Purification and Characterization of DNA Polymerase α-Associated Replication Protein A-Dependent Yeast DNA Helicase A†

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ABSTRACT: A novel, eukaryotic, hexameric DNA helicase that was earlier identified as a component of the multiprotein polymerase α complex [Biswas et al. (1993) *Biochemistry 32*, 13393−13398] has been purified to homogeneity and characterized. Thus far, our studies demonstrated that helicase A shares certain unique features of two other hexameric DNA helicases: the DnaB helicase of *Escherichia coli* and the T-antigen helicase of the SV40 virus. The helicase activity was stimulated by yeast replication protein A (RPA) and to a lower extent by *E. coli* single-stranded DNA binding protein (SSB). The helicase had an apparent molecular mass of 90 kDa, as determined by its mobility on sodium dodecyl sulfate−polyacrylamide gel electrophoresis. A tryptic peptide fragment of the polypeptide was sequenced followed by a BLAST search of GenBank with the tryptic peptide sequence. The search identified a 1.8 kb open reading frame previously designated as ykl017c on chromosome XI, that codes for a 78.3 kDa (683 amino acid) polypeptide. The important features of the polypeptide sequence of helicase A included a type I ATP/GTP binding motif, and a *K E E R R* L N V A M T *R P R R* sequence at the C-terminus that may be indicative of a nuclear localization signal which is required of a nuclear DNA helicase. The polypeptide sequence of helicase A appears to have homology to the DnaB helicase of *E. coli* (∼25%). The facts that these two helicases are vastly separated by evolution and retained similar structural and functional features, as demonstrated here, point to a possible significance of this limited homology. Although the amount of purified helicase A was limited, we have carried out necessary enzymatic characterization so that these data could be correlated with that of immunoaffinity-purifed helicase A and recombinant helicase A expressed in heterologous systems.

It is well-established that the replication of chromosomal DNA requires a large number of proteins and enzymes with a variety of enzymatic activities, in addition to DNA polymerases (Kornberg & Baker, 1992; DePamphilis, 1993a,b). During DNA replication, only the DNA helicases carry out the pivotal role of unwinding the duplex DNA so that the replication fork can proceed. A number of DNA helicases have been purified and characterized in prokaryotes and eukaryotes, including several from yeast (Kornberg & Baker, 1992; Matson, 1994; Thommes et al., 1992; Tuteja et al., 1995; Tuteja & Tuteja, 1996). Some of these helicases have been shown to be involved in DNA replication, such as DnaB protein, Rep protein, and n′, while others have been implicated in DNA repair or transcription. Eukaryotic RAD3 protein from *Saccharomyces cerevisiae* has now been established as the DNA helicase involved in nucleotide excision repair in yeast (Prakash et al., 1993; Sung et al., 1987, 1994).

DnaB helicase of *Escherichia coli* has been shown to function in chromosomal DNA replication (Lebowitz & McMacken, 1986; Baker et al., 1987). The DnaB protein acts as the major helicase in the replication of *E. coli* origin of replication, *OriC*, (Baker et al., 1987; Lebowitz & McMacken, 1986). It is stimulated by the *E. coli* single-stranded DNA binding protein (SSB)[1] and primase of *E. coli* (Kornberg & Baker, 1992). The bacteriophage T7-encoded gene 4A-B helicase/primase protein complex is involved in the replication of T7 phage DNA replication. The bacteriophage T4 DNA helicase (gp41) is involved in the replication of bacteriophage T4 genome (Bernstein & Richardson, 1989; Dong & von Hippel, 1996; Venkatesan et al., 1982). The eukaryotic homologue of DnaB helicase has yet to be identified, although the T-antigen of SV40 virus functions as a DNA helicase in the replication of SV40 genome and can be utilized by eukaryotic DNA polymerases during *in vitro* replication of SV40 viral DNA (Goetz et al., 1988; Scheffner et al., 1989). All of these helicases, which are involved in the replication of their respective genomes, have been shown to have similar structural and enzymatic characteristics. The DnaB protein is hexameric and requires Mg^{2+} ion. Bacteriophage T7 gene 4 and T4 gp41, as well as the SV40 viral T-antigen, are all hexameric DNA helicases (Dong et al., 1995; Dong & von Hippel, 1996; Notarnicola et al., 1995; Dean et al., 1992). The hexameric state is normally dynamic and requires specific conditions such as Mg^{2+} and ATP (Arai & Kornberg, 1981). In some cases, the hexameric structure is assembled on the fork itself (Dong

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[1] Abbreviations: aa, amino acid; HcsA, helicase A; RPA, replication protein A; pol α, DNA polymerase α; Tris, tris(hydroxymethyl)-aminomethane; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; RF-C, replication factor C; SSB, single-stranded DNA binding protein of *Escherichia coli*, and TCA, trichloroacetic acid.

