DNA-Dependent ATPase from HeLa Cells Is Related to Human Ku Autoantigen†

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ABSTRACT: A 150-kDa DNA-dependent ATPase composed of 83/68-kDa subunits was previously reported to cofractionate with a 21S complex of enzymes for DNA synthesis from HeLa cells (Vishwanatha, J. K., & Baril, E. F. (1990) *Biochemistry 29,* 8753–8759). The DNA-dependent ATPase was purified to electrophoretic homogeneity from a HeLa cell homogenate by a modified procedure that involves subcellular fractionation, poly(ethyleneglycol) precipitation of the combined nuclear extract/cytosol, and chromatography on Q-Sepharose and native and denatured DNA/celluloses followed by Mono-S fast protein liquid chromatography. The purified enzyme showed equimolar amounts of 83- and 68-kDa polypeptides following polyacrylamide gel electrophoresis under denaturing conditions. Sequence analysis of peptide fragments derived from the separated 83- and 68-kDa polypeptides showed 90–100% homology with the corresponding 80- and 70-kDa subunits of human Ku protein. Immunoblot analysis of the ATPase during the course of its purification and immunoprecipitation with antibodies to the 80- and 70-kDa subunits of human Ku protein confirmed the relationship of the 83- and 68-kDa polypeptides of the human DNA-dependent ATPase to the subunits of human Ku protein. Both the 83- and 68-kDa polypeptides are phosphorylated by a DNA-dependent protein kinase that cofractionates with the ATPase. The DNA-dependent ATPase activity is up regulated by phosphorylation.

The human Ku autoantigen is a heterodimeric protein composed of equimolar amounts of 80- and 70-kDa subunits (Mimori *et al.*, 1981, 1986; Reeves, 1985; Yaneva *et al.*, 1985). Full-length cDNA clones have been obtained for both subunits of the human (Reeves & Sthoeger, 1989; Yaneva *et al.*, 1989; Mimori *et al.*, 1990) and murine (Chan *et al.*, 1989; Porges *et al.*, 1990; Falzon & Kuff, 1992) Ku proteins. The actual molecular masses for the deduced primary sequences of the respective subunits are 83 kDa (Mimori *et al.*, 1990) and 70 kDa (Reeves & Sthoeger, 1989). Autoantibodies to both subunits of the Ku protein are produced in large amounts in individuals with systemic lupus erythematosus (SLE) and related disorders (Mimori *et al.*, 1981; Reeves, 1985; Francoeur *et al.*, 1986). Ku protein binds to the terminal ends (Mimori & Harding, 1986; de Vries et al., 1989) and nicks (Blier *et al.*, 1993) of double-stranded DNA. This finding led to the proposal that Ku may function in DNA metabolism such as replication, repair, recombination, or transposition (Mimori & Hardin, 1986; Mimori *et al.*, 1986; de Vries *et al.*, 1989; Anderson, 1993). Ku protein has recently been reported to interact with the transcription factor USF and to bind to a region of human DNA containing a replication origin (Toth *et al.*, 1993). Also, disruption of the gene for the yeast homologue of Ku protein, i.e. the 85/70-kDa high-affinity DNA binding factor (Hdf), results in a temperature-sensitive phenotype for growth (Feldmann & Winnacker, 1993). At the nonpermissive temperature of 37 °C the growth-arrested cells accumulated an abnormally high content of DNA suggestive of a defect in the regulation of DNA replication coupled with an arrest in G_2 or M phase of the cell cycle. The actual function of Ku protein in DNA metabolism, however, remains obscure.

Some recent evidence suggests that Ku antigen may also be involved in transcriptional regulation (Reeves & Sthioeger, 1989; Thompson *et al.*, 1989; Knuth *et al.*, 1990; Dvir *et al.*, 1992; Gottlieb & Jackson, 1993). Ku was reported to be related to a transcription activator protein that binds to the proximal sequence element of the human promoter for the U1 small nuclear RNA (Knuth *et al.*, 1990). Also, Ku protein is a target for phosphorylation by a DNA-dependent protein kinase (DNA-PK[1]) in human cells (Lees-Miller *et al.*, 1990; reviewed by Anderson, 1993). The DNA-PK is reported to interact with Ku protein in the phosphorylation of promoter-associated RNA polymerase II (Dvir *et al.*, 1992) and the transcription factor Sp1 (Gottlieb & Jackson, 1993; Anderson, 1993) from HeLa cells. Ku protein was reported to be required in addition to DNA for full phosphorylating activity of the DNA-PK from HeLa cells on RNA polymerase II (Dvir *et al.*, 1992). It was postulated that the Ku protein recruits the DNA-PK to the DNA template for phosphorylating the components of the transcription complex by a bind–slide mechanism.

A 150-kDa DNA-dependent ATPase (DNA-ATPase[1]) cofractionates with a complex of enzymes for DNA synthesis in HeLa cells (Vishwanatha & Baril, 1990). The highly

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[1] Abbreviations: AAN, aminoacetonitrile hemisulfate; BAP, bacterial (*Escherichia coli*) alkaline phosphatase; BSA, bovine serum albumin; C10-agarose, Affi-Gel 10 *N*-hydroxysuccinimide cross-linked agarose; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DNA-ATPase, DNA-dependent ATPase; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; EDTA-Na_3, ethylenediaminetetraacetic acid, pH 7.5; EGTA-Na_3, ethylenebis(oxyethylenenitrilo)-tetraacetic acid, pH 7.5; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; NE, 0.15 M KCl nuclear extract; PEG, poly(ethyleneglycol); PEI, polyethyleneimine; PMSF, (phenylmethyl)sulfonyl fluorophosphate; PS, resulting supernatant solution following poly(ethyleneglycol) precipitation of the combined nuclear extract/post-microsomal supernatant solution; PVP, polyvinylpyrrolidone-40; S-3, postmicrosomal supernatant solution; SDS, sodium dodecyl sulfate; SV40, simian virus (40); T-ag, simian virus (40) large, tumor (T) antigen; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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purified ATPase is composed of two polypeptides of 83 and 68 kDa. Photoaffinity labeling experiments showed that both polypeptides contained ATP binding sites. The ATPase was shown to stimulate DNA polymerase α activity on primed, single-stranded DNA templates by overcoming a lag in the initiation of synthesis (Vishwanatha & Baril, 1990).

In this paper we present evidence that the previously described 150-kDa DNA-ATPase from HeLa cells has physical and immunological properties identical to those of the human Ku autoantigen. Moreover, the activity of the ATPase is enhanced by phosphorylation by the HeLa cell 350-kDa DNA-PK.

