Characterization of the DNA-Dependent ATPase and a DNA Unwinding Activity Associated with the Yeast DNA Polymerase α Complex

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ABSTRACT: We have analyzed the ATPase and dATPase activities associated with the yeast DNA polymerase α complex. The ATPase/dATPase was primarily a single-stranded DNA-dependent ATPase. Analysis of the stimulatory effect of a large number of DNA substrates demonstrated that polynucleotides longer than 60 nucleotides (nts) had the maximal effect. The stimulation by oligonucleotides smaller than 60 nts, in general, decreased proportionally with decreased length of the oligomer. Poly- or oligopyrimidines were twice as stimulatory as the poly- or oligopurines of the same length. In addition to DNA, replication protein A (RP-A), a single-stranded DNA (ssDNA) binding protein, also stimulated the ATPase activity. Photocross-linking of the ATP binding component of the pol α complex to [α-^{32}P]ATP at 0 °C resulted in the exclusive labeling of a 90-kDa polypeptide. The labeling was inhibited by ATP and dATP but not by any other ribo- or deoxynucleotides, which suggests that the 90-kDa polypeptide is specific for ATP/dATP binding and possibly the active site for the ATPase/dATPase. We have also reported here a novel DNA unwinding activity associated with the multiprotein complex of DNA polymerase α. The complex was able to unwind M13mp19 ssDNA hybridized to an oligonucleotide (17–60 nucleotides long) with a protruding 3′-terminus. Regardless of the size of the duplex, the DNA unwinding was significantly stimulated by RP-A, while RP-A itself did not have any DNA unwinding activity. Consequently, it appeared that the DNA polymerase α complex possessed a putative RP-A-dependent helicase activity. The results presented here and in the preceding paper [Biswas, E. E., Chen, P.-H., Gray, W., Li, Y. H., Ray, S., & Biswas, S. B. (1993) *Biochemistry* (preceding paper in this issue)], when taken together, suggest that the multiprotein DNA polymerase α complex has all of the activities, including DNA unwinding, necessary to propel the replication fork and simultaneously carrying out replication of the lagging strand.

Studies on the replication of phage, plasmid, and chromosomal DNA in *Escherichia coli* during the last few decades established a comprehensive understanding of the basic mechanism of DNA replication (Kornberg, 1980, 1982). A large number of proteins and enzymes with a variety of enzymatic activities are required for efficient replication of the chromosomal DNA. Several DNA-dependent adenosinetriphosphatases (ATPases)[1] have been identified in prokaryotes and eukaryotes, and many of them have been shown to be involved in DNA replication: DnaB protein, Rep protein, n′, and the τ subunit of DNA polymerase III holoenzyme. The DnaB and Rep proteins are both known to have DNA helicase activity (Lebowitz, 1986; Matson, 1991; Matson & Kaiser-Rogers, 1990). Similar DNA helicases have been shown to be involved in the replication of bacteriophages T4 (Venkatesan et al., 1982) and T7 (Matson et al., 1983). In vitro studies with λ and *E. coli* DNA replication indicate that the DnaB protein acts as a helicase in the replication of λ and plasmids containing *E. coli* origin of replication, *Ori C* (Fuller et al., 1981; Lebowitz & McMacken, 1986). DnaB helicase has been shown to be stimulated by the single-stranded DNA binding protein (SSB) and primase of *E. coli*. Three separate yeast DNA helicases have been reported in the last few years. The 89-kDa RAD3 gene product has been shown to be a DNA-dependent ATPase and helicase (Sung et al., 1987a,b; Harosh et al., 1989). Sugino et al. (1986) have reported the purification of a 63-kDa helicase. The 134-kDa RADH gene product has the sequence homologous to a number of known helicases. However, none of these proteins appear to require the replication protein A (RP-A) which has been shown to be involved in DNA replication and recombination in yeast and other eukaryotes (Wold & Kelly, 1987; Heyer & Kolodner, 1989; Heyer et al., 1990; Erdlie et al., 1991). As there are eight separate DNA helicases in *E. coli*, it is quite likely that there will be a number of DNA helicases in eukaryotic cells with unique functions and mechanisms of action (Lahue & Matson, 1988; Matson & Morton, 1991; Matson, 1991; Wood & Matson, 1987).

In addition, several DNA-dependent eukaryotic ATPases have been reported recently (Vishwanath & Baril, 1990; Tsurimoto & Stillman, 1989; Lee et al., 1991; Fien & Stillman, 1992). Replication factor C (RF-C) is known to be a stimulator of DNA polymerase δ, functions in the replication of the leading strand (Lee et al., 1991), and is stimulated by the 3′-OH termini of primers in a primed DNA template. Vishwanath and Baril (1990) have described another ssDNA-dependent ATPase that stimulates HeLa cell DNA polymerase α.

Studies on SV40 DNA replication showed the virus-encoded T antigen acts as the helicase to unwind the viral DNA in a RP-A-dependent manner and forms a complex with the large subunit of DNA polymerase α (Wold et al., 1987; Goetz et al., 1987; Dornreiter et al., 1990, 1992). However, the cellular homolog of SV40 T antigen, that functions in the unwinding of the chromosomal DNA in the replication fork, remains unknown. These findings regarding the T-antigen helicase perhaps suggest that the cellular helicase might also have

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[1] Abbreviations: pol α, DNA polymerase α; pol δ, DNA polymerase δ; pol ε, DNA polymerase ε; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF-C, replication factor C; RP-A, replication protein A.