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& von Hippel, 1996). The DNA helicases have characteristic polarities of movement, and the DNA replication helicases, such as DnaB, have 5′ → 3′ polarity. In addition, these replicative helicases all appear to interact with the polymerase and/or primase. T4 gp41 helicase interacts with T4 primase (gp61); DnaB helicase interacts with DNA primase and the τ subunit of DNA polymerase III holoenzyme (Kim, et al., 1996); and SV40 T-antigen associates with the pol α−primase (Dornreiter et al., 1990, 1992). All of these helicases require a single-stranded DNA binding protein for efficient function. In eukaryotes, the replication protein A (RPA) is the primary single-stranded DNA binding protein and has been shown to be involved in replication and recombination (Wold et al., 1987; Wold & Kelly, 1988; Brill & Stillman, 1989; Erdile et al., 1991; Alani et al., 1992). Thus, the chromosomal DNA replication helicases appear to have common modes of action.

Previously, we reported the purification of a multienzyme complex of DNA polymerase *S. cerevisiae* which has both DNA-dependent ATPase and RPA-dependent DNA helicase activities (Biswas et al., 1993a,b). Using pol α immunoaffinity chromatography, we were able to isolate the helicase/ATPase (helicase A; Biswas et al., 1993b). This helicase was distinguishable from the 127 kDa helicase B (Biswas et al., 1995) as well as the recently identified 170 kDa yeast Dna2 helicase (Budd et al., 1995). Our previous studies demonstrated that the helicase correlated with a 90 kDa polypeptide by ATP photo-cross-linking. Although the immunoaffinity purification scheme was invaluable in further identification of the helicase protein, the yields of protein obtained were insufficient for further biochemical and structural characterizations. As a consequence, we needed to develop a scheme of large-scale purification for yeast helicase A. In this report we describe the purification to homogeneity, genetic identification, and molecular characterization of yeast DNA helicase A. An accompanying report (Biswas et al., 1997) describes expression of functional yeast helicase A in *E. coli* and the characterization of the recombinant protein.

MATERIALS AND METHODS

Yeast. Protease-deficient yeast, *S. cerevisiae*, BJ 2168 strain was from the Yeast Genetic Stock Center (Berkeley, CA) and was grown in a laboratory fermenter to midlog phase, following which the cells were chilled to 4 °C and harvested by centrifugation. The cells were then resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 10% glycerol to OD 400 (λ = 600 nm) and stored frozen at −80 °C until further use.

Nucleic Acids, Enzymes, and Other Reagents. Oligonucleotides were synthesized by Oligos Etc. (Portland, OR). Ultrapure deoxy- and ribonucleotides were obtained from Pharmacia-LKB (Piscataway, NJ) and were used without further purification. [α-^{32}P]ATP, [α-^{32}P]dATP, and [γ-^{32}P]-ATP were obtained from Dupont/NEN (Boston, MA). RPA used in this study was purified to homogeneity from wild-type yeast as described by Brill and Stillman (1989). *E. coli* SSB and terminal deoxynucleotidyltransferase were purchased from U.S. Biochemical Corp. (Cleveland, OH). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). All chemicals used to prepare buffers and solutions were reagent-grade and were purchased from Fisher Scientific Co. (Pittsburgh, PA). Protease inhibitors were from Bachem (Los Angeles, CA). Poly(ethylenimine)−cellulose strips for thin-layer chromatography were from Fisher Chemical Co.

Buffers. Buffer A contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.01% (v/v) NP40, 1 mM EDTA, and NaCl as indicated. Buffer B was analogous to buffer A, except that it contained 25 mM Hepes (pH 7.5) instead of Tris-HCl. Buffer C contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT. 1× TBE buffer was 89 mM Tris−borate and 2.5 mM EDTA (pH 8.3).

ATPase Assays. The ATPase assays were carried out as previously described (Biswas et al., 1993b). The standard reaction mixture contained buffer C, 10 mM $MgCl_2$, 200 pmol of M13mp18 ssDNA, 100 μM [^{32}P]ATP (1000−2000 cpm/pmol), and helicase protein, as indicated. 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. Aliquots (1−2 μL) were applied to poly(ethylenimine)−cellulose strips that were prespotted with ADP−ATP marker. The strips were developed with 1 M formic acid and 0.5 M LiCl and dried. The ADP−ATP spots were located by UV fluorescence at 254 nm. The portions containing ATP and ADP were excised and counted in a liquid scintillation counter using a toluene-based scintillator.

Helicase Assays. The helicase assays were based on the methods described by Matson et al. (1983) and Biswas et al. (1993b).

(A) Preparation of the Helicase Substrate. A synthetic 45-mer oligonucleotide, complementary to a 35 bp sequence between nucleotides 6268 and 6302 of M13mp19 ssDNA, contained a 10 nucleotide tail on its 3′ terminus. The oligonucleotide was labeled at its 5′ end using T4 polynucleotide kinase. The oligonucleotide was hybridized to M13mp19 as previously described (Biswas et al., 1993b). Excess unhybridized labeled oligomer was removed by spin column purification (Promega Biotech, Madison, WI). The purified substrate was diluted to 17 fmol/μL with 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA.

(B) Assay Conditions. Reaction mixtures were set up on ice as follows. A standard 20 μL reaction volume contained buffer C, 10 mM $MgCl_2$, 3.4 mM ATP, helicase substrate, and the indicated amount of DNA helicase. 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 (60%) of each reaction mixture was analyzed on 8% polyacrylamide gels in TBE and 0.1% SDS. The electrophoresis was carried out in TBE and 0.1% SDS for 1 h at 160 V. Following electrophoresis, the gels were dried and exposed to Fuji XAR-5 film for 12 h at −80 °C.