MATERIALS AND METHODS

Materials. Radioactively labeled nucleotides were obtained from Amersham or Dupont-NEN. Unlabeled nucleotides, Q-Sepharose, ATP-agarose (type 3), CNBr-activated Sepharose 4-B, protein G-Sepharose, and Mono-S HR5/5 (1 mL) columns were from Pharmacia LKB Biotechnology Inc. Poly(dT)$_{1000}$ and poly[(dA)·(dT)] were purchased from Midland Certified Reagents. Calf thymus DNA, bacterial (*Escherichia coli*) alkaline phosphatase (BAP[1]), alkaline phosphatase conjugated goat anti-human IgG (whole molecule), affinity purified sheep, anti-mouse IgG, polyvinylpyrrolidone-40 (PVP[1]), (phenylmethyl)sulfonyl fluorophosphate (PMSF[1]), and CHAPS[1] were from Sigma. Hydrogenated Triton X-100 was from Calbiochem. Aminoacetonitrile hemisulfate (AAN[1]) was obtained from Pfaltz-Bauer Inc. Polyethyleneimine- (PEI[1]-) impregnated MN 300 cellulose sheets for thin-layer chromatography (TLC[1]) were obtained from Brinkmann Instruments Inc. Long-fiber CF1 cellulose powder was from Whatman. Chemicals and standards used for protein sequencing were purchased from Applied Biosystems. HPLC grade solvents used for peptides purification were purchased from EM Science. Sequencing grade trypsin was obtained from Promega. Affi-Gel 10 *N*-hydroxysuccinimide cross-linked agarose (C10-agarose[1]) and all electrophoresis reagents were purchased from Bio-Rad. The 15-amino acid polypeptide MPEETQTQDQP-MEEE was synthesized at the Worcester Foundation Protein Chemistry Facility. HeLa S_3 cells were grown and synchronized by the double-thymidine block technique as previously described (Chiu & Baril, 1975). Mouse monoclonal antibodies 111 and N3H10 (purified IgG fractions) to the 80- and 70-kDA subunits, respectively, and human autoimmune serum, from patient JM with systemic lupus erythroderma, that reacts with both the 80- and 70-kDa subunits of human Ku proteins were generous gifts from Dr. Westley Reeves (University of North Carolina School of Medicine, Chapel Hill, NC). Monoclonal antibody D6D8 (purified IgG fraction) to the 83-kDa subunit of human Ku protein was a gift from Dr. Ann Black (Boehringer Mannheim Corp., Indianapolis, IN) and Dr. Harris Busch (Baylor College of Medicine, Houston, TX). Mouse ascites fluid containing secreted monoclonal antibody RZ-2 to the 83-kDa subunit of human Ku protein was a kind gift from Dr. James Freeman (Lucille Markey Cancer Center, Lexington, KY). The antibody (IgG_1) fraction was purified from the mouse ascites fluid by chromatography on a protein G-linked Sepharose column. A mix of purified mouse IgGs containing monoclonal antibodies 18–2, 25–4, and 42–26 to human DNA-PK was a generous gift from Dr. Timothy Carter (St. John's University, New York). Native and denatured DNA/cellulose was prepared by UV cross-linking native or heat-denatured calf thymus DNA to washed CF1 cellulose powder according to the procedure of Litman (1968). Purified BAP (5 mg) was cross-linked to 5 mL of CNBr-activated Sepharose 4B overnight at 4 °C in 0.1 M $NaHCO_3$, pH 8.5, by the procedure recommended by Pharmacia LKB Inc. All other reagents that were used have been described in recent publications from this laboratory (Vishwanatha & Baril, 1990; Li *et al.*, 1993, 1994).

Purification of DNA-ATPase. The DNA-ATPase was purified from the 21S complex of enzymes for DNA synthesis in HeLa cell extracts by modification of a published procedure (Vishwanatha & Baril, 1990). HeLa S_3 cells in suspension culture were synchronized by the double-thymidine block technique and harvested in the mid-S phase of the cell cycle. A 30% homogenate in an isotonic buffer was fractionated into a crude nuclear extract (NE[1]) and cytosolic postmicrosomal supernatant solution (S-3[1]) according to a published procedure (Li *et al.*, 1993). The combined NE and S-3 (NE/S-3) was subjected to poly(ethyleneglycol) (PEG[1]) precipitation, and the resulting supernatant (PS) was subjected to Q-Sepharose chromatography for isolation of the 21S complex of enzymes for DNA synthesis. The 21S complex elutes from Q-Sepharose at a KCl concentration between 0.15 and 0.25 M (Malkas *et al.*, 1990). Fractions from Q-Sepharose containing the 21S complex were dialyzed against 50 mM Tris[1]-HCl, pH 7.5, 1 mM each of EDTA-Na_3[1] and EGTA-Na_3[1] (buffer A) containing 0.05 M KCl and loaded onto a native DNA/cellulose column (10 cm^3) that was equilibrated with the dialysis buffer. Preliminary fractionations with coupled columns of native and denatured DNA/cellulose showed that 80% or more of the DNA-ATPase and DNA-PK activities associated with the 21S complex bind to the native DNA/cellulose matrix (Cao, Q.-P., & Pitt, S. Unpublished results). After being washed with 8 column volumes of the dialysis buffer, the column was eluted with a gradient of increasing KCl concentration from 0.05 to 1.0 M in buffer A. One-milliliter fractions were collected at a flow rate of 0.5 mL/min. The collected fractions were assayed for DNA-ATPase activity, and the active fractions were pooled and dialyzed against buffer A containing 0.05 M KCl. The dialyzed active fractions from native DNA/cellulose were loaded onto a 1-cm^3 Q-Sepharose column that was equilibrated with the dialysis buffer. One-milliliter fractions were collected at a flow rate of 0.5 mL/min. After being washed with 8 column volumes of the dialysis buffer, the column was eluted with a 20 column volume gradient of increasing KCl concentration from 0.05 to 1 M in buffer A. The eluted fractions containing DNA-ATPase activity were pooled and dialyzed against a buffer containing 50 mM Tris-HCl, pH 6.8, 0.05 M KCl, 1 mM EDTA, and 1 mM EGTA. The dialyzed sample was subjected to fast protein liquid chromatography (FPLC[1]) on a Mono-S (HR 5/5, 1 cm^3) column equilibrated with the same buffer. After being loaded at a flow rate of 0.5 mL/min, the column was washed with the equilibration buffer. Elution was with a 20-mL gradient of increasing pH from 8 to 8.9 and increasing KCl concentration from 0.05 to 0.5 M through addition of 0–100% of a buffer containing 50 mM Tris-HCl, pH 8.8, 0.5 M KCl, 1 mM EDTA, and 1 mM EGTA. One-milliliter fractions were collected at a flow rate of 0.5 mL/min. The fractions containing DNA-ATPase activity were pooled and dialyzed against buffer A containing 0.05 M KCl. The dialyzed fraction was loaded onto a denatured DNA/cellulose column (1 mL) equilibrated with the dialysis buffer and eluted with a 20-mL gradient of increasing KCl concentration from 0.05 to 0.5 M. Fractions containing DNA-ATPase activity were pooled, dialyzed against buffer A containing 0.15 M KCl and 10% glycerol, and stored at −80 °C.

Peptide Sequencing of the 83- and 68-kDa ATPase Subunits in Situ. Approximately 8 μg of the purified ATPase (eluted fraction 6 from the Mono-S step of the purification) was electrophoresed at 200 V for 40 min on a 7% polyacrylamide gel under denaturing conditions (Lamothe *et al.*, 1981). Electrotransfer of the separated protein bands to Immobilon-PVDF membranes (Millipore) was performed in the cold room at 70 V for 1 h in the presence of 100 mM CHAPS buffer, pH 11, according to the Millipore recommended procedure. The transferred proteins were visualized by staining the membrane for 1 min with Ponceau S and destaining for 1 min with distilled water. The membrane sections containing the stained 83- and 68-kDa bands, respectively, were sliced with a razor blade.