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similar functions and interactions with DNA polymerase α. In the preceding paper, we have described the purification of a multiprotein polymerase α complex which, in addition to its polymerase and primase, exhibited ATPase and 5′ → 3′ exonuclease activities (Biswas et al., 1993). The presence of an ATPase activity in the highly purified polymerase α complex and the fact that the SV40 T antigen interacts physically with pol α led us to explore the possibility of any associated helicase-like activity. In this paper, we have presented evidence that the multiprotein pol α complex has a strong DNA unwinding activity that was dependent on the participation of RP-A, the eukaryotic single-stranded DNA binding protein. The polymerase-associated helicase exhibited properties similar to that of DnaB helicase of *E. coli* and SV40 T antigen, suggesting a similar role in DNA replication.

MATERIALS AND METHODS

Yeast. Saccharomyces cerevisiae, wild-type bakers' yeast, was obtained as a gift from the American Yeast Corp., Baltimore, MD, and was removed from the fermentor at midlog phase, chilled to 4 °C, and harvested by centrifugation. Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia and were used without further purification. [α-^{32}P]-ATP, [α-^{32}P]dATP, and [γ-^{32}P]ATP were obtained from Amersham. All chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Chemical Co. Protease inhibitors were from Bachem. Poly-(ethylenimine)–cellulose TLC strips were from J. T. Baker. All electrophoretic supplies were of molecular biology grade and were purchased from Fisher Chemical Co.

Nucleic Acids and Enzymes. Synthetic homopolymers and oligomers were from Pharmacia—LKB (Piscataway, NJ); other oligomers were synthesized locally. DNA polymerase α complex was purified from yeast as described in Biswas et al. (1993). RP-A used in this study was purified from wild-type yeast to homogeneity as described by Brill and Stillman (1989). Immunoaffinity-purified yeast DNA polymerase α was purified using an immunoaffinity column obtained as a kind gift from Dr. Lucy M. S. Chang of the Uniformed Services Health Sciences University and was carried out as described (Plevani et al., 1985).

Buffers. 1× TBE contained 89 mM Tris–borate (pH 8.3) and 2.5 mM EDTA. 1× TAE contained 40 mM Tris–acetate (pH 8.3) and 2 mM EDTA. Buffer A contained 10% glycerol, 25 mM Tris-HCl (pH 7.5), 160 μg/mL BSA, 8 mM DTT, and 8 μg/mL each pepstatin A and leupeptin.

Enzymatic Assays. DNA polymerase assays were carried out as described earlier (Biswas et al., 1993), and protein was assayed as described by Bradford (1976).

ATPase Assays. The ATPase assays were carried out as previously described (Biswas et al., 1986; Biswas & Biswas, 1987). The standard reaction mixture contained 10 mM $MgCl_2$, 200 pmol of M13mp18 ssDNA, 100 μM [α-^{32}P]ATP (1000–2000 cpm/pmol), and polymerase α complex (0.5 unit of ATPase activity, Fr V, unless otherwise indicated) in buffer A. The reactions were incubated at 37 °C for 30 min (unless stated otherwise) and terminated by the addition of 2 μL of 200 mM EDTA followed by chilling on ice. Two-microliter aliquots were applied to poly(ethylenimine)–cellulose strips which were prespotted with ADP–ATP marker. The strips were developed with 1 M formic acid/0.5 M LiCl and dried. The ADP–ATP spots were located by UV fluorescence. The portions containing ATP and ADP were excised and counted in a liquid scintillation counter using a toluene-based scintillator.

Photo-Cross-Linking of the ATP Binding Subunit of the pol α Complex. Photo-cross-linking was carried out essentially as described earlier (Biswas & Biswas, 1987; Biswas & Kornberg, 1984). Standard ATPase reaction mixtures were set up on ice, and challenge nucleotides, if any, were added as indicated. The mixtures were incubated at 30 °C for 1 min in order to form a stable protein–nucleotide complex. Photo-cross-linking of the ATP binding subunit of the pol α complex was carried out at 0 °C by irradiating the reaction mixtures with UV light for 15 min at 1 nE mm^{-2} min^{-1} as previously described (Biswas & Kornberg, 1984). The samples were then loaded on a 10% SDS–PAGE (Laemmli, 1971). Following electrophoresis, the gel was stained with Coomassie blue, destained, dried, and autoradiographed at −80 °C using Fuji RX-G film.

Helicase Assays. The helicase assays were designed following the procedure of Matson et al. (1983) and LeiBowitz and McMacken (1986) as described below.

