Structural and Enzymological Characterization of a Deoxyribonucleic Acid Dependent Adenosine Triphosphatase from KB Cell Nuclei†

Linda Minium Boxer and David Korn*

ABSTRACT: We have purified to near homogeneity the single DNA-dependent ATPase activity that we have identified in extracts of KB cell nuclei. The protein structure of the enzyme was defined by sodium dodecyl sulfate gel electrophoresis, which revealed a single protein band of 75 000 daltons that was coincident with the profile of ATPase activity resolved by the final step of agarose–ATP chromatography or by isoelectric focusing. The enzyme has a p*I* of 8.5, a Stokes' radius by gel filtration of 3.8 nm, and a sedimentation coefficient in high salt of 5.3 S. At low ionic strength the enzyme activity sediments at 7.0 S, suggesting that it may dimerize under these conditions. The purified enzyme has a specific activity of 5.9×10^5 nmol of ATP hydrolyzed per h per mg of protein and is devoid of endonuclease, exonuclease, RNA or DNA polymerase, nicking–closing, and gyrase activities at exclusion limits of 10^{-6}–10^{-8} of the ATPase activity. The enzyme can hydrolyze only ATP or dATP, to generate ADP or dADP plus P_i, but the other NTPs and dNTPs are competitive inhibitors of the enzyme with respect to ATP. A divalent cation ($Mg^{2+} > Mn^{2+} > Ca^{2+}$) as well as a nucleic acid cofactor is required for activity. Single-stranded DNA or deoxyhomopolymers are most effective, but blunt-ended linear and nicked circular duplex DNA molecules are also used at V_{max} values ~20% of that obtained with single-stranded DNA. Intact duplex DNA and polyribonucleotides are unable to support ATP hydrolysis. Velocity gradient sedimentation studies corroborate the interpretations of the kinetic analyses and demonstrate enzyme binding to single-stranded DNA and nicked duplex DNA but not to intact duplex DNA. Although we have not succeeded directly in demonstrating DNA unwinding by this protein, preliminary results suggest that in the presence of ATP, the ATPase can stimulate the reactivity of homogeneous human DNA polymerases α and β on nicked duplex DNA substrates.

During the past few years, a combination of genetic and biochemical approaches has led to the isolation from prokaryotes of a number of distinct DNA-dependent ATPase activities that have been shown to be essential components of in vitro reconstituted *Escherichia coli* phage T4 and phage T7 DNA replication systems. Although these ATPases differ substantially among themselves with respect to their structures, specific catalytic properties and modes of interaction with DNA polymerases and other replication factors on a variety of single-stranded and duplex DNA templates, they can be grouped for purposes of this presentation in three major categories: (1) those that participate primarily in the formation of appropriate "initiation complexes", e.g., *E. coli* factor Y, *dna Z* and *dna* B proteins, and gyrase (Wickner & Hurwitz, 1975; Wickner, 1976; Reha-Krantz & Hurwitz, 1978a,b; McMacken et al., 1977; Arai & Kornberg, 1979; Gellert et al., 1976; Marians et al., 1977); (2) those that possess intrinsic primase activity, e.g., T7 gene 4 protein and T4 gene 41 protein (Scherzinger et al., 1977; Romano & Richardson, 1979a,b; Morris et al., 1979; Nossal, 1979); (3) those that facilitate DNA duplex unwinding, as initially recognized by their required participation in strand displacement synthesis catalyzed by their cognate DNA polymerases on nicked duplex DNA substrates, e.g., T7 gene 4 protein, T4 gene 44/62 and 45 complex, and *E. coli rep* protein (Kolodner & Richardson, 1978; Kolodner et al., 1978; Nossal & Peterlin, 1979; Piperno & Alberts, 1978; Piperno et al., 1978; Scott et al., 1977; Scott & Kornberg, 1978; Kornberg et al., 1978; Yarranton & Gefter, 1979). Of this last group of proteins, only the *rep* protein has been shown by direct assay to be capable of unwinding DNA in the absence of the coupled DNA polymerase (*E. coli* pol III) reaction (Scott et al., 1977; Yarranton & Gefter, 1979).