Photo-Cross-Linking of the ATP Binding Domain of the Helicase Protein. Photo-cross-linking was carried out essentially as described earlier (Biswas & Kornberg, 1984; Biswas et al., 1993b). This photo-cross-linking is highly specific as described in these publications.

Peptide Sequencing. The helicase A polypeptide (Fr V) was resolved by SDS 5→18% gradient polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membrane. The polypeptide was visualized with 1% Ponceau S, and the stained band was excised. The immobilized polypeptide was subjected to *in situ*

digestion and peptide sequencing as described earlier (Biswas et al., 1995).

Other Methods. Protein concentrations were estimated according to the method of Bradford (1976), using bovine serum albumin as a standard. SDS−PAGE was carried out following the method described by Laemmli (1970).

RESULTS

Purification of Helicase A. All purification steps were carried out at 4 °C. Unless otherwise indicated, all buffers used in the various chromatographic steps contained 5 mM DTT and the following protease inhibitors: 1 μg/mL each leupeptin, pepstatin A, antipain, and chymostatin; 0.1 mM each benzamidine hydrochloride, and $NaHSO_3$; and 2.5 μg/mL each TPCK and TLCK. A flowchart of the purification is shown below (Scheme 1).

Scheme 1

yeast extract (fraction I)

↓

phosphocellulose chromatography (fraction II)

↓

ssDNA−cellulose chromatography (fraction III)

↓

POROS-HQ/H HPLC (fraction IV)

↓

POROS-HS/H HPLC (fraction V)

Yeast extract was prepared from ∼400 g of log-phase protease-deficient yeast (strain BJ2168, Yeast Genetic Stock Center, Berkeley, CA) as previously described (Biswas et al., 1993a). The initial purification of DNA polymerase α−primase complex with associated activities was carried out as described earlier (Zhu et al. 1997). The isolation of helicase A was then carried out as follows.

The fractions from the phosphocellulose chromatography containing DNA polymerase α activity were pooled (Fr II) and dialyzed against buffer A-0 until the conductivity was that of buffer A-50 and loaded onto a 10 mL ssDNA−cellulose column. The column was washed with buffer A-100 and eluted with a 120 mL gradient of buffers A-100→1000. These fractions were assayed for helicase and ATPase. The active fractions were dialyzed against A-0 until the conductivity was that of buffer A-50 and then loaded onto a 2 mL (0.46 × 10 cm) POROS HQ/H column (PerSeptive Biosystems Inc., MA) equilibrated with buffer A-50. The column was washed with 50 mL of buffer A-50 and eluted with a 50 mL gradient of buffer A-100→A-500. Under these conditions, the resolution of this column appeared to be optimal. Helicase A eluted from the column in a single peak at a salt concentration of ∼300 mM NaCl, and the helicase activity appeared to comigrate with a ∼90 kDa polypeptide (Figure 1). The fractions were reasonably pure at this stage. The major helicase/ATPase fractions were pooled (Fr IV) and dialyzed to adjust the conductivity of the pool to that of buffer B-100. Fraction IV was then loaded onto a 1 mL POROS HS/H column (PerSeptive Biosystems, Cambridge, MA) equilibrated with buffer B-100. The helicase was eluted