In situ enzymatic digestion of the 83- and 68-kDa subunits following electrotransfer to the Immobilon PVDF (Millipore) membrane was performed according to a published procedure (Fernandez *et al.*, 1992). Briefly, 50 pmols each of the Ponceau stained 83- and 68-kDa bands on the PVDF membrane were treated with 0.5 mL of 0.2% PVP-40/methanol (w/v) at room temperature for 30 min followed by the addition of 0.5 mL of distilled water. The PVDF bands were transferred to a clean microfuge tube (1.5 mL) and washed eight times with 1-mL aliquots of distilled water. The washed membranes were transferred to clean microfuge tubes, cut into 1-mm^2 pieces, and immersed in 50 μL of digestion buffer (100 mM $NaHCO_3$, 10% acetonitrile, and 1% hydrogenated Triton X-100). One microgram of sequencing grade trypsin in 2 μL of 50 mM acetic acid was added to each tube, and digestion proceeded overnight at 37 °C. The released peptides were recovered in the supernatant following centrifugation of the samples in a microfuge. The pieces of PVDF membrane were washed once with 50 μL of digestion buffer and once with 50 μL of 0.1% trifluoroacetate (TFA[1]). The washes were pooled with the original supernatant from the digestion, and the pooled sample was subjected to high-pressure liquid chromatography (HPLC[1]) for resolution of the peptides.

HPLC separations were performed on a Hewlett Packard 1090M system equipped with a UV diode array detector. The tryptic digests were injected onto a Brownlee Aquapore C8 microbore column (1 × 250 mm^2), using 0.1% TFA as solvent A and 0.08% TFA in acetonitrile/water (70:30 v/v) as solvent B. A linear gradient was developed from 0 to 55% solvent B over a period of 90 min at a flow rate of 50 μL/min. The peptide peaks were collected manually in 1.5-mL microfuge tubes.

Three peptide fractions were chosen from each digest (Figure 4) and subjected to automated Edman degradation by applying the respective fraction directly to an Applied Biosystems 477A sequencer equipped with a Model 120A in-line PTH analyzer. The sequence information obtained was then used to search the Swiss Prot Database using the FASTA search program.

Polyacrylamide Gel Electrophoretic Analysis of Proteins. Polyacrylamide gel electrophoretic analysis of proteins was essentially as described previously (Lamothe *et al.*, 1981). Any modifications of the procedure are described with the individual experiments. The protein-stained bands were developed by Coomassie blue or silver staining.

Immunoblot Analysis. Immunoblot analysis was performed as previously described (Malkas *et al.*, 1990) with minor modifications. Electroblotting of the electrophoresed proteins was performed essentially according to the procedure of Towbin and co-workers (1979) using nitrocellulose membranes. After blocking and washing, the membrane was incubated at room temperature for 1 h with 1:500 dilutions in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS[1]) of autoimmune serum (Porges *et al.*, 1990) to human 80/70-kDa Ku protein (SLE patient JM) or murine monoclonal antibodies 111 and N3H10 to the human Ku 80- and 70-kDa subunits, respectively (Porges *et al.*, 1990). This was followed by a 1-h incubation at room temperature with a 1:1000 dilution in TBS of either alkaline phosphatase-conjugated goat anti-human rabbit IgG (whole molecule), for patient JM serum, or alkaline phosphatase-conjugated sheep anti-mouse IgG (whole molecule), for murine antibodies 111 and N3H10. Color was developed by incubating in a diethanolamine buffer, pH 9.8, containing 4 mM $MgCl_2$, 0.005% 5-bromo-4-chloro-3-indolyl phosphate, and 0.01% nitroblue tetrazolium.

Immunodepletion of ATPase Activity. Purified mouse monoclonal antibody RZ-2 (reacting with the 83-kDa subunit of human Ku autoantigen) was linked to C10-agarose (4 mg of IgG_1/mL of gel) by the Bio-Rad recommended procedure. Purified sheep anti-mouse IgG was linked to C10-agarose (2 mg/mL of gel) by the same procedure. Aliquots of the purified DNA-ATPase (600 ng of protein) were incubated with varying amounts of the C10 agarose-linked antibody RZ-2 (0–80 μg of protein) or C10 agarose-linked anti-mouse IgG (0–80 μg of protein) control at 4 °C for 3 h. Following the incubation the samples were centrifuged in a microfuge. Aliquots (100 ng) of the supernatant for each sample were assayed for DNA-ATPase activity and for immunoblot analysis of Ku protein.

Photoaffinity Labeling with [^{32}P]ATP. DNA-ATPase (400 ng) was added to a 40-μL reaction at 4 °C containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, and 10 μM [α-^{32}P]ATP (12.5 mCi/mmol). A 20-μL aliquot was transferred to a microfuge tube at 4 °C and irradiated at 254 nm for 10 min with a radiation intensity of 2500 μW/cm^2 using a General Electric G15T8, 15-W germicidal lamp. The remaining 20-μL aliquot (nonirradiated control) was left at 4 °C during the irradiation. The irradiated and control samples were subjected to sodium dodecyl sulfate–(SDS[1]–)polyacrylamide gel electrophoresis using an 8% cross-linked gel. Following electrophoresis, the protein bands were electrotransferred to the nitrocellulose membrane. The membrane was subjected to immunoblot analysis using antibodies to human Ku protein and exposed to XAR-5 film for detection of the [^{32}P]ATP cross-linked proteins by autoradiography.

Dephosphorylation and Rephosphorylation of ATPase. Dephosphorylation and rephosphorylation of the purified DNA-ATPase was carried out according to the following three-step procedure.

Step 1. An aliquot of the purified DNA-ATPase (1 μg)

$$\text{DNA-ATPase} \xrightarrow{\text{BAP-sepharose}} \text{dephosphorylated DNA-ATPase}$$

was equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM $ZnCl_2$, and 1 mM magnesium acetate by dialysis. After dialysis the enzyme was incubated for 1 h at 4 °C with 25 μL of Sepharose 4B-linked BAP that was equilibrated with the dialysis buffer. After incubation the Sepharose-linked BAP was removed by centrifugation in a spin column (Quik Sep) and the excluded fraction containing the ATPase was dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA-Na_3, 1 mM EGTA-Na_3, 150 mM KCl, and 10% glycerol. Aliquots of the dialyzed sample were used for assays of DNA-PK activity and for SDS–polyacrylamide gel electrophoretic analysis.

Step 2. Two aliquots of the dialyzed sample (165 ng each) were also incubated under conditions for rephosphorylation

$$\text{dephosphorylated DNA-ATPase} \xrightarrow{\text{DNA-PK}} \text{rephosphorylated DNA-ATPase} + \text{DNA-PK}$$

of the dephosphorylated DNA-ATPase by the partially purified DNA-PK (Mono-S FPLC fraction 8 in Figure 1). Incubation was at 35 °C for 30 min in a reaction (25-μL volume) containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, 165 ng of purified DNA-ATPase, and 100 ng of the partially purified DNA-ATPase or buffer containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM DTT, and 1 mM ATP (minus DNA-PK control).

Step 3. An excess of monoclonal antibody (purified IgG)

$$\text{DNA-ATPase} + \text{DNA-PK} \xrightarrow{\text{anti-DNA-PK-sepharose}} \text{DNA-ATPase}$$

to human DNA-PK (Carter *et al.*, 1990) was incubated with C10-agarose-linked sheept anti-mouse IgG at 4 °C for 1 h. The C10-agarose/antibody complex was separated from the free DNA-PK antibody by centrifugation. The C10 agarose/antibody complex was added to the rephosphorylation and control reactions in step 2 to remove the DNA-PK. Incubation was continued at 4 °C for an additional hour. The C10-agarose/antibody/DNA-PK complex was removed by centrifugation in a spin column, and the excluded fraction was assayed for DNA-ATPase activity. The remainder of the dephosphorylated ATPase was used in the incubation for rephosphorylation as described in steps 2–3 above but in a reaction containing 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) to give a specific activity of 80 mCi/mmol of ATP. After the incubation the samples were subjected to SDS–polyacrylamide gel electrophoresing conditions.

