA-dependent ATPases. Further characterization of the ATPase activity and the accompanying RP-A-dependent DNA unwinding activity is presented in the following paper (Biswas et al., 1993).

In summary, the purified polymerase α complex had a number of enzymatic activities that would enable the complex to (i) synthesize RNA primers, (ii) extend the primers to form nascent DNA fragments, and (iii) move the complex by the energy of ATP hydrolysis generated by the ATPase activity.

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