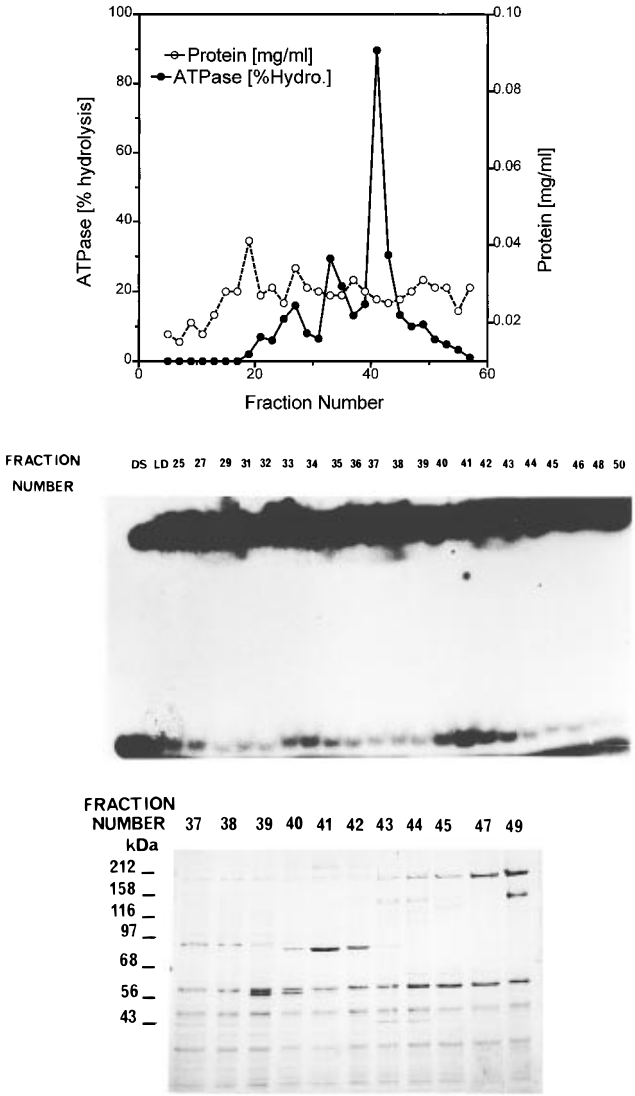

FIGURE 1: Anion-exchange chromatographic fractionation of helicase A. Details of the POROS HQ/H chromatographic procedure are given in Results. Aliquots of the indicated chromatographic fractions were assayed for protein, ATPase, helicase activities, and by SDS−PAGE. (A, top panel) Analysis of protein and ATPase activities. A standard ATPase assay was carried out using 2 μL of the indicated fractions. The percent hydrolysis represents that obtained during a 30 min assay. (B, middle panel) Analysis of the helicase activity. A standard helicase assay was carried out using 1 μL of the indicated fractions, and the autoradiogram of the helicase products is shown. (C, bottom panel) SDS−PAGE analysis of the HQ/H fractions. Aliquots of the indicated fractions were resolved on an SDS polyacrylamide gel (5→18%) followed by silver staining.

with a 20 mL gradient of buffers B-100→B-500. The active fractions (Fr V) were pooled and stored at −80 °C. SDS−PAGE analysis of helicase A (Fr V) is shown in Figure 2. The final chromatographic step, POROS HS/H cation-exchange chromatography, appeared to purify helicase A to homogeneity (Figure 2). The amount of protein obtained from this procedure was quite low. Approximately 5 μg of the helicase protein was obtained from ∼400 g of yeast cells. As a result, extensive enzymological studies as well as structure−function analysis of helicase A were quite difficult. SDS−PAGE analysis of the purified helicase is shown in Figure 2B, and it clearly demonstrated its homogeneity. The molecular weight of ∼90 kDa was extrapolated from a least-squares linear regression analysis of the molecular weight markers. It should be noted that previous purification of

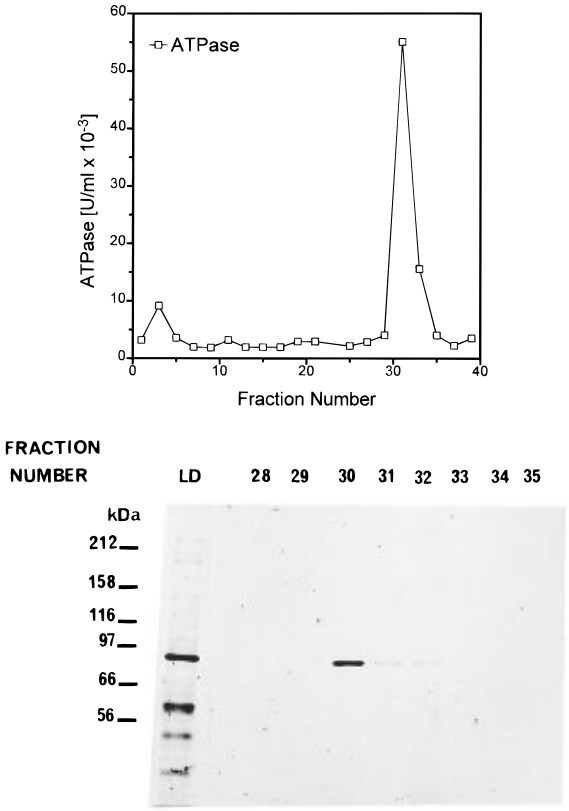

FIGURE 2: Profile of HS/H fractionation of native helicase A. Aliquots of the indicated fractions from the POROS HS/H cation-exchange chromatography were analyzed for protein, ATPase, and by SDS−PAGE. (A, top panel) Analysis of the ATPase activities. A standard ATPase assay was carried out using 1 μL of the indicated fractions. The percent hydrolysis represents that obtained during a 30 min assay. (B, bottom panel) SDS−PAGE analysis. Aliquots (100 μL) of the indicated fractions were TCA-precipitated and then resolved on an SDS polyacrylamide gel (5→18%) followed by silver staining.