In addition to this diverse set of proteins that have been implicated in DNA replication, there is a separate group of prokaryotic DNA-dependent ATPases (DNA helicases) that can unwind duplex DNA in the absence of additional protein components, but under assay conditions that require large molar ratios (10^2–10^5) of helicase to substrate DNA (Abdel-Monem & Hoffmann-Berling, 1976; Abdel-Monem et al., 1976, 1977a,b; Richet & Kohiyama, 1976, 1978; Purkey & Ebisuzaki, 1977; Krell et al., 1979; Kuhn et al., 1979). Although one of these enzymes is known to be the T4 gene *dda* product, it appears to be a nonessential phage function (Krell et al., 1979), and at the present time the physiological roles of these DNA helicases are unknown.

Recent reports from this laboratory have presented a detailed description of some of the inherent catalytic properties of near homogeneous human DNA polymerases α and β on a variety of DNA primer–templates of defined structure (Fisher & Korn, 1977; Wang et al., 1977; Eichler et al., 1977; Fisher et al., 1979; Korn et al., 1978; Fisher & Korn, 1979a,b; Wang & Korn, 1980). A goal of those studies has been to define mechanistically interpretable model assay systems which, by analogy to the results with prokaryotes, might prove useful to the identification of candidate replication factors from human tissues. In accord with that objective, we have examined the DNA-dependent ATPase activities in logarithmically growing KB cells, and in this paper we describe the structure and catalytic properties of the single such activity that we have thus far identified in extracts of purified KB nuclei. The near homogeneous enzyme fraction is devoid of nuclease, polymerase, and topoisomerase activities. Although initial efforts to detect DNA unwinding activity directly have been unsuccessful, we have succeeded in preliminary experiments in demonstrating an ATP-dependent stimulation by the ATPase of the activity of DNA polymerases α and β on nicked duplex DNA substrates.

† From the Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305. *Received January 21, 1980.* These studies were supported by Grant CA-14835 and Training Grant GM-09122 (L.M.B.) from the National Institutes of Health.

0006-2960/80/0419-2623$01.00/0

Materials and Methods

Unlabeled ribo- and deoxyribonucleotides were from P-L Biochemicals and Boehringer, [^{3}H]ATP and [^{3}H]dTTP were from New England Nuclear, and [γ-^{32}P]ATP, [α-^{32}P]dTTP, and iodo[^{14}C]acetamide were from Amersham/Searle. Calf thymus and salmon sperm DNAs were Calbiochem A grade. Yeast sRNA was from Sigma Chemical Co. Pancreatic DNase I and micrococcal nuclease were from Worthington; *E. coli* RNA polymerase was from New England Bio Labs; bacterial alkaline phosphatase was from Worthington and was further purified according to Weiss et al. (1968); polynucleotide kinase was from Boehringer, nuclease S1 was from BRL, and *E. coli* DNA polymerase I [Sephadex G-100 fraction (Jovin et al., 1969)] was a gift from Dr. L. A. Loeb, University of Washington. Restriction endonuclease *Hae*III was from New England Bio Labs and was used according to the manufacturer's instructions. Poly(ethylenimine)-impregnated cellulose thin-layer plates (polygram Cel 300 PEI) were purchased from Brinkmann Instruments; Sephadex G-25 and Sephadex G-200 were from Pharmacia; DEAE-cellulose (DE-52) was from Whatman; Bio-Rex 70 was from Bio-Rad; agarose–ATP type 4 was from P-L Biochemicals. LKB ampholytes were used for isoelectric focusing. Materials for polyacrylamide gel electrophoresis were from Bio-Rad. Electrophoresis-grade agarose was from VWR Scientific; BDH sodium dodecyl sulfate was from Gallard-Schlesinger; Coomassie brilliant blue was from Schwarz/Mann. Standard proteins included the following: 7S γ-globulin and bovine serum albumin from Sigma; chymotrypsinogen, ovalbumin, pancreatic RNase, and aldolase from Pharmacia; β-galactosidase, catalase, phosphorylase *a*, and pyruvate kinase from Worthington. DNA–cellulose was prepared as described (Alberts & Herrick, 1971) with heat-denatured or native calf thymus DNA. Poly(dA), poly(dC), and poly(dG) were from Collaborative Research, and poly(A) and poly(dA-dT) were from Miles. Oligo(dT)$_{\overline{200}}$, oligo(dT)$_{\overline{34}}$, oligo(dT)$_{\overline{16}}$, and oligo(dT)$_{\overline{200}}$–[^{3}H](dT)$_x$ (16 800 cpm/pmol of terminal dTMP residue) were synthesized as described (Wang et al., 1974), with calf thymus terminal transferase that was a generous gift from Dr. R. L. Ratliff, Los Alamos Scientific Laboratory. Activated DNA was prepared by digestion of salmon sperm DNA with pancreatic DNase I to 6% acid solubility. Activated salmon sperm [5′-^{32}P]DNA was prepared according to Weiss et al. (1968). PM2 form I [^{3}H]DNA (8.1 cpm/pmol) was prepared by T. S.-F. Wang according to Espejo et al. (1969); singly nicked PM2 DNA was prepared as described by Greenfield et al. (1975); relaxed PM2 DNA was prepared with KB cell nicking–closing enzyme, purified by T. S.-F. Wang according to Vosberg & Vinograd (1976) and demonstrated to be nuclease free. Multiply nicked KB [^{3}H]DNA was prepared and characterized as described by Wang & Korn (1980). M13 [^{3}H]DNA (18.2 cpm/pmol) was prepared by T. Bonura (Stanford) according to Marco et al. (1974), and *E. coli* plasmid pMS 4375, a recombinant plasmid containing a 1980 base-pair EcoRI restriction fragment of mouse mtDNA cloned into pBR 322, was the generous gift of J. Battey and D. A. Clayton (Stanford).