(*A*) *Preparation of the 17-mer Primed Substrate.* A 17-mer oligonucleotide (5′-GTTTCCCAGTCACGAC-3′) complementary to M13mp19 ssDNA (nucleotides 6311–6326) was extended three to five nucleotides at the 3′-terminus using terminal transferase and dATP and subsequently labeled with ^{32}P at the 5′-terminus using T4 polynucleotide kinase. This 5′-labeled oligonucleotide was then hybridized to M13mp19 ssDNA as follows. Ten micrograms of ssDNA was added to 400 ng of labeled oligonucleotide in 200 μL of a solution of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 25 mM NaCl. The mixture was incubated at 37 °C for 30 min followed by chilling on ice. Free 17-mer was removed from the primed DNA by ethanol precipitation. The precipitated DNA was washed once with 70% ethanol, dried, and resuspended in 50 μL of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA. One microliter of the resuspended DNA was counted, and was on average 25 000 cpm/μL.

(*B*) *Preparation of the 60-mer Primed Substrate.* The 60-mer helicase substrate was prepared as described above, with the following modifications: a 60-mer oligonucleotide whose first and last five residues were noncomplementary and 50 nucleotides (6–55) were complementary to the 50 bp sequence between nucleotides 6268 and 6317 of M13mp19 ssDNA. The 60-mer oligonucleotide was labeled at its 5′-terminus using T4 polynucleotide kinase. Hybridization was carried out as described above, except that the annealing mixture was heated at 65 °C for 5 min and allowed to slow-cool to 30 °C prior to ethanol precipitation. The precipitated DNA was resuspended in 50 μL of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA. On the average, the substrate had activity of 10 000 cpm/μL. The directionality substrate was prepared by digesting the labeled DNA with *SacI* restriction enzyme followed by dephosphorylation with calf alkaline phosphatase and rephosphorylation using T4 polynucleotide kinase.

(*C*) *Assay Conditions.* Reaction mixtures were set up on ice as follows. A standard 20-μL reaction volume would contain 10 mM $MgCl_2$, 1 mM ATP, 10 000–20 000 cpm/μL of labeled substrate, and the indicated amount of pol α enzyme complex in buffer A. The mixtures were incubated at 30 °C for the times indicated, and the reactions were terminated by the addition of 4 μL of 1% SDS, 60 mM EDTA, and 1% bromophenol blue. A fraction (25%) of each reaction mixture was analyzed on both 0.8% agarose and 3–8% gradient native polyacrylamide gels. The agarose gel electrophoresis was carried out in 1× TAE for 3 h at 65 V. The native polyacrylamide gel electrophoresis was carried out in 1× TBE

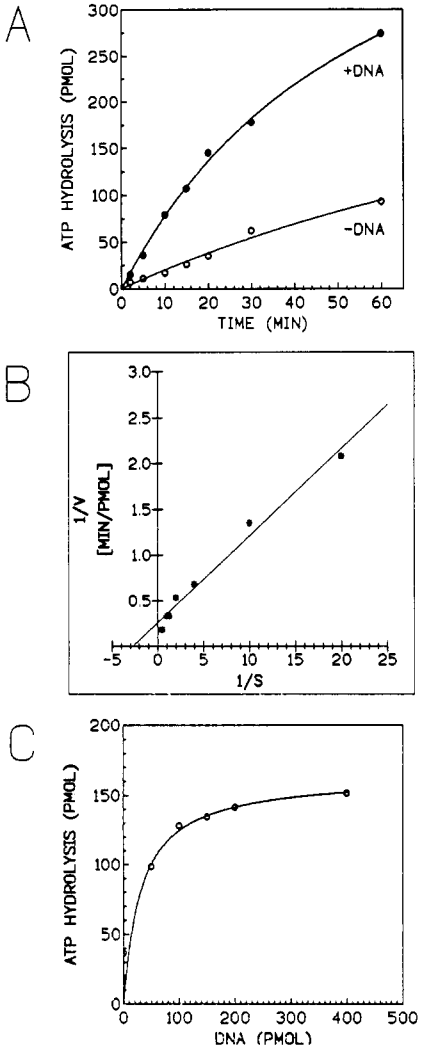

FIGURE 1: (A) ATPase activity of polymerase α complex in the absence or presence (as shown) of 200 pmol of M13mp18 ssDNA. (B) Linweaver–Burk plot of ATPase activity. (C) DNA dependence of polymerase α complex ATPase activity. The assay was carried out in the presence of 0, 50, 100, 150, 200, and 400 pmol of M13mp18 ssDNA. The details of the ATPase assay have been described under Materials and Methods. Each curve was generated by nonlinear (A and C) or linear (B) regression analysis of the data.

for 1.5 h at 160 V. Following electrophoresis, the gels were dried and autoradiographed at −80 °C for 12 h.