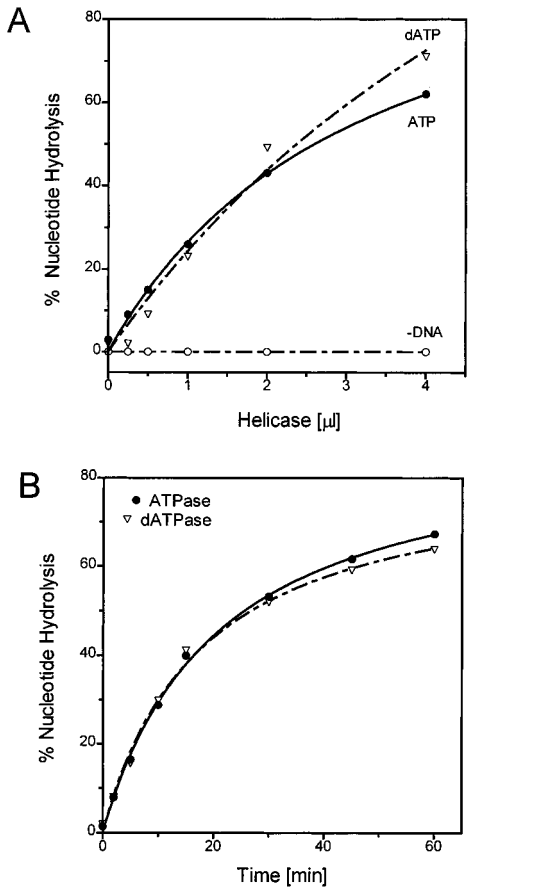

FIGURE 3: Characterization of the ATPase activity of helicase A. (A) Titration of ATPase and dATPase activities. Protein titration of ATPase and dATPase activities in the presence of 200 pmol of M13mp19 ssDNA and the ATPase activity in the absence of DNA (−DNA) is shown. (B) Time-course analysis of the ATPase and dATPase activities. Standard ATPase/dATPase assays were carried out in the presence of 200 pmol of M13mp19 ssDNA and the reaction was terminated at the indicated time points. The curves were generated by nonlinear regression analysis.

the helicase by immunoaffinity chromatography (Biswas et al., 1993c) also identified a 90 kDa polypeptide. Thus, the results of the present studies are consistent with the results of our earlier studies with helicase A.

Nucleotidase Activities of Purified Helicase A. Previous studies with partially purified helicase A indicated that it can hydrolyze both ATP and dATP and that the nucleotidase activity is DNA-dependent. Our present studies involving purified helicase A demonstrated its ATPase and dATPase activities (Figure 3A). Both of these activities are DNA-dependent. Unlike many other DNA-dependent ATPases, helicase A had no measurable ATPase activity in the absence of DNA, and thus, the DNA dependence appeared absolute. The rates of hydrolysis of ATP and dATP were indistinguishable (Figure 3B). With ~20 ng of purified helicase A, the rates of hydrolysis of ATP and dATP were 32 and 31 pmol/min respectively. The turnover number for helicase A would be approximately 17−18 nucleotides s^{-1} monomer^{-1} for both ATP and dATP.

A Lineweaver−Burk analysis of the purified helicase revealed that the purified enzyme had K_m values in the range of 50−100 μM, and the V_{max} was approximately ~2 × 10^6 pmol min^{-1} mg^{-1} (Figure 4). Thus the K_m and V_{max} values of helicase A were comparable to those observed earlier with partially purified enzyme (Biswas et al., 1993b,c).

ATP Photo-Cross-Linking to Helicase A. In our previous studies of a complex of DNA polymerase α with an associated helicase activity, we had demonstrated that the

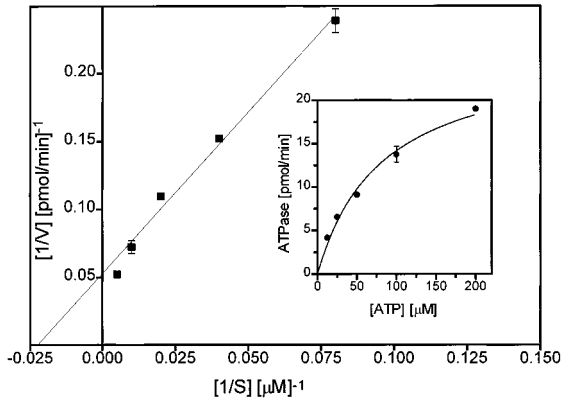

FIGURE 4: Analysis of the kinetics of DNA-dependent ATP hydrolysis. The ATPase assays were carried out as described in Materials and Methods except substrate concentrations were altered as indicated in the ATPase (V) vs ATP concentration ([S]) plot (inset). The Lineweaver−Burk ($1/V$ vs $1/[S]$) plot was generated from the plot in the inset and the best-fit line was determined by linear least-squares regression analysis of the data.

ATP binding/ATPase activity of the helicase resides within a 90 kDa polypeptide (Biswas et al. 1993a). ATP cross-linking using purified helicase A indicated that a 90 kDa protein cross-linked to ATP (Figure 5). These results are consistent with those previously observed with the pol α-associated DNA helicase A (Biswas et al., 1993b,c).

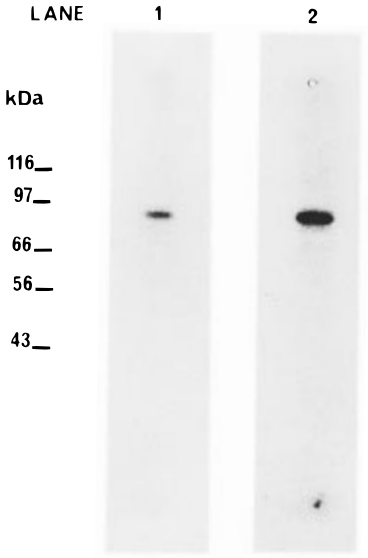

FIGURE 5: ATP cross-linking to helicase A. Cross-linking of helicase A to [^{32}P]ATP was carried out as described in Materials and Methods using 185 ng (lane 1) and 740 ng (lane 2) of helicase A Fr V.