DNA-ATPase Assay. DNA-ATPase activity was assayed according to a published procedure (Vishwanatha & Baril, 1990) with modifications. The reaction (25-μL volume) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT[1]), 1 mM ATP, 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) to give a specific activity of 80 mCi/mmol of ATP, and 5 μg of heat-inactivated bovine serum albumin (Lamothe *et al.*, 1981), plus or minus 0.5 μg of poly(dT), heat-denatured calf thymus DNA, or M13 DNA as a cofactor. Incubation was usually for 30 min at 35 °C. The reaction was stopped by placing the tubes at 4 °C and adding 1 μL of 0.1 M EDTA to the reaction. A 2-μL aliquot of the reaction product was applied to the origin of a prewashed 20 × 20 cm^2 PEI/cellulose TLC plate overlaid with 2 μL of a mixture of mobility markers containing 2 mg each of ATP, ADP, and AMP per milliliter. After drying, the plate was developed by ascending chromatography at room temperature in 0.75 M KH_2PO_4, pH 5, as the solvent. After completion of the chromatography the plates were air-dried and the areas corresponding to the nucleotide mobility markers were scraped off and the radioactivity was determined by liquid scintillation counting. One unit[2] of ATPase activity is defined as the hydrolysis of 1 nmol of ATP in 1 h at 35 °C.

DNA-Dependent Protein Kinase Assay. DNA-PK activity was routinely assayed using a synthetic 15-mer peptide containing the amino terminal sequence MPEETQTQDQP-MEEE that contains two of the phosphorylation sites in the human heat shock 90 protein (hsp90) for the DNA-PK (Lees-Miller & Anderson, 1989). The reaction (25-μL volume) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) to give a specific activity of 0.8 mCi/mmol of ATP, 0.1 μg of calf thymus DNA, and 10 μg of the 15-mer peptide. Incubation was for 1 h at 35 °C, and the reaction was stopped by addition of 1 μL of 0.1 M EDTA. The sample was applied to a DE-81 disk which was washed three times with 0.3 M ammonium formate, pH 7.5, for 5 min each, and once with 95% ethanol and dried under a heat lamp. The radioactivity retained on the disk was measured by liquid scintillation counting. One unit of protein kinase activity equals 1 pmol of phosphate incorporated into the 15-mer peptide per minute at 35 °C.

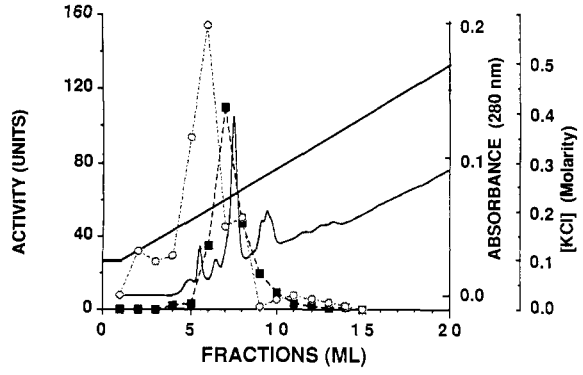


FIGURE 1: Mono-S FPLC of DNA-ATPase. Elution profiles of the DNA-ATPase (open circles and dotted line) and DNA-PK (solid boxes and dashed line) and activities and absorbance (280 nm) profiles of the protein (narrow, continuous line) eluted by the gradient of increasing KCl concentration (heavy continuous line) and pH (not shown). The procedures for chromatography and assays were as described in Materials and Methods.

Other Assays. SV40 *in vitro* DNA replication activity was assayed according to the procedure of Wold and Kelly (1988). DNA polymerase α, primase, DNA ligase, and topoisomerase I activities were assayed according to published procedures from this laboratory (Lamothe *et al.*, 1981; Vishwanatha & Baril, 1986; Li *et al.*, 1993, 1994). Protein was assayed by the procedure of Bradford (1976) using bovine serum albumin (BSA[1]) as the standard.

RESULTS

Purification of the DNA-ATPase. Most (85% or more) of the 150-kDa DNA-ATPase in the HeLa cell extract is associated with a 21S complex of enzymes that functions in T-antigen- (T-ag[1]-) dependent SV40 DNA replication *in vitro* (Li *et al.*, 1993). Chromatography of the 21S enzyme complex on coupled columns of native and denatured DNA/cellulose dissociates the complex into subassemblies (Li *et al.*, 1993, 1994). One of the subassemblies that binds to native DNA/cellulose contains DNA ligase I, topoisomerase I (Li *et al.*, 1994), as well as the DNA-ATPase and DNA-PK (Carter *et al.*, 1990; Lees-Miller *et al.*, 1990). The DNA-ATPase and DNA-PK coelute from the native DNA/cellulose at a KCl concentration between 0.3 and 0.4 M. Mono-S FPLC, using an ascending gradient of pH and KCl concentration, separates the DNA-Pk from the ATPase (Figure 1). The ATPase elutes between pH 8.2 and 8.3 and a KCl concentration between 0.15 and 0.16 M. The DNA-PK elutes at a pH between 8.4 and 8.5 and a KCl concentration between 0.19 and 0.20 M. SDS–polyacrylamide gel electrophoresis of the eluted fractions showed that the enzyme is about 80% pure at this stage in the purification (fractions 5–6 in Figure 2A). Denatured DNA/cellulose chromatography of pooled fractions 5 and 6 from the Mono-S column (Figure 1) removes the minor contami-

[2] The unit for the DNA-dependent ATPase activity reported by Vishwanatha and Baril (1990) was incorrect. The correct value is one unit equals the hydrolysis of 1 nmol of ATP/h at 30 °C.

Table 1: Purification of HeLa Cell 150-kDa DNA-ATPase[a]

fraction	volume (mL)	total protein (mg)	total activity (units)	specific activity (units/mg)	x-fold purification
(I) Ne + S-3	130	1690			
(II) PS	150	930			
(III) Q-Sepharose eluate	16	72			
(IV) native DNA/cellulose	11	4.2	8452	2012	1
(V) Mono-S FPLC	4	0.08	496	6200	3
(VI) denatured DNA/cellulose	3	0.03	2954	98 466	48

[a] The starting material was 25 g (wet weight) of synchronized HeLa cells in S phase. The native DNA/cellulose stage is taken as step I in the purification because earlier stages contained interfering materials such as DNA-independent ATPase activity. Half of the DNA-ATPase activity eluting from the Q-Sepharose column was used for the purification. The reported values were extrapolated to the use of the entire Q-Sepharose-eluted DNA-ATPase in the purification.

nants associated with the ATPase eluting from the Mono-S column. The DNA-ATPase binds to denatured DNA/cellulose and elutes at a KCl concentration between 0.45 and 0.50 M. SDS–polyacrylamide gel electrophoresis of the ATPase eluted from the denatured DNA/cellulose column showed the presence of only two protein bands of 83 and 68-kDa by Coomassie blue and silver staining (Figure 2B). These polypeptides were present in approximately equimolar amounts, suggesting that the enzyme is a heterodimer of 83- and 68-kDa subunits. The DNA-ATPase was purified about 50-fold from the native DNA/cellulose to the denatured DNA/cellulose chromatographic steps in the purification with 35% recovery of the activity (Table 1). Purification of the enzyme from the crude HeLa cell extract is undoubtedly much higher than 50-fold. We were unable to calculate, however, the DNA-ATPase activity reliably in fractions prior to the native DNA/cellulose chromatographic step due to the presence of DNA-independent ATPase and other competing activities.