RESULTS

ATPase and dATPase Activities of DNA Polymerase α Complex. We have shown earlier that a single-stranded DNA-dependent ATPase activity is tightly associated with a high molecular weight complex of yeast DNA polymerase α. ATPase activity was stimulated from 2- to 5-fold by single-stranded DNA (Figure 1). Kinetic analysis of the DNA-stimulated ATPase activity (Figure 1B) showed that the K_m for ATP is low, 0.375 μM, indicating that the ATPase bound ATP very tightly. Complete saturation of DNA stimulation was observed at a low DNA concentration. Half-maximal stimulation was observed at 2 nmol (as nucleotide) of ssDNA/mL. Complete saturation was observed at 10 nmol/mL (Figure 1C). RP-A somewhat stimulated the DNA-dependent ATPase (Figure 2A,B). In the presence of single-stranded M13mp18 ssDNA cofactor, the ATPase activity was stimulated only ~20% by RP-A (Figure 2A). RP-A did not have

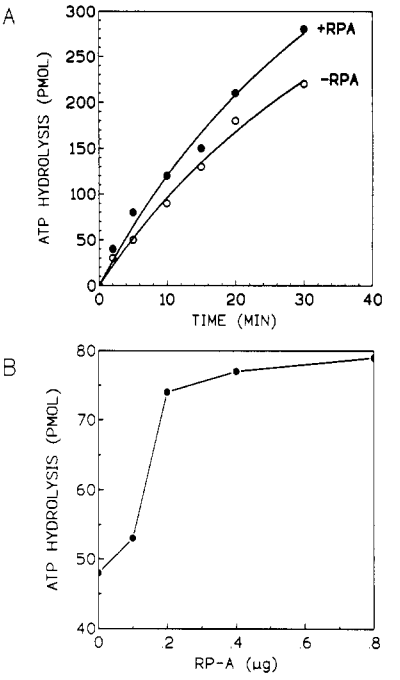

FIGURE 2: ATPase activity of polymerase α complex: (A) with 200 pmol of M13mp18 ssDNA in the absence or presence of 240 ng of RP-A; (B) with 200 pmol of poly(dT), 0.1 units of pol α complex and RP-A (amounts as indicated).

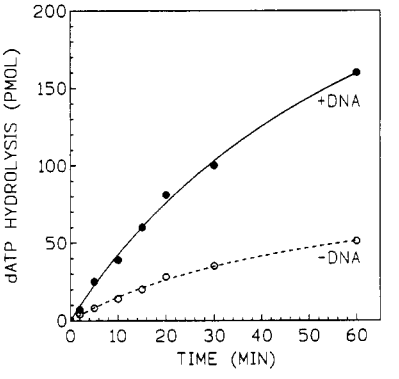

FIGURE 3: dATPase activity of the polymerase α complex in the absence or presence of 200 pmol of M13mp18 ssDNA. The dATPase assay was carried out as described under Materials and Methods.

any effect on the ATPase activity in the absence of ssDNA or in the presence of native double-stranded DNA (data not shown).

The ATPase also hydrolyzed dATP with a reduced rate (Figure 3). The ATPase required the presence of a divalent cation for activity. Both Mg^{2+} and Mn^{2+} proved to be effective metal cofactors for the ATPase. Maximal activity with either cation was at 1.5 mM $MgCl_2$ or $MnCl_2$ (Figure 4). The activity decreased only slightly with increasing Mg^{2+} concentration. However, with Mn^{2+}, the activity was inhibited by Mn^{2+} at concentrations higher than 1.5 mM.

Analysis of Size and Composition of DNA Stimulators of the ATPase. We have analyzed a series of synthetic and native oligomers and homopolymers in order to understand the influence of DNA on the ATPase activities (Table I). Native single-stranded DNA with length greater than 35 nts could stimulate the ATPase activity very effectively. ATPase responded to various single-stranded homopolymers and oligomers very differently depending on the structure and length of the effector. In general, oligopyrimidines were twice as effective as oligopurines of the same size. The same was

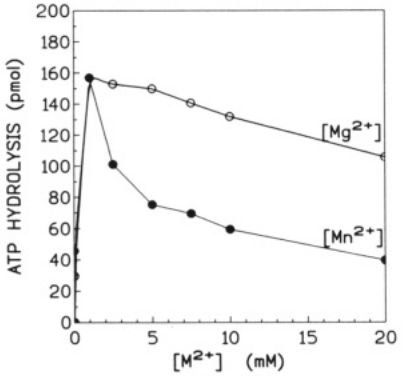

FIGURE 4: Influence of $[Mg^{2+}]/[Mn^{2+}]$ on ATPase activity. The standard ATPase assay was carried out in the presence of varying amounts of Mg^{2+} or Mn^{2+} using pol α complex.

Table I: Influence of Various DNA Effectors on ATPase Activity

DNA	% activity
ssM13mp18	100
poly(dC)	121
$poly(dT)_{1000}$	82
poly(dA)	42
activated calf thymus DNA	124
oligonucleotide 60-mer	102
oligonucleotide 35-mer	79
$(dA)_{20}$	32
$(dT)_{20}$	59
$(dT)_{16}$	59
$(dA)_{10}$	15
$(dT)_{10}$	34
$(dT)_8$	25
$(dA)_4$	12
poly(dG)/poly(dC)	41