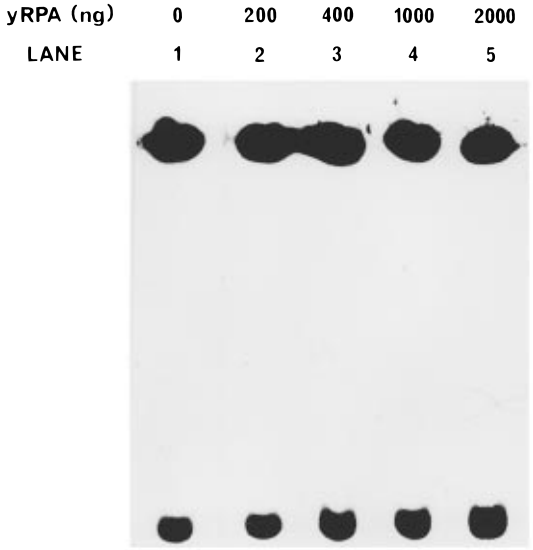

FIGURE 6: Influence of yRPA on DNA unwinding. Standard helicase assays were carried out in the presence of increasing amounts of yRPA as indicated.

DNA Helicase Activity of Helicase A and Its Modulation by RPA. The DNA unwinding activity of helicase A was stimulated by yeast RPA (Figure 6). The stimulation was approximately 3-fold, as determined by scanning densitometry (data not shown). *E. coli* SSB also stimulated yeast helicase A; however, the extent of stimulation was lower. Modulation of the helicase activity of immunoaffinity-purified helicase A was closely comparable (Biswas et al., 1993c).

Protein Sequencing and Identification of the Gene for Helicase A. In order to determine the true identity of the purified helicase/ATPase, we have analyzed the peptide sequence of the 90 kDa polypeptide. Following immobilization on PVDF membrane, the polypeptide was subjected to *in situ* digestion with trypsin. The peptides were separated by microbore reversed-phase chromatography, and the individual peptide peaks were collected. One of the purified peptides was sequenced by automated Edman degradation using an Applied Biosystems gas-phase peptide sequencer. The mass of the tryptic fragment was determined using a

A. SEQUENCE OF THE TRYPTIC PEPTIDE:

NH_2-F L S S I K H E R E Q D I Q T-COOH

B. SEQUENCE OF THE YEAST HELICASE A:

MNKELASK*FL SSIKHEREQD IQ*TTSRLLTT LSIQQLVQNG LAINNIHLEN
Peptide Sequence
IRSGLIGKLY MELGPNLAVN DKIQRGDIKV GDIVLVRPAK TKVNTKTKPK

VKKVSEDSNG EQAECSGVVY KMSDTQITIA LEESQDVIAT TFYSYSKLYI

LKTTNVVTYN RMESTMRKLS EISSPIQDKI IQYLVNERPF IPNTNSFQNI

KSFLNPNLND SQKTAINFAI NNDLTIIH**GP PGTGKT**FTLI ELIQQLLIKN
ATP Binding Motif
PEERILICGP SNISVDTILE RLTPLVPNNL LLRIGHPARL LDSNKRHSLD

ILSKKNTIVK DISQEIDKLI QENKKLKNYK QRKENWNEIK LLRKDLKKRE

FKTIKDLIIQ SRIVVTTLHG SSSRELCSLY RDDPNFQLFD TLIIDEVSQA

MEPQCWIPLI AHQNQFHKLV LAGDNKQLPP TIKTEDDKNV IHNLETTLFD

RIIKIFPKRD MVKFLNVQYR MNQKIMEFPS HSMYNGKLLA DATVANRLLI

DLPTVDATPS EDDDDTKIPL IWYDTQGDEF QETADEATIL GSKYNEGEIA

IVKEHIENLR SFNVPENSIG VISPYNAQVS HLKKLIHDEL KLTDIEISTV

DGFQGREKDV IILSLVRSNE KFEVGFL*KEE* ***RR****LNVAMTR*P ***RR***QLVVVGNI
Nuclear Localization Signal
EVLQRCGNKY LKSWSEWCEE NADVRYPNID DYL

FIGURE 7: Peptide sequence analysis of helicase A. Helicase A (Fr V) was resolved by SDS−PAGE transferred to PVDF membrane, and the 90 kDa band was excised and microsequenced, as described in Materials and Methods. (A) Sequence of the tryptic peptide; (B) deduced polypeptide sequence of helicase A. Polypeptide sequence of helicase A was derived from the sequence of the ORF [ykl017c] obtained from GenBank. The sequence of the tryptic peptide is in italic type; the type I ATP binding motif and the nuclear localization signal are underlined and in boldface type.

MALDI-TOF apparatus. The sequence of the peptide is shown in Figure 7A. A GenBank search using the peptide sequence indicated that this protein is identical to the hypothetical gene product of the 1.8 kb open reading frame (ORF) ykl017c (accession number Swiss-Prot P34243) located on chromosome 11 of the yeast *S. cerevisiae* (Wiemann et al., 1993). Although the purified protein migrated in SDS−PAGE as 90 kDa, the ORF codes for a 683 amino acid polypeptide which corresponded to a 78.3 kDa protein. The mass spectral data from MALDI-TOF analysis also matched exactly with the predicted tryptic peptide fragmentation pattern of this polypeptide.