The DNA-ATPase that is associated with the 21S complex of enzymes for DNA synthesis has identical enzymatic and physical properties to those previously reported for the 150-kDa DNA-ATPase in HeLa cells (Vishwanatha & Baril, 1990).

DNA-ATPase Has Very Similar Properties to Those of the Human Ku Autoantigen. To understand better the structure/function of the 150-kDa DNA-ATPase from HeLa cells, its primary structure was partially determined and compared to sequences for known proteins.

Partial Amino Acid Sequence Analysis. Sequences of selected peptide fragments from the tryptic digest of the electroblotted 83- and 68-kDa polypeptides of the purified ATPase were determined by microsequencing (Figure 3). The sequence information obtained from a computer search of the Swiss Prot Database indicated 90–100% identity of sequences in the selected tryptic peptide fragments of the 83- and 68-kDa polypeptides of the purified HeLa cell DNA-ATPase to sequences in corresponding domains of the 80- and 70-kDa subunits, respectively, of human Ku protein (Figure 3, lower panel). The sequences in three peptide fragments derived from the 83-kDa ATPase polypeptide (HPLC peaks 1, 2, and 3 in Figure 3, upper panel) showed 100% identity to sequences at residues 655–660, 185–192, and 544–558 plus 316–325, respectively, in the 80-kDa subunit of human Ku protein (Figure 3, lower panel). Also, sequences in two of the peptide fragments derived from the 68-kDa polypeptide of the ATPase (peaks 4 and 6 in Figure 3, upper panel) showed greater than 90% identity to sequences at residues 165–180 and 474–486, respectively, in the 70-kDa subunit of human Ku protein (Figure 3, lower panel). The differences being E in the sequence of one peptide fragment (peak 4) of the ATPase 68-kDa polypeptide in place of H at residue 175 in the deduced sequence of the Ku protein 70-kDa subunit. Also, in the other sequenced peptide fragment (peak 6 in Figure 3, upper panel),

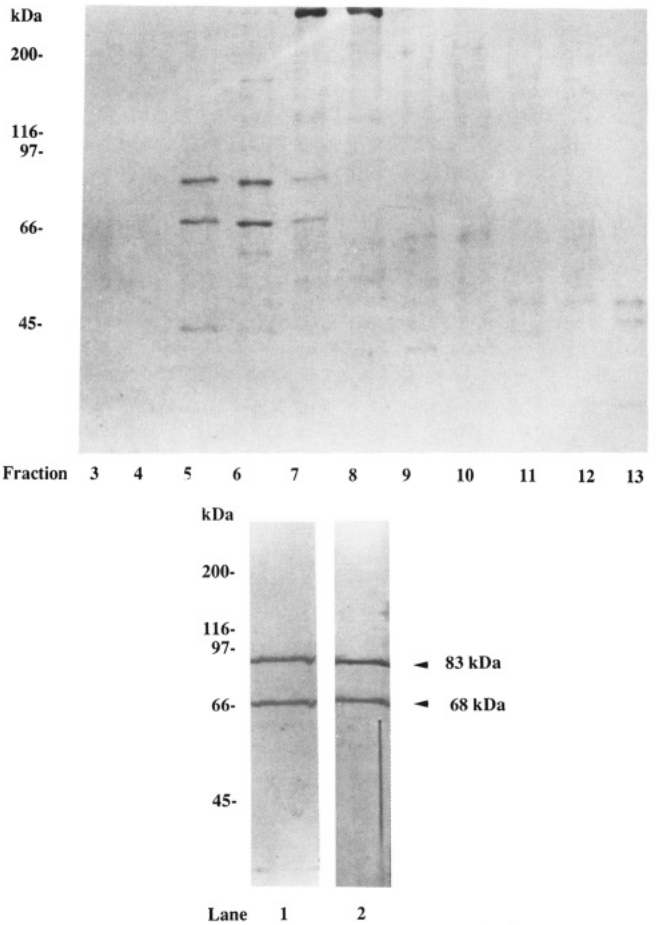
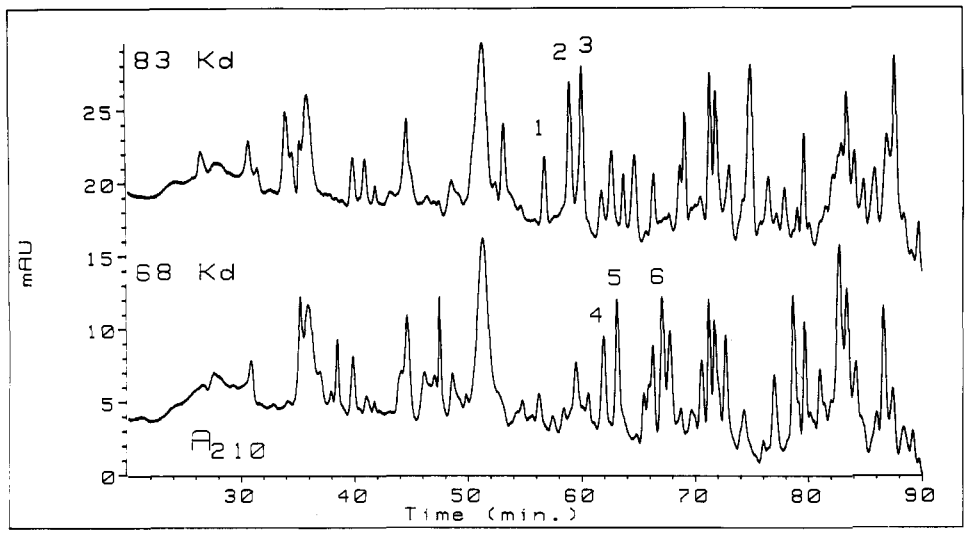


FIGURE 2: SDS polyacrylamide gel electrophoresis of the ATPase eluted during the Mono-S FPLC (step 5) and denatured DNA/cellulose (step 6) steps in the purification. (A, top) Aliquots (8 μL each) of eluted fractions 3–13, respectively, from Mono-S FPLC (Figure 1) were subjected to SDS-polyacrylamide gel electrophoresis according to the procedures described in Materials and Methods. Protein bands were visualized by Coomassie blue staining. (B, bottom) SDS-polyacrylamide gel electrophoresis of the DNA-ATPase eluted from denatured DNA/cellulose (step 6). The purified ATPase (0.6 μg) was electrophoresed on an 8% polyacrylamide gel in the presence of SDS. Protein bands were visualized by Coomassie blue (lane 1) and silver staining (lane 2). Molecular mass markers included myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), and ovalbumin (45 kDa).

three residues did not appear in the sequencing that correspond to S, D, and H in the deduced sequence for this domain of the 70-kDa subunit of human Ku protein. We were unable to obtain the sequence of the third selected peptide fragment of the 68-kDa ATPase polypeptide (peak 5 in Figure 3) probably due to a blocked NH_2 terminus.