Growth and Harvest of KB Cells. The KB cells were grown and harvested as described (Fisher & Korn, 1977).

Standard ATPase Assay. The assay measures the release of [^{3}H]ADP from [^{3}H]ATP or of $^{32}P_i$ from [γ-^{32}P]ATP by thin-layer chromatography on PEI–cellulose. The 20-μL reaction mixture contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2mM $MgCl_2$, 2mM ATP, 200 μg/mL bovine serum albumin, 0.1 mM (nucleotide) heat-denatured calf thymus DNA, [^{3}H]ATP at 50 mCi/mmol or [γ-^{32}P]ATP at 20 mCi/mmol, and enzyme. Incubation was at 37 °C. Aliquots of 2 μL were spotted on PEI–cellulose strips (0.6 × 10.0 cm), marked with ADP, and developed in 1 M formic acid–0.5 M LiCl for 35 min. The strips were taped with scotch tape, cut into sections, and counted in toluene–Omnifluor. One unit of ATPase activity is defined as the amount of enzyme that can hydrolyze 1 nmol of ATP per h at 37 °C. Specific activity is expressed as units per milligram of protein.

Velocity Gradient Centrifugation. Sedimentation studies were performed in the SW 60 Ti rotor in linear 15–40% (v/v) glycerol gradients containing 20 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 1 mM EDTA, and ±300 mM NaCl. The sample load was 150 μL. Centrifugation was for 24 h at 50 000 rpm at 5 °C. Fractions were collected from the bottom of the tube and were assayed immediately.

Sephadex G-200 Gel Filtration. A column (1.5 × 75 cm) of Sephadex G-200 was poured, equilibrated, and developed in 20 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 1 mM EDTA, 10% glycerol, and 300 mM NaCl. The column was calibrated with blue dextran (V_0), bovine γ-globulin (M_r 160 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), chymotrypsinogen (M_r 25 000), and pancreatic RNase (M_r 13 700).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gels were formulated and run essentially as described by Laemmli (1970), using a 4% stacking gel. The sample was prepared by precipitation with 10% trichloroacetic acid in the presence of 1% (w/v) sodium dodecyl sulfate. Precipitates were solubilized in Laemmli stacking gel buffer plus 1% (w/v) sodium dodecyl sulfate, 1% (v/v) 2-mercaptoethanol, and 10% glycerol and were boiled for 5 min. Gels were stained with Coomassie brilliant blue.

Agarose Gel Electrophoresis. Electrophoresis of DNA samples on nondenaturing 0.8% agarose gels or on alkaline (denaturing) 2% agarose gels was performed according to Keller (1975) and McDonnell et al. (1977), respectively.