Analysis of Sequence Homology with Other DNA Helicases. The polypeptide sequence of helicase A revealed several important structural motifs (Figure 7B): (i) a type I ATP/GTP binding motif (Walker et al., 1982) that is a common feature of many DNA helicases, including the DnaB helicase of *E. coli*, and (ii) a nuclear localization signal (NLS), *K E R R* L N V A M T *R P R R*, which is present at the C-terminus and indicates that helicase A is a nuclear protein.

A sequence homology search of various protein databases demonstrated that helicase A is homologous to a number of DNA helicases including a human DNA helicase. Most notable is its homology (∼25%) with *E. coli* DnaB helicase (Figure 8A), which is still the prototype of all replicative DNA helicases. The sequence similarities between helicase A and DnaB were limited (∼25%); however, structural and functional similarities described in this and the accompanying paper (Biswas et al., 1997) point to a possible significance of this limited homology. The complete amino acid (aa) sequence of DnaB helicase comprising of 471 residues was

A

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hcsa  12.SIKHEREQDIQTTSR.26..37.VQNGLAINNIHLENIR.52..135.QDVIATTFYSYSKLYIL.151
Dnab  10.QQAEPRERDPQVAGL.24..37.VLGGLMLDNERWDDVA.52...53.ERVVADDFYTRPHRHIF.69

hcsa  168.KLSEISSPIQDKIIQ.182..192.PNTNSFQNIKSFLNPN.207..214.TAINFAINN.222
Dnab   74.RLQESGSPIDLITLA.88...111.KNTPSAANISAYADIV.126..205.TGVNTGYDD.213

hcsa  223.DLTIIHGPPGTGKTFTLIELIQQLL.247..248.IKNPEERILICGPS.261..277.PNNLLLRIGH.286
Dnab  225.DLIIVAARPSMGKTTFAMNLVENAA.249..261.LEMPSEQIMMRSLA.274..279.VDQTKIRTGQ.288

hcsa  301.ILSKKNTIVKDISQ.314..324.KKLKNYKQR.332..335.NWNEIKLLRKDLK.347
Dnab  304.LLEKRNIYIDDSSG.317..321.TEVRSRARR.329..341.MIDYLQLMRVPAL.353

hcsa  391.TLIIDEVSQAMEPQC.405..406.WIPLIAHQNQFHKLV.420..426.KQLPPTIKT.434
Dnab  358.TLEIAEISRSLKALA.372..419.YRDEVYHENSDLKGI.433..440.KQRNGPIGT.448

hcsa  442.HNLETTLFDR.451..506.DATPSEDDD.514
Dnab  453.FNGQWSRFDN.462..463.YAGPQYDDE.471
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B

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hcsa  127.ITIALEESQ...204.LNPNLNDSQKTAINFAIN...225.TIIHGPPGTGKTFTLIELIQQLLIK..
hhcs  124.FQLSLDREN...188.FNTCLDTSQKEAVLFALS...210.AIIHGPPGTGKTTTVVEIILQAVKQ..

hcsa  261.SNISVDTILERLTPL...339.IKLLRKDLKKRE...358.IIQSRIVVTTLHGS...391.TLIIDEVSQAMEP.
hhcs  244.SNIAVDNLVERLALC...323.IKLLRKELKERE...342.LTSANVVLATNTGA...371.VVVIDECAQALEA.

hcsa  420.VLAGDNKQLPPTI...443.NLETTLFDRIIK...462.VKFLNVQYRMNQKIME...480.SHSMYNGKLLADA.
hhcs  396.ILAGDHKQLPPTT...417.GLSLSLMERLAE...435.VRTLTVQYRMHQAIMR...453.SDTMYLGQLTAHS.

hcsa  563.NVPENSIGVISPYNAQVSHLKKL...597.ISTVDGFQGREKDVIILSLVRSNEKFEVGFL..
hhcs  529.GVPARDIAVVSPYNLQVDLLRQS...561.IKSVDGFQGREKEAVILSFVRSNRKGEVGFL..

hcsa  631.RRLNVAMTRPRRQLVVVG...661.LKSWSEWCE
hhcs  595.RRINVAVTRARRHVAVIC...623.LKTLVEYFT
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FIGURE 8: Homology analysis of the amino acid sequences of helicase A and other helicases. The polypeptide sequences were aligned by matchbox server multialign algorithm. Protein sequence alignments were carried out using the supercomputer facilities at the Baylor College of Medicine [http://dot.imgen.bcm.tmc.edu:9331 and http:// www.fundp.ac.be matchbox server]. The matched domains shown were predicted as statistically significant. The residues that appear to be conserved between the helicases in both A and B are highlighted manually in boldface type. (A) Sequence alignment of helicase A and *E. coli* DnaB helicase; (B) sequence alignment of helicase A and human DNA helicase.

found to be homologous to the N-terminal 502 aa sequence between residues 12 and 514 of helicase A (Figure 8). The C-terminal 169 aa domain of helicase A did not show any homology with DnaB helicase, suggesting that this domain could be involved in specific interactions with other cellular proteins. The residues that appear to be conserved in these two proteins may have important roles in the structure and function of these two helicases. A putative human DNA helicase (accession number gi|908917) with a 993 aa polypeptide appeared to have significant homology (≥40%) to the yeast DNA helicase A. An alignment of protein sequences from yeast helicase A (*hcsa*) and human DNA helicase (*hhcs*) demonstrated that there are 15 separate domains of sequence homology (Figure 8B).