Immunoblot Analysis. As shown in Figure 4, immunoblot analysis of the crude NE, crude S-3, and purified DNA-ATPase (i.e. fraction 6 in the eluted ATPase from Mono-S



150 kDa ATPase			Ku protein		
poly-peptide	HPLC peptide fragment	amino acid sequence	subunit	residue number	deduced amino acid sequence
	(peak)				
			80 kDa	655-660	FNNFLK
83 kDa	1	FNNFLK		185-192	LGGHPSF
	2	LGGHGPSF		544-558	KKKDQVTAQEIFQDNH
	3	KKDQVTAQEIFQDNH + YGSDIVPFSK		316-325	YGSDIVPFSK
			70 kDa	165-180	IMLFTNEDNPHGNDSA
68 kDa	4	no sequence			
	5	IMLFTNEDNPEGNDSA		474-486	SDSFENPVLQQHF
	6	(X)(X)SFENPVLQQ(X)F			

FIGURE 3: Sequence comparison of tryptic peptide fragments derived from the 83- and 68-kDa ATPase polypeptides and domains in the corresponding 80- and 70-kDa subunits of human Ku protein. (Upper panel) Comparison of the HPLC profiles of tryptic digests of the 83- and 68-kDa polypeptides from PVDF electroblots of the DNA-ATPase. On the basis of the initial yields of 1–3 pmoles of PTH amino acids, it is estimated that 5 pmoles or less of the corresponding peptides was purified. The procedures and conditions used were as described under Materials and Methods. (Lower panel) Comparison of amino acid sequences (single-letter code) in tryptic peptide fragments of the ATPase 83- and 68-kDa polypeptides and the deduced amino acid sequences in domains of the corresponding 80- and 70-kDa subunits of human Ku protein. The procedures used for sequencing and search of a protein database are given under Materials and Methods. X represents unidentifiable residues during the sequence call. The deduced amino acid sequences for the 80-kDa subunit (Mimori *et al.*, 1989; Yaneva *et al.*, 1989) and 70-kDa (Reeves & Sthoeger, 1989) subunits of human Ku protein were from published results.

FPLC, Figure 1) using monoclonal antibodies to the 80-kDa (antibody P6D8) and 70-kDa (antibody N3H10) subunits of human Ku proteins (Porges *et al.*, 1990) showed the presence of two protein bands of about 83 and 70 kDa. Immunoblot analysis of fractions containing the DNA-ATPase activity during the course of purification of the enzyme using autoimmune sera to the human Ku protein 80/70-kDa subunits (Porges *et al.*, 1990) also showed the presence of the 83- and 70-kDa proteins throughout the purification (data not shown). This corresponds well to the two bands of about 83 and 68 kDa observed for the purified enzyme by Coomassie blue and silver staining following polyacrylamide gel electrophoresis of the purified ATPase under denaturing conditions (Figure 2B).

ATPase Activity Is Intrinsic to Ku Protein. Both polypeptides of the DNA-ATPase bind ATP by photo-cross-linking (Vishwanatha & Baril, 1990) and UV-cross-linking (data not shown).

Immunoprecipitation of the ATPase Activity and Ku Protein. The 80- and 70-kDa subunits of the human Ku protein are tightly associated (Mimori *et al.*, 1985; Yaneva & Busch, 1986; Porges *et al.*, 1990). To ascertain if the DNA-ATPase activity is associated with the Ku protein, immunoprecipitation analysis was performed on the purified ATPase using C10-agarose-linked monoclonal antibody RZ-2 to the 80-kDa subunit of human Ku protein. Incubation of aliquots of the purified ATPase with increasing amounts of C10-agarose-linked RZ-2 antibody followed by centrifugation of the matrix-linked antibody resulted in a linear decrease of the ATPase activity in the resulting supernatant solution (Figure 5, lower panel). Under these conditions, incubation of the ATPase with C10-agarose alone or with C10-agarose-linked normal mouse IgG resulted in less than 3% depletion of the DNA-ATPase activity in the resulting supernatant solution (Figure 5, lower panel).

The bound ATPase was dissociated from the sedimented C10-agarose-linked RZ-2 antibody by treatment with the denaturing polyacrylamide gel electrophoresis loading buffer followed by centrifugation. SDS–polyacrylamide gel electrophoresis of the resulting supernatant showed the presence of two protein staining bands of 83 and 68 kDa that increased in intensity with the corresponding decrease of ATPase activity in the supernatant (Figure 5, upper panel). Taken together these results strongly indicate that the ATPase activity is

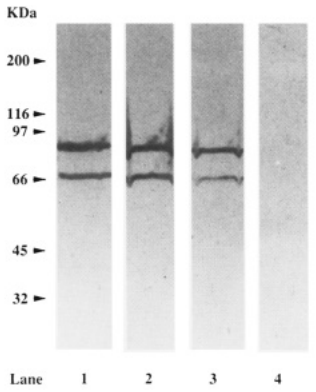


FIGURE 4: Immunoblot analysis of the purified ATPase unsing monoclonal antibodies to the human Ku protein: (lanes 1 and 4) 0.6 μg of the purified ATPase (step 6), (lane 2) 0.6 μg of the crude cytosol fraction, and (lane 3) 0.6 μg of the crude NE were electrophoresed under denaturing conditions. The separated polypeptides were electrotransferred to nitrocellulose according to a published procedure (Towbein *et al.*, 1979). Immunoblotting of the transferred polypeptides was performed using 1:500 dilutions in TBS (lanes 1–3) of monoclonal antibodies 111 (IgG_1) and N3H10 (IgG_{2b}) to the 80- and 70-kDa subunits, respectively, of human Ku protein (Porges *et al.*, 1990) and a 1:500 dilution in TBS (lane 4) of sheep anti-mouse IgG (control). Molecular mass markers included myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (32 kDa). The procedures used for electrophoresis and immunoblot analysis were as described under Materials and Methods.

intrinsic to the 83- and 68-kDa polypeptides that are coprecipitated by monoclonal antibody to the human Ku protein 80-kDa subunit.

ATPase Activity Is Up Regulated by Phosphorylation. The DNA-ATPase and DNA-PK activities cofractionate to the penultimate step in the purification procedure. The protein kinase has been reported to phosphorylate Ku protein (Lees-Miller *et al.*, 1990), as well as other nuclear proteins. It has also been reported that the DNA-PK and Ku protein directly interact in binding to DNA (Dvir *et al.*, 1992; Gottlieb & Jackson, 1993). In light of the proposed interactions of these proteins and our observed cofractionation of the proteins from a subassembly of the 21S complex of enzymes for DNA synthesis, it was of interest to ascertain the effect of phosphorylation by the protein kinase on the catalytic activity of the DNA-ATPase.

The purified DNA-ATPase activity was dephosphorylated by incubation with Sepharose-linked BAP. After removal of the Sepharose-BAP by centrifugation, the DNA-ATPase activity in the resulting supernatant solution was assayed. Aliquots of the supernatant were also incubated with the partially purified DNA-PK or buffer (control) under conditions for rephosphorylation of the dephosphorylated ATPase. The DNA-PK was removed by first incubating with mouse monoclonal antibody (purified IgGs of monoclonal antibodies 18–2, 25–4, and 42–26) to human DNA-PK (Carter *et al.*, 1990) followed by incubation with C10-agarose-linked goat anti-mouse IgG. The control sample was treated in the same manner. The matrix-bound antibody complexes were removed by centrifugation, and the DNA-ATPase activity in the resulting supernatants were measured. Parallel experiments were performed in which the rephosphorylation of the dephosphorylated ATPase was performed using [γ-^{32}P]ATP and the rephosphorylated ATPase was analyzed by SDS–polyacrylamide gel electrophoresis.