true for polypyrimidines. $(dT)_{20}$ stimulated the ATPase to 59%, and the extent of stimulation decreased with an apparent linearity with the size of the oligomer. It would appear that oligonucleotides greater than 35 bp in length and containing an average number of pyrimidine nucleotides would stimulate the ATPase effectively. Several groups have described the purification of DNA-dependent ATPases that perfer multiprimed ssDNA over ssDNA alone. The notion of specialized stimulation by the 3′-hydroxyl end of the primed DNA template would seem quite plausible given their role in DNA replication. Consequently, we have also analyzed the effects, if any, of the primer-template junctions using poly(dA)/$(dT)_{20}$ and poly(dT)/$(dA)_{20}$ with increasing primer to template ratio. With either DNA, it appeared that an increase in the primer concentration, with the total DNA remaining constant, did not lead to an increase in the stimulation of the activity (Figure 5). In fact, an increase in the primer:template ratio led to a decrease in the stimulation. At a 1:1 ratio, where the DNA will be virtually double stranded, the stimulation was low and comparable to the minimal effects of dsDNA on the ATPase activity. Thus, the concentration of 3′-OH termini of primers present in the primed DNA template had no positive effect on the ATPase of the pol α complex. It was also evident from this analysis that the poly(dT), as a single or partially single-stranded DNA, was a better stimulator of the ATPase activity. However, when poly(dT) or poly(dA) were hybridized with oligomers to near-saturation, they became equally ineffective DNA stimulators.

Photo-Cross-Linking of DNA Polymerase α to ATP. In order to identify the likely subunit of the multiprotein DNA polymerase α complex that might function as an ATPase, we have photo-cross-linked the complex to [α-^{32}P]ATP. The only polypeptide that cross-linked with reasonable efficiency was

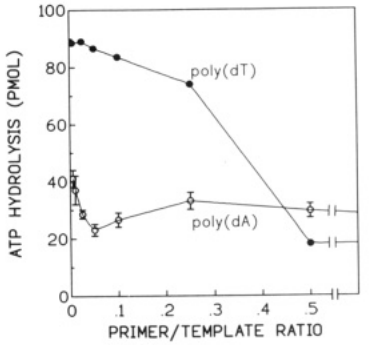

FIGURE 5: Effect of primer:template ratio on ATPase stimulation. The standard ATPase assay was carried out using 200 pmol of poly(dT) and increasing amounts of oligo(dA) primer as indicated.

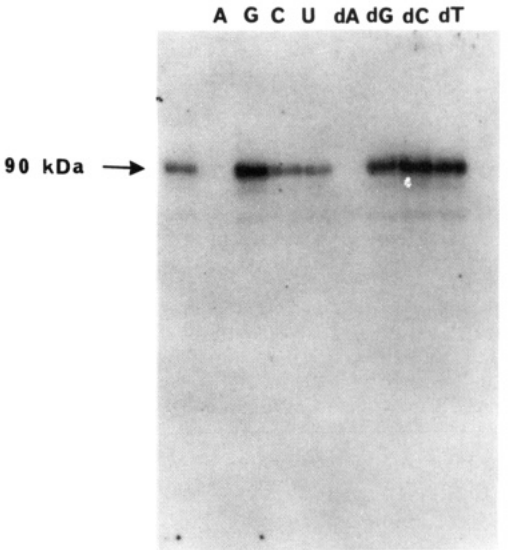

FIGURE 6: ATP photo-cross-linking of the ATPase subunit of the polymerase α complex and inhibition of ATP photo-cross-linking by ribo- and deoxynucleotides (25 μM). The experimental details are given under Materials and Methods.

the 90-kDa polypeptide (p90) (Figure 6). We have further examined the specificity of p90 for nucleotide binding by challenging the ATP cross-linking with various ribo- and deoxynucleotides. The ATP incorporation was totally inhibited by unlabeled ATP and dATP, but other ribo- and deoxynucleotides were completely ineffective in competing out the ATP incorporation. We observed some increase in ATP incorporation in the presence of the nonspecific nucleotides which is likely due to decreased nonspecific binding and concomitant loss of [α-^{32}P]ATP during the cross-linking process.

DNA Unwinding Associated with the DNA Polymerase α Complex. Hübscher and Stalder (1985) have reported the association of a DNA-dependent ATPase and helicase with a form of DNA polymerase α from calf thymus. This study was based on the use of a synthetic fork with an oligo(dT)-tailed pentadecamer primer hybridized to a M13mp8 ssDNA. One of the problems associated with a small primer is that the melting energy of the duplex is low, and as a result, a simple displacement of the primer may occur. Consequently, we have prepared a 60-mer with a 5 bp tail, in addition to a heptadecamer substrate, prepared according to Hübscher and Stalder (1985). Synthetic forks were created involving each of these primers and M13mp19 ssDNA.

We have analyzed the purified multiprotein DNA polymerase α complex and the immunoaffinity-purified four-subunit complex for helicase activity in the presence and