Labeling of Proteins with Iodo[^{14}C]acetamide. Labeling was performed by the procedure described by Fisher (1979). After precipitation of protein samples with trichloroacetic acid as described above, the pellets were resolubilized in 10 μL of 250 mM Tris-HCl, pH 8.2, and 0.5% sodium dodecyl sulfate, and any excess acid was neutralized by addition of 2 M Tris base. The sample was boiled for 5 min, 4 μCi (40 mM) of iodo[^{14}C]acetamide was added, and the sample was incubated in the dark for 2 h at 37 °C. The reaction was stopped by addition of 90 μL of 62.5 mM Tris-HCl, pH 6.8, 1% (w/v) sodium dodecyl sulfate, and 1% (v/v) 2-mercaptoethanol, and protein was precipitated with 10% trichloroacetic acid to remove the unreacted iodoacetamide. The pellet was then solubilized in Laemmli stacking buffer, boiled, and electrophoresed. After staining and destaining, we fluorographed the gels (Bonner & Laskey, 1974) using preflashed (Laskey & Mills, 1975) Kodak XR-5 film that was exposed at −70 °C.

Isoelectric Focusing. Isoelectric focusing was carried out as described (Wang et al., 1974), using a 5–50% glycerol gradient instead of sucrose to stabilize the pH gradient.

3′→5′-Exonuclease Assays. Assays with oligonucleotide substrates were performed as described by Wang et al. (1974) in reactions (100 μL) containing 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, ±2 mM ATP, 200 μg/mL bovine serum albumin, 30 units of enzyme, and 8.7 μM (dT)$_{\overline{200}}$–[^{3}H](dT)$_x$ ± 43.5 μM poly(dA). The lower limit of detection was 27 fmol of dTMP hydrolyzed per h.

5′→3′-Exonuclease Assays. The reaction mixture (130 μL) contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, ±2 mM ATP, 200 μg/mL bovine serum albumin, 65 μM activated salmon sperm [5′-^{32}P]DNA, and 45 units of enzyme. The lower limit of detection was 40 fmol of [^{32}P]dNMP hydrolyzed per h.

Endonuclease Assays. Assay for duplex DNA endonuclease activity was performed in a reaction (180 μL) containing 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, ±2 mM ATP, 200 μg/mL bovine serum albumin, 0.76 μg of PM2 form I [^{3}H]DNA, and 40 units of enzyme. After incubation for 60 min at 37 °C, reaction products were analyzed by ethidium bromide–cesium chloride buoyant density centrifugation to detect conversion of form I to form II molecules (Radloff et al., 1967). The lower limit of detection was 0.7 fmol of phosphodiester bonds cleaved per h. Assay for single-stranded DNA endonuclease activity was carried out in a reaction (50 μL) identical with the above, except the substrate was 0.62 μg of M13 [^{3}H]DNA and 14 units of enzyme was added. After incubation at 37 °C for 60 min, the conversion of closed circular DNA molecules to linear forms was analyzed by alkaline agarose gel electrophoresis. The lower limit of detection was 1.5 fmol of phosphodiester bonds incised per h.

Polymerase Assays. The DNA polymerase assay (150 μL) contained 50 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 200 μg/mL bovine serum albumin, 100 μM each of dATP, dGTP, dCTP, and [^{3}H]dTTP (1042 cpm/pmol), 1 mg/mL activated salmon sperm DNA, and 24 units of enzyme. After incubation for 1 h at 37 °C, reactions were terminated and processed as described (Wang et al., 1977). The lower limit of detection was 0.1 pmol of dTMP incorporated per h. The RNA polymerase reaction (150 μL) contained 40 mM Tris-HCl, pH 7.5, 40 mM $(NH_4)_2SO_4$, 1.6 mM $MnCl_2$, 20 mM 2-mercaptoethanol, 0.4 mM EDTA, 200 μg/mL bovine serum albumin, 0.4 mM each of GTP, UTP, CTP, and [^{3}H]ATP (1042 cpm/pmol), 1 mg/mL denatured salmon sperm DNA, and 24 units of enzyme. After incubation for 1 h at 37 °C, reactions were terminated and processed as described above. The lower limit of detection was 0.1 pmol of dAMP incorporated per h.