As shown in Figure 6, dephosphorylation reduced the catalytic activity of the purified DNA-ATPase over 99%. Rephosphorylation of the dephosphorylated ATPase by

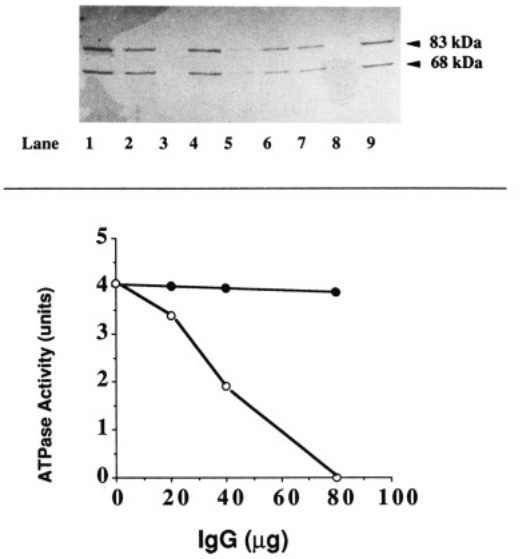
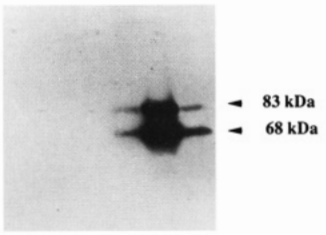


FIGURE 5: Immunoprecipitation of the 83/68-kDa polypeptides and ATPase activity by monoclonal antibody to human Ku protein. (Lower Panel) Immunodepletion of the ATPase activity by monoclonal antibody RZ-2 to the 80-kDa subunit of human Ku protein. Aliquots (0.6 μg) of the purified ATPase (step 6) were incubated with C10-agarose-linked monoclonal antibody RZ-2 IgG_1 (open circles) or C10-agarose-linked sheep anti-mouse IgG (control, closed circles). Following the incubation, the agarose-linked antibodies were removed by a spin column. Aliquots (5 μL) of the filtrate were assayed for DNA-ATPase activity or subjected to SDS-polyacrylamide gel electrophoresis. (Upper Panel) SDS-polyacrylamide gel electrophoretic analysis of the resulting supernatant and immunoprecipitate fractions from immunodepletion of the ATPase activity by monoclonal antibody to human Ku protein. SDS-polyacrylamide gel electrophoresis was performed on the resulting supernatant (20 μL) and the spin column isolated precipitates that were solubilized in the SDS-polyacrylamide gel loading buffer. Following electrophoresis the protein bands were visualized by Coomassie blue staining: (lane 1) 0.6 μg of purified ATPase; (lane 2) supernatant from reaction of ATPase with C10-agarose-linked IgG of sheep anti-mouse IgG (control); (lane 3) precipitate from reaction of ATPase with agarose-linked sheep anti-mouse IgG (control); (lanes 4, 6, and 8) supernatants from reaction of ATPase with 20, 40, and 80 μg, respectively, of C10-agarose-linked IgG of monoclonal antibody RZ-2; (lanes 5, 7, and 9) immunoprecipitates from reaction of ATPase with 20, 40, and 80 μg, respectively, of C10-agarose-linked IgG_1 of monoclonal antibody RZ-2. Procedures for electrophoresis, immunoprecipitation, and assay of ATPase activity were as described under Materials and Methods.

incubation with the DNA-PK completely restored the catalytic activity of the enzyme. The DNA-ATPase activity of the control BAP-dephosphorylated DNA-ATPase incubated in the absence of DNA-PK during the rephosphorylation incubation period and subsequently treated with the anti-DNA-PK and C10-agarose-linked goat anti-mouse IgG antibody was less than 0.5% (0.05 units) of the purified DNA-ATPase activity. Also, the partially purified DNA-PK contained less than 0.3% of the initial DNA-ATPase activity (Figure 6, lower panel). Thus, it appears that the increase in the activity of the dephosphorylated DNA-ATPase upon rephosphorylation by DNA-PK is directly attributable to activation by phosphorylation.

Electrophoretic analysis of the rephosphorylated ATPase indicated that both the 83- and 68-kDa subunits of the enzyme were phosphorylated (Figure 6, upper panel, lanes 4–6). The phosphorylation was enhanced over that of poly(dT) in the presence of calf thymus DNA or the absence of DNA. This is in agreement with the reported dependence of the protein kinase activity on DNA as a cofactor and with single-stranded DNA being less efficient as a cofactor (Carter *et al.*, 1990). There was no phosphorylation of the dephosphorylated ATPase



condition and enzyme	ATPase activity (units)	percent of initial activity
ATPase[a]	13.9	100%
BAP-dephosphorylated ATPase	0.04	0.3%
Rephosphorylated ATPase[b]		
Control (minus DNA-PK)	0.03	0.2%
plus DNA-PK	15.0	107%
DNA-PK	0.04	0.3%

FIGURE 6: ATPase activity up regulated by phosphorylation. (Lower panel) The effect of dephosphorylation and rephosphorylation on the activity of the purified ATPase. [a]The purified ATPase was from the denatured DNA/cellulose (step 6) stage of the purification. [b]The partially purified DNA-PK was fraction no. 8 eluted from the Mono-S column (Figure 2). The procedures for dephosphorylation, rephosphorylation, and assay of DNA-ATPase activity were described under Materials and Methods. (Upper panel) Autoradiogram from SDS-polyacrylamide gel electrophoresis of the purified DNA-ATPase following phosphorylation in the presence of different DNA cofactors. (Lanes 1–3): Incubation of the ATPase in the presence of (1) no DNA, (2) 0.5 μg of native calf thymus DNA, and (3) 0.5 μg of poly(dT) but in the absence of DNA-PK. (Lanes 4–6): Incubation of the ATPase in the presence of (4) no DNA, (5) 0.5 μg of native calf thymus DNA, and (3) 0.5 μg of poly(dT) and DNA-PK. The procedures for phosphorylation and SDS-polyacrylamide gel electrophoresis were as described under Materials and Methods.

in the absence of the DNA-PK in the rephosphorylation reaction (Figure 6, upper panel, lanes 1–3).

These results clearly indicate that the DNA-ATPase activity is up regulated by phosphorylation. They also suggest that the DNA-PK that cofractionates with this ATPase can function in the phosphorylation that up regulates the ATPase activity.

DISCUSSION

A DNA-ATPase was reported to be associated with a complex of enzymes for DNA synthesis in HeLa cells (Vishwanatha & Baril, 1990). The purified enzyme had an apparent molecular mass of about 150 kDa and was composed of polypeptides of 83 and 68 kDa. The DNA-ATPase required single-stranded DNA as a cofactor, and poly(dT) was preferred over other single-stranded polydeoxynucleotide homopolymers or denatured calf thymus DNA.

In this paper we presented evidence that indicates that the 150-kDa HeLa cell DNA-ATPase, purified to apparent homogeneity from the complex of enzymes for DNA synthesis, is identical to or has very similar properties to human Ku autoantigen. The evidence for this is as follows. First, the Ku protein (Mimori *et al.*, 1981, 1986; Reeves, 1985; Yaneva *et al.*, 1985) and DNA-ATPase (Figure 2) are both heterodimeric proteins composed of equimolar amounts of dissimilar subunits of comparable molecular mass. Second, amino acid sequence analysis of tryptic peptide fragments of the separated 83- and 68-kDa polypeptides from the purified DNA-ATPase shows almost 100% identity to the deduced amino acid sequences for domains in the corresponding 80- and 70-kDa human Ku protein subunits. Third, the 83- and 68-kDa polypeptides of the DNA-ATPase react in immunoblot analysis with monoclonal antibodies directed to the 80- and 70-kDa subunits of human Ku protein. Finally, antibody to the human Ku protein precipitates both the ATPase activity and 83/68-kDa polypeptides associated with the purified DNA-ATPase.