DISCUSSION

DNA helicases play the unique role of unwinding the DNA double helix during various cellular functions such as DNA replication, repair, etc. We have previously isolated a multiprotein pol α complex with 5′ → 3′ exonuclease, DNA-dependent ATPase, and DNA unwinding activities (Biswas et al., 1993a,b). We have recently shown that the 5′ → 3′ exonuclease component of the complex is the *RTH1* nuclease (Zhu et al., 1997). Using a modified pol α immunoaffinity chromatography, we were also able to purify the ATPase/helicase (Biswas et al., 1993c). However, this procedure did not allow isolation of sufficient quantities of the enzyme for further characterization, in particular, the identification of its gene. Due to a lack of availability of this chromatography matrix, it became difficult to scale up this procedure to generate a sufficient quantity. Consequently, we opted to purify helicase A by conventional chromatography employing high resolution and advanced separation technologies, which proved useful in the purification of the pol α-associated 5′ → 3′ exonuclease (Zhu et al. 1997). In this paper, we have presented the purification method for helicase A and describe the identification and characterization of its gene. In the accompanying paper (Biswas et al., 1997), we have reported the high-level expression, purification, and enzymatic characterization of the recombinant helicase A.

The methodology described here enabled us to obtain a small amount of highly purified helicase A from the yeast *Saccharomyces cerevisiae*. This allowed for preliminary characterization of the enzymatic activities of the helicase and, more importantly, for generating protein sequence information that enabled us to identify and isolate its gene. This purification method is reproducible and could be optimized and/or scaled up in order to generate larger quantities of the helicase. However, as described in the accompanying paper, recombinant helicase A, expressed in *E. coli*, provided a more convenient source for this enzyme at the present time (Biswas et al., 1997).

The helicase A is absolutely DNA-dependent in its ATPase activity. Unlike other DNA-dependent ATPases such as DnaB helicase, no nucleotide hydrolysis was observed in the absence of a DNA cofactor. The ATPase and dATPase activities were kinetically indistinguishable. These results correlated reasonably well with the finding of our earlier

studies (Biswas et al., 1993a,b). Photo-cross-linking of helicase A with [^{32}P]ATP also demonstrated that the 90 kDa polypeptide is the helicase A. The K_m for ATP was approximately 50−100 μM and the V_{max} was $\sim 2 \times 10^6$ pmol^{-1} min^{-1} mg^{-1}. The purified helicase A was a RPA-dependent DNA helicase. The helicase activity was stimulated severalfold by RPA and to a lesser extent by *E. coli* SSB. Immunoaffinity-purified helicase A (Biswas et al., 1993c) was stimulated identically and to a similar extent by RPA and SSB. Thus, with respect to both size and mechanistic features, the helicase A purified by conventional chromatography presented here and the pol α immunoaffinity-purified helicase A reported earlier (Biswas et al., 1993c) appeared to be identical.

Helicase A (Fr V) was homogeneous, as evident from the SDS−PAGE analysis. However, in order to unambiguously determine the identity of the helicase A by peptide sequencing, we isolated very precisely the 90 kDa band by SDS−PAGE followed by electrophoretic transfer to PVDF membrane. The sequence of one of the isolated peptides was used to search the GenBank database and the ykl017c ORF was identified. This ORF codes for a polypeptide of molecular weight 78 300. Although this is slightly lower than that determined by SDS−PAGE, the difference could be due to a number of reasons including aberrant mobilities in SDS−PAGE, posttranslational modification, etc. Henceforward we have referred to the ykl017c ORF as the *HcsA* gene.

The polypeptide sequence of helicase A contained a type I ATP/GTP binding motif, which is a feature of most DNA helicases (Walker et al., 1982). The sequence also contained a nuclear localization signal (Figure 7B). We have compared this sequence with the DnaB helicase sequence. Although the homology of these two protein sequences was limited (∼25%), there were 17 homologous domains shared between the two proteins (Figure 8A). These two proteins may have an evolutionary relationship. Interestingly, a significant sequence homology (≥40%) was observed with a putative human DNA helicase (Figure 8B). Alignment of the protein sequences indicated that helicase A shared with the human helicase 15 separate domains of sequence homology. As other eukaryotic genomes are not yet completely sequenced, more homologous helicases may exist.

Due to the modest amount of enzyme obtained from this purification scheme, it has been difficult to explore the mechanism of action of this enzyme in detail. However, we have carried out necessary enzymatic characterization, as described here, so that these data could be correlated with that of immunoaffinity-purified helicase A (Biswas et al., 1993c) and recombinant helicase A expressed in heterologous systems.

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