Assays for Nicking–Closing Activity. Reactions (50 μL) contained 10 mM Tris-HCl, pH 8.0, 20 mM KCl, 5 mM EDTA, 50 μg/mL bovine serum albumin, 1 mM spermidine, 0.14 μg of PM2 form I [^{3}H]DNA, and 20 units of enzyme. Reactions were also carried out under the standard assay conditions for ATPase activity, in the presence of 2 mM ATP. Products were analyzed by nondenaturing agarose gel electrophoresis to detect conversion of form I to form IV (relaxed, closed circular) DNA species.

Assays for DNA Gyrase Activity. Reactions (50 μL) contained 35 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 18 mM KPO_4, pH 7.5, 1.4 mM ATP, 5 mM 2-mercaptoethanol, 90 μg/mL yeast sRNA, 5 mM spermidine, 50 μg/mL bovine serum albumin, 0.14 μg of PM2 form IV [^{3}H]DNA, and 20 units of enzyme. Reactions were also carried out under standard ATPase assay conditions. Products were analyzed on nondenaturing agarose gels to score the conversion of form IV to form I DNA species.

Pancreatic DNase I Treatment of Calf Thymus DNA. Reactions (1 mL) contained 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 175 μg of heat-denatured calf thymus DNA, and 1 μg of DNase I. Incubation was for 10 min at 23 °C. The digestion was terminated by addition of EDTA to 10 mM, and the reaction mixture was extracted with redistilled phenol. The DNA products were then recovered by filtration on a Bio-Gel A-5m column equilibrated and developed with 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA.

Micrococcal Nuclease Treatment of Calf Thymus DNA. The reaction (1 mL) contained 10 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$, 100 μg of denatured calf thymus DNA, and 3 ng of micrococcal nuclease. After incubation for 10 min at 37 °C, the DNA products were recovered as described above.

Pancreatic DNase I Treatment of PM2 DNA. The reaction (1 mL) contained 20 mM Tris-HCl, pH 8.0, 62.4 mM NaCl, 300 μg/mL bovine serum albumin, 10 mM $MgCl_2$, 65 μg of PM2 form I [^{3}H]DNA, and 10 ng of DNase I. After incubation for 10 min at 20 °C, the mixture was extracted with redistilled phenol and then ether, and the DNA was recovered by ethanol precipitation. The average number of nicks per DNA molecule was estimated to be 10 by alkaline sucrose gradient sedimentation (Studier, 1965).

Micrococcal Nuclease Treatment of PM2 DNA. The reaction (1 mL) contained 10 mM Tris-HCl, pH 8.9, 10 mM $CaCl_2$, 200 μg/mL bovine serum albumin, 310 μg of PM2 form I [^{3}H]DNA, and 5 ng of micrococcal nuclease. After incubation at 22 °C for 50 min, the DNA products were recovered and analyzed as described above. The average number of nicks per DNA molecule was determined to be 10.

Preparation of Blunt-End Duplex DNA. Blunt-end duplex DNA was prepared from plasmid pMS 4375 in a reaction (1.0 mL) that contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 200 μg/mL bovine serum albumin, 150 μg of plasmid [^{3}H]DNA, and 37.5 units of *Hae*III restriction endonuclease. After incubation at 37 °C for 4 h, the DNA products were separated by agarose gel electrophoresis. An ~1950 base-pair fragment of mouse mtDNA was located by staining a portion of the gel with ethidium bromide and was recovered by electroelution, followed by ethanol precipitation and dialysis against 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM EDTA.