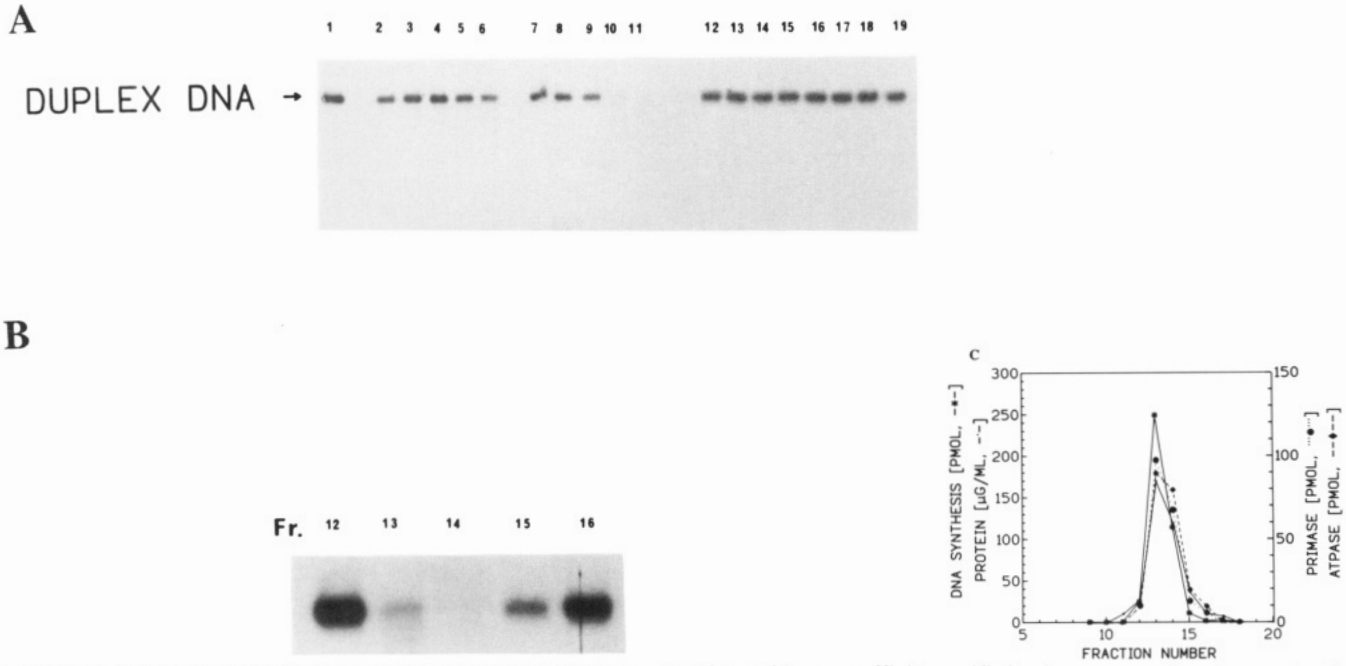

FIGURE 7: (A) Unwinding of a 17-mer primer by the polymerase α complex and immunoaffinity-purified polymerase α. Equal amounts of activated calf thymus activity were used in each case. Lane 1, 17-mer helicase substrate. Lanes 2–6, unwinding by the pol α complex in the absence of yeast RP-A. Lanes 7–11, unwinding by the pol α complex in the presence of 160 ng of yeast RP-A. Unwinding activity of the immunoaffinity-purified pol α, in the absence (lanes 12–15) and the presence (lanes 16–19) of 400 ng of yeast RP-A. Reaction times were as follows: lanes 2, 7, 12, and 16, 0 min; lanes 3 and 8, 2 min; lanes 4, 9, 13, and 17, 5 min; lanes 5, 10, 14, and 18, 15 min; lanes 6, 11, 15, and 19, 30 min. (B) Unwinding activity of HPLC fractions of polymerase α complex. The reactions were carried out for 15 min at 30 °C and analyzed on an 0.8% agarose gel as described under Materials and Methods. (C) HPLC elution profile of the multiprotein polymerase α complex. The details of HPLC fractionation were as described earlier (Biswas et al., 1993).

absence of purified RP-A, which we thought may play a role similar to *E. coli* single-stranded DNA binding protein (SSB) in helicase action. We used oligodeoxynucleotide-tailed heptadecamer primer hybridized to M13mp19 DNA for an absolute comparison of the helicase activity of the multiprotein complex of polymerase α and immunoaffinity-purified polymerase α and RP-A (Figure 7A). Immunoaffinity-purified pol α could not unwind the heptadecamer in the absence (lanes 12–15) or presence (lanes 16–19) of RP-A. Thus, unwinding of even the simplest helicase substrate was not an inherent property of the immunoaffinity-purified pol α or RP-A. However, the DNA polymerase α complex displayed a weak unwinding activity (lanes 2–6) which was significantly enhanced when 400 ng of RP-A was present in the reaction (lanes 7–11). The unwinding activity comigrated with the polymerase activity of the pol α complex in the HPLC fractionation (Figure 7B,C). We have further examined this unwinding activity of the DNA polymerase α complex with and without RP-A utilizing a 60-mer substrate (Figure 8). The reaction products were analyzed on 0.8% agarose (Figure 8A) and 3 to 8% gradient native polyacrylamide gels (Figure 8B). The use of these gels in conjunction with one another allowed us to obtain a comlpete picture of the duplex DNA and the 60-mer ssDNA after unwinding. The agarose gel was used for clearly analyzing the duplex DNA, but the primer DNA was difficult to detect in the agarose gel. The 3–8% polyacrylamide gel permitted the visualization of the single-stranded primer generated as a result of duplex unwinding. DNA polymerase α complex alone carried out unwinding of the substrate, although weakly (lanes B–E). RP-A was unable to carry out any unwinding by itself (lanes J–L). However, when RP-A was added to the DNA polymerase α complex, the DNA unwinding was very efficient (lanes F–I). In fact, all of the input DNA was unwound in approximately 2 min. This result is highly comparable to that observed with the T