From the alignment of homologous sequences in subunits of several ATP synthases and other adenine nucleotide binding proteins, Walker and co-workers (Walker *et al.*, 1982) established two domains of highly conserved sequences in these proteins. The A-type ATP binding motif contained the conserved sequence GXXXXGK[T/S]XXXXX[I/V] while the B-type motif contained the conserved sequence of R/KXXXGXXXLUUUD (Table 2). Koonin (1993) recently reported three conserved sequence motifs in DNA-ATPases that deviate from the A- and B-type motifs of Walker and co-workers (Walker *et al.*, 1982). The DNA-ATPases include prokaryotic transcription regulators (e.g. NtrC related), RNA and DNA helicases, and yeast minichromosome maintenance (MCM) protein that functions in the initiation of yeast chromosomal DNA replication (Chen *et al.*, 1992). As shown in Table 2, a general conserved sequence motif shared by the yeast MCM protein and prokaryotic DNA-ATPases is UUUXGXXXX[G,A,S]K[S,T,E]. The 80- and 70-kDa Ku protein subunits show reasonably good homology to the Koonin (1993) conserved sequence motif of DNA-ATPases. As shown in Table 2, the homology occurs at amino acid residues 677–689 (IVVQDGITLITKE) in the 80-kDa subunit while for the 70-kDa subunit the homology occurs at amino acids 527–538 (LVYPPDYNPEGK). As mentioned above, the Koonin DNA-dependent ATPase sequence motifs deviate from the highly conserved A- and B-type sequence motifs reported by Walker and co-workers (Walker *et al.*, 1982) for ATP synthases. It is possible that DNA-dependent ATPases, including helicases, deviate from the conserved A- and B-type motifs because of their performance of unique functions. Variation in sequence motifs among cellular ATPases may not be unusual, since considerable variation in DNA in the sequence recognition motifs in the family of Zn^{2+} finger proteins has recently been reported (Pavietich & Pabo, 1993). A direct sequence analysis of the ATP binding sites in the 83- and 68-kDa polypeptides of the purified DNA-ATPase is required, however, to establish their actual relationship to the Walker A- and B-type conserved sequence motifs. Such an analysis is being undertaken.

A second important finding in this study is that the catalytic activity of the purified DNA-ATPase/Ku protein is up regulated by phosphorylation by the DNA-PK that cofractionates with it (Figure 6). Phosphorylation of the 80- and 70-kDa subunits of the Ku protein from human cells by DNA-PK had been reported by Lees-Miller and co-workers (Lees-Miller *et al.*, 1990). No effect of the phosphorylation on the properties of the Ku protein was shown in that study, however. Interestingly, both the DNA-PK (Lees-Millers *et al.*, 1990; Carter *et al.*, 1990) and Ku protein bind to double-stranded DNA. The binding to DNA by both DNA-PK (Carter *et al.*, 1990) and Ku protein (de Vries *et al.*, 1989; Blier *et al.*, 1993) is believed to require free ends, and at least for Ku protein, the binding was reported to be energy- and sequence-independent. The DNA-ATPase activity that is intrinsic to the Ku-like protein reported here requires single-stranded DNA as a cofactor. The activation of the ATPase activity

Table 2: Adenine Nucleotide Binding Motifs in Some ATP-Requiring Enzymes

enzyme(s)	binding sequence motif[a]	reference
Walker, ATP binding		
type A	GXXXXGKT/SXXXXXXI/V	Walker *et al.*, 1982
type B	R/KXXXGXXXLUUUUD	Walker *et al.*, 1982
motif A in nucleic-dependent ATPases of prokaryotes and yeast human Ku protein	$(U)_n$(G)XX(G)XGK[STE]	Koonin, 1993
80 kDa subunit	IVVQDGITLITKE (amino acids 677–689)	Mimori & Hardin, 1986; Yaneva *et al.*, 1989
70-kDa subunit	ELVYPDYNPEGK (amino acids 527–538)	Reeves & Sthoeger, 1989

[a] X signifies any residue. U signifies a hydrophobic residue. Square brackets enclose all of the possible residues in a given position.

through phosphorylation by DNA-PK, on the other hand, requires double-stranded DNA for the activation.

In two independent studies Ku protein was shown to interact with DNA-PK and it was proposed that Ku protein functions as a targeting protein for DNA-PK by recruiting its binding to DNA (Dvir *et al.*, 1992; Gottlieb & Jackson, 1993). Ku protein binds cooperatively to DNA (de Vries *et al.*, 1989), and there have been reports that, once bound to the ends, Ku protein slides processively to internal sequences in the DNA (Paillard & Strauss, 1991). No evidence was presented in those studies for the energetics for movement of Ku protein along the DNA. The DNA-dependent ATPase activity associated with the Ku-like DNA-ATPase reported here could provide energetics for such a sliding mechanism.

We have not detected DNA helicase activity with the DNA-ATPase using a variety of substrates (Vishwanatha & Baril, 1990; Cao, Q.-P. Unpublished data). This is consistent with the lower catalytic activity of the DNA-ATPase compared to that observed for helicases (Matson & Kaiser-Rogers, 1991; Biswas *et al.*, 1993) and the absence of the typical helicase "deadbox" sequences in the deduced amino acid sequences of the Ku 80- and 70-kDa subunits (Reeves & Sthoeger, 1989; Yaneva *et al.*, 1989; Mimori *et al.*, 1990). It seems probable, therefore, that the DNA-ATPase activity has a different function(s) than a helicase in DNA metabolism related to protein/DNA and/or protein/protein interactions.

The association of Ku protein with the 21S complex of enzymes for DNA synthesis suggests that, in addition to its possible role in transcription, Ku autoantigen may also participate in DNA replication. The transcription factor ENBP-80 that is related to Ku autoantigen binds to gapped DNA, single-stranded DNA tails, replication or transcription bubbles, as well as the usual linear duplex DNA (Falzon *et al.*, 1993). Another transcription factor USF from human cells has recently been shown to interact with human Ku protein in binding to a region of human DNA containing a replication origin adjacent to a promoter (Toth *et al.*, 1993). Also, a DNA binding protein from yeast (high-affinity DNA binding factor, HDF), that appears to be a yeast homologue of human Ku protein, was shown by gene disruption experiments to be essential for the initiation of DNA replication in yeast (Feldman & Winnacker, 1993).

The possible involvement of Ku protein in the process of DNA replication, as well as in transcription, is not that improbable. Ku is an abundant protein in human cells (about 400 000 molecules per cell) and may likely participate in several aspects of macromolecular metabolism. Also, transcription has been shown to activate the initiation of SV40 DNA replication *in vivo* and *in vitro* by the action of transcription factors at auxiliary sequences adjacent to the core replication origin (reviewed by DePamphilis, 1993). There is also evidence for the participation of transcription factors in the initiation of replication of papilloma virus DNA *in vitro* (Yang *et al.*, 1991) and yeast chromosomal DNA *in vivo* (Lue & Kornberg, 1993). Our ability to isolate human Ku protein in tight association with DNA-PK in a subassembly of the 21S complex of enzymes for DNA synthesis will now allow the study of its role in SV40 DNA replication *in vitro* using a cell-free system.

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