Assay of Unwinding of Duplex DNA. The method of Abdel-Monem et al. (1976) was used. Partially duplex M13 DNA was prepared by RNA-primed DNA synthesis. The reaction (0.5 mL) contained 67 mM Tris-HCl, pH 7.5, 6.7 mM $MgCl_2$, 0.66 mM 2-mercaptoethanol, 16.6 mM NaCl, 200 μg/mL bovine serum albumin, 10 μM each of ATP, CTP, GTP, and UTP, 50 μM each of dATP, dGTP, and dCTP, 2 μM [α-^{32}P]dTTP (16 Ci/mmol), 8 nmol (nucleotide) of M13 [^{3}H]DNA, 10 μg of *E. coli* RNA polymerase, and 2.2 μg of *E. coli* DNA polymerase I. After incubation for 60 min at 35 °C, the reaction mixture was extracted with redistilled phenol, and the products were recovered by filtration through a Bio-Gel A-5m column in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The reaction (50 μL) for duplex unwinding contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 2 mM ATP, 200 μg/mL bovine serum albumin, 100 pmol of M13 [^{3}H]DNA base-paired to 12 pmol of ^{32}P-labeled complementary DNA, and 10 units of ATPase (~12 molecules of enzyme per molecule of partially duplex DNA). After incubation at 37 °C for 45 min, a sample (35 μL) was taken from each reaction mixture and diluted into 240 μL of 30 mM sodium acetate buffer, pH 4.5, 20 mM NaCl, 5 mM $ZnSO_4$, and 50 units/mL single-strand-specific S1 nuclease. The latter reaction was incubated for 45 min at 45 °C and then chilled. Carrier calf thymus DNA and an equal volume of 20% trichloroacetic acid were added, and after 30 min at 0 °C, the solution was filtered through GF/C filter disks to quantitate the recovery of

acid-precipitable ^{32}P-labeled DNA.

Assays for Primase Activity. (1) The reaction with DNA polymerase α (70 μL) contained 40 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 200 μg/mL bovine serum albumin, 40 μM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (6040 cpm/pmol), 500 μM each of ATP, GTP, CTP, and UTP, 4 mM $MgCl_2$, 100 μM M13 [^{3}H]DNA, 1 unit of KB DNA polymerase α, fraction VIII (Fisher & Korn, 1977), and 10 units of ATPase. The reaction with DNA polymerase β contained, in 70 μL, 50 mM Tris-HCl, pH 8.9, 300 μg/mL bovine serum albumin, 25 μM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (5830 cpm/pmol), 500 μM each of the rNTPs, 2 mM $MnCl_2$, 100 μM M13 [^{3}H]DNA, 0.13 unit of human hepatic DNA polymerase β, isoelectric focused fraction (Wang et al., 1977), and 10 units of ATPase. Incubations were at 37 °C for 60 min. The reactions were processed for DNA polymerase activity as described by Wang et al. (1977). Since neither of the human DNA polymerases is capable of utilizing single-stranded circular M13 DNA, the assay depends on the formation of oligonucleotide primer species by the ATPase.

(2) Chromatography on DEAE-cellulose was used to separate product oligoribonucleotides from the labeled ATP precursor. The reaction (25 μL) contained 50 mM Tris-HCl, pH 7.5, 20 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 500 μM each of GTP, UTP, CTP, and [^{3}H]ATP (440 cpm/pmol), 200 μg/mL bovine serum albumin, 50 μM heat-denatured calf thymus DNA, and 10 units of ATPase. Incubation was at 37 °C for 60 min. Then 1 unit of bacterial alkaline phosphatase was added and the incubation was continued for 120 min. The reaction mixture was loaded onto a 2.5 × 0.5 cm column of DEAE-cellulose, and the column was rinsed with water to remove the labeled nucleoside. The column was then eluted in steps with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 200 mM NaCl, and 500 mM NaCl to remove any bound oligoribonucleotides.

Other Methods. The pH and ionic strength of buffers were measured at room temperature with a Corning Digital pH meter and a Radiometer conductivity meter, respectively. Protein was estimated spectrophotometrically by measuring absorbance at 260 and 280 nm or was assayed by the technique of Schaffner & Weissmann (1973) with bovine serum albumin as the standard. Fluorograms of polyacrylamide gels were scanned at 505 nm with a Transidyne RFT densitometer.