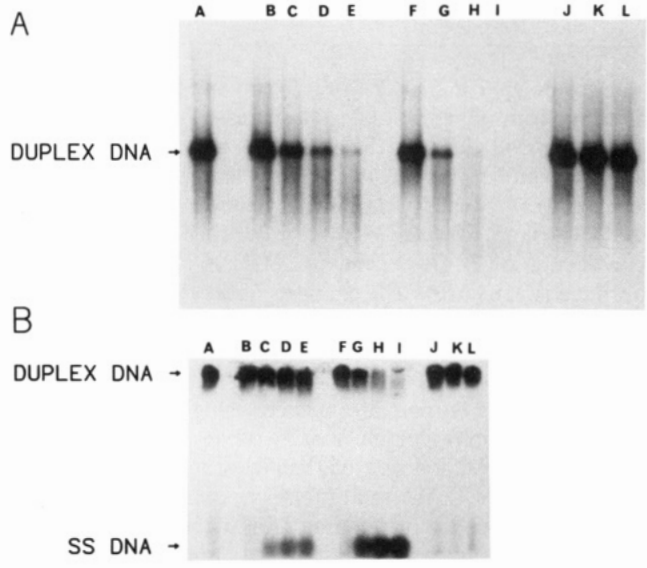

FIGURE 8: Effect of RP-A on the DNA unwinding activity of polymerase α complex. The assays were carried out using M13mp19 ssDNA primed with the 60-mer oligonucleotide. Unwinding activity of 150 ng of pol α complex (Fr V) in the absence and presence of 160 ng of yeast RP-A. (A) 0.8% agarose gel analysis of the reaction mixtures. Lanes were as follows: A, duplex DNA alone; B–E, contained pol α complex; F–I, contained pol α and RP-A; J–L, RP-A alone. Incubation periods, at 30 °C, were as follows: B and F, 0 min; C, G, and J, 2 min; D, H, and K, 5 min; E, I, and L, 10 min. (B) A 3–8% native polyacrylamide gel analysis of the reaction mixtures; lanes were as described in panel A.

antigen and DnaB protein, both of which are known helicases. *E. coli* SSB had a marginal effect on the helicase activity (data not shown).

We have explored the directionality of DNA unwinding by using a linear M13mp19 DNA hybridized to a 43-mer at the 5′-terminus and to a 17-mer at the 3′-terminus (Figure 9).

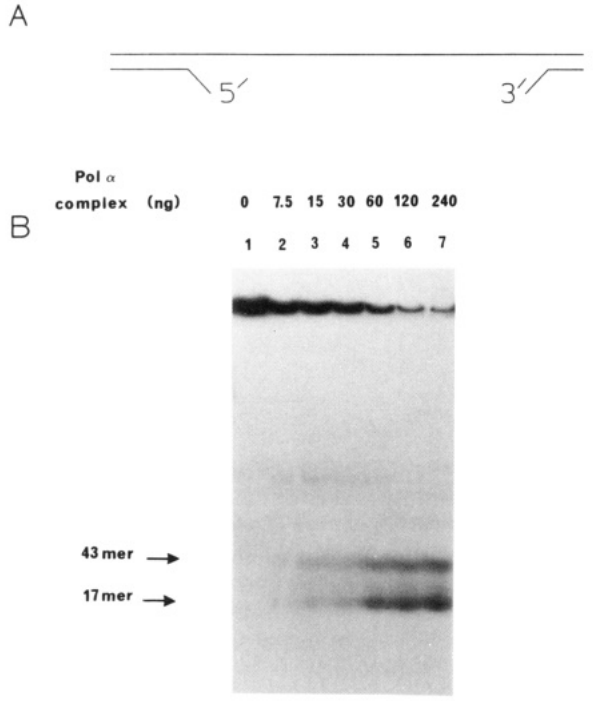

FIGURE 9: (A) Linear unwinding substrate with duplex ends consisting of a 43-mer at the 5′-end and a 17-mer at the 3′-end. (B) Unwinding of the linear substrate, as shown above, by the polymerase α complex. The unwinding assays were carried out as described under Materials and Methods using 200 ng of RP-A and pol α complex as indicated for 15 min at 30 °C.

Titration of the pol α complex in the unwinding of this substrate failed to show any unique directionality.

DISCUSSION

We have analyzed the DNA-dependent ATPase activity associated with the DNA polymerase α complex (Biswas et al., 1993). THe fact that the DNA-dependent ATPase is associated with polymerase α and this class of enzymes is known to play important roles in various stages of replication makes analysis of this ATPase particularly intriguing (Kornberg, 1980, 1982; Lebowitz & McMacken, 1986; Matson et al., 1983, Matson, 1991; Venkatesan et al., 1982). We have characterized the general features of the ATPase including the probable activity site, substrate requirements, specificity, its DNA cofactors, and kinetic parameters so that the ATPase could be compared with other known ATPases. We have also examined the probable DNA unwinding activity of the ATPase. The ATPase hydrolyzed ATP and dATP quite efficiently. The rate of hydrolysis of dATP was approximately 50% of that observed with ATP. The DnaB protein of *E. coli*, which is an ssDNA-dependent ATPase, is also capable of hydrolyzing dATP with a diminished rate. In both ATP and dATP hydrolysis, the hydrolysis was stimulated significantly by DNA. The stimulation of ATPase was observed with poly- and oligonucleotides of various sizes. It was very clear from our analysis that the polymerase-associated ATPase has a sequence preference (Table I). Poly- and oligopyrimidines were more effective stimulators of ATPase than the corresponding poly- and oligopurines. Recently, Kim et al. (1992) have shown that human RP-A and yeast RP-A preferentially bind to pyrimidine-rich strands with 50-fold higher affinity with a binding site size of 30 nucleotides. We have shown earlier that the yeast DNA primase alone or in a complex with polymerase α synthesized longer (multimeric) and larger amounts of primer with polypyrimidines than with native DNA templates (Biswas & Biswas, 1988). Consequently, it appears that many eukaryotic replication proteins have higher affinity for pyrimidine-rich DNA than purine-rich DNA. With regard to the size of the effector DNA, our results indicated that the optimal size of the DNA effector would be between 35 and 60 nucleotides. At 35 nucleotides, the stimulation of ATPase is 80%. We have analyzed the effect of 3′-OH termini of the primed DNA template on the ATPase activity. With both poly(dA) and poly(dT) DNA, increasing the primer:template ratio did not stimulate the activity, and the activity was actually diminished with an increase in the ratio which is likely due to the conversion of ssDNA to dsDNA. Therefore, this ATPase was purely ssDNA-dependent and did not have any preference for the primer-template junction like that reported for RF-C (Fien & Stillman, 1992).

A 90-kDa (p90) polypeptide in the DNA polymerase α large complex bound ATP and dATP with high specificity. The ATP cross-linking to p90 could be inhibited only by ATP or dATP. Other nucleotides were completely ineffective. Thus, the nucleotide binding pattern of p90 is consistent with the ATPase and dATPase activities of this enzyme and most likely the p90 is the activity site of the ATPase. It has been reported that the 40-kDa (or 41-kDa) polypeptides in both human and yeast RF-C are the ATP binding and the ATPase activity site. Consequently, the polymerase α associated ATPase appears to be distinct from RF-C in both physical and mechanistic characteristics.

Several helicases from *E. coli* have been extensively characterized. The bacterial helicases are all DNA-dependent ATPases. One of these helicases, the DnaB helicase, is an ATPase/dATPase, and its helicase action requires single-stranded DNA binding protein (SSB) and perhaps other replication proteins such as primase (Lebowitz & McMacken, 1986). The DnaB protein is likely the helicase for replication of the *E. coli* and λ genomes. The association of the polymerase, primase, and the ATPase in the complex encouraged us to explore any DnaB-like duplex DNA unwinding activity of this complex (Lebowitz & McMacken, 1986). We have designed two substrates for examining the unwinding activity: a 17-mer or a 60-mer oligonucleotide hybridized to M13mp19 ssDNA with protruding 3′-OH termini. Both yeast RP-A and immunoaffinity-purified four-subunit polymerase α were inactive in unwinding either of these substrates, alone or together. The purified polymerase α complex could unwind either of the two substrates, and the unwinding was stimulated significantly by yeast RP-A. *E. coli* SSB stimulated the unwinding less significantly (data not shown). RP-A has been shown to be involved in a variety of cellular functions such as DNA recombination and replication (Heyer & Kolodner, 1989; Heyer et al., 1990; Erdlie et al., 1991), similar to that observed with *E. coli* SSB. Our studies did not show any preference for the direction of translocation of the helicase activity. The association of an ATPase with the multiprotein yeast DNA polymerase α, with an RP-A-dependent duplex unwinding activity, could have important implications in the function of this multiprotein complex in chromosomal DNA replication. Three helicases have recently been described (Sugino et al., 1986; Sung et al., 1987a,b; Abussekhara, 1989; Harosh et al., 1989). Two independent helicases from mammalian sources have been described; however, the structure and function of these helicases are still not complete (Seo et al., 1991; Hubscher & Stalder, 1986). Seo et al. (1991) reported the purification of a DNA helicase from HeLa cells that is uniquely human RP-A-dependent. Further studies

are necessary in order to establish whether the observed DNA unwinding activity associated with the multiprotein pol α complex represented the true cellular helicase activity, especially in light of the fact that in *E. coli* there are multiple SSB-dependent helicases (Kornberg, 1980, 1982; Matson & Kaiser-Rogers, 1990; Matson 1991), out of which perhaps the DnaB helicase is the only one that is indispensable in the replication of chromosomal DNA.

In summary, purification and characterization of the multiprotein complex with ssDNA-dependent ATPase, unique $5' \rightarrow 3'$ exonuclease, and a putative RP-A-dependent DNA unwinding activity suggested that this polymerase assembly can carry out many of the functions required for both replication fork movement and replication of the lagging strand in the presence of RP-A.

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