Results

Purification Protocol. By the procedure that is summarized in Table I, we have isolated a single species of DNA-dependent ATPase activity from purified KB nuclei and have succeeded in purifying the enzyme to near homogeneity. To separate DNA-dependent ATPases from the plethora of ATPase activities that are expected to be present in KB extracts, we imposed the requirement of adsorbability at low ionic strength (0.07 M NaCl) to a column of denatured DNA–cellulose. We selected this criterion even though we recognize that not all DNA-dependent ATPases of possible interest need necessarily satisfy it, e.g., the phage T7 gene 4 protein (Scherzinger et al., 1977). Logarithmically growing KB cells were harvested and separated into the standard cytoplasmic (Fisher & Korn, 1977) and purified nuclear fractions (Wang et al., 1977). The cytoplasmic crude extract and both standard (0.2 M KPO_4, pH 8.5) (Wang et al., 1977) and 2 M NaCl extracts of the nuclear fraction were separately chromatographed on a column of denatured DNA–cellulose. By this procedure, only the standard nuclear extract was found to contain a peak of ATPase activity that adsorbed to the column, and this extract was therefore selected as the starting material (fraction I) for the purification procedure. All subsequent steps were performed at 4 °C. The crude nuclear extract was dialyzed against buffer A (20 mM Tris-HCl, pH 8.1, 20% glycerol, 20 mM 2-mercaptoethanol, and 1 mM EDTA) containing 70 mM NaCl for 15 h. A precipitate that formed was removed by centrifugation, and the supernatant was loaded onto a column (150 mL) of DEAE-cellulose that had been equilibrated with buffer A containing 70 mM NaCl and was connected in series to a 30-mL column of native DNA–cellulose. The columns were washed with 500 mL of buffer A containing 70 mM NaCl. The DNA-dependent ATPase activity flows through DEAE-cellulose (fraction II), adsorbs to native DNA–cellulose, and is eluted with a 180-mL linear gradient of 70–500 mM NaCl in buffer A. The activity is recovered in a broad peak centered at 230 mM NaCl (fraction III). This fraction is loaded directly onto a 12-mL column of denatured DNA–cellulose, and the enzyme is eluted with a 72-mL linear gradient of 200–500 mM NaCl in buffer A, followed by a step with the 500 mM NaCl buffer (fraction IV). The peak of enzyme activity is recovered at 480 mM NaCl. At this stage of the procedure large losses of activity have resulted from dialysis of the enzyme into buffer A with 70 mM NaCl. To avoid dialysis, we diluted fraction IV 1:1 with buffer A containing 200 μg/mL bovine serum albumin and loaded it onto a 4-mL column of denatured DNA–cellulose that overlays a 1-mL plug of DEAE-cellulose. The enzyme is stepped off the column with 5 column volumes of buffer A containing 500 mM NaCl (fraction V). (In the absence of the DEAE-cellulose plug, ATP hydrolysis by fraction V is not completely dependent on added DNA, most probably because of leaching of DNA fragments from the DNA–cellulose column.) Fraction V is desalted on a column of Sephadex G-25 (fraction VI), and the enzyme is loaded immediately onto a 6-mL column of Bio-Rex 70 equilibrated with buffer A containing 70 mM NaCl. The ATPase is recovered by elution with a 36-mL linear gradient of 70–350 mM NaCl in buffer A; the peak of activity is centered at 200 mM NaCl (fraction VII). Fraction VII is immediately diluted 1:1 with buffer A containing 200 μg/mL bovine serum albumin and is loaded onto a 2-mL column of agarose–ATP type 4. The column is washed with 10 mL of buffer A containing 1 mM GTP, which removes two major protein contaminants of about 105000 and 60000 daltons that are revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1B), and the ATPase is then eluted with a linear gradient of 70–500 mM NaCl in buffer A. The peak of enzyme activity is recovered at 220 mM NaCl (fraction VIII). The final preparation is extremely labile, with ~20% of the activity remaining after 24 h at 4 °C, and we

Table I: Purification of KB Cell DNA-Dependent ATPase[a]

	fraction	protein (mg)	act. (units)	sp act. (units/mg)	yield (%)
(I)	crude extract	198	367 000	1 850	
(II)	DEAE-cellulose				
(III)	native DNA–cellulose	5.3	37 100	5 110	(100)[b]
(IV)	denatured DNA–cellulose	0.88	22 400	25 400	83
(V)	second denatured DNA–cellulose		19 000		70
(VI)	Sephadex G-25	0.70	17 200	24 600	63
(VII)	Bio-Rex 70	0.058	12 300	212 000	45
(VIII)	agarose–ATP	0.014	8 200	586 000	30

[a] Purification from 15 L of KB cells. [b] Fraction III is the first fraction with which it is possible to identify DNA-dependent ATPase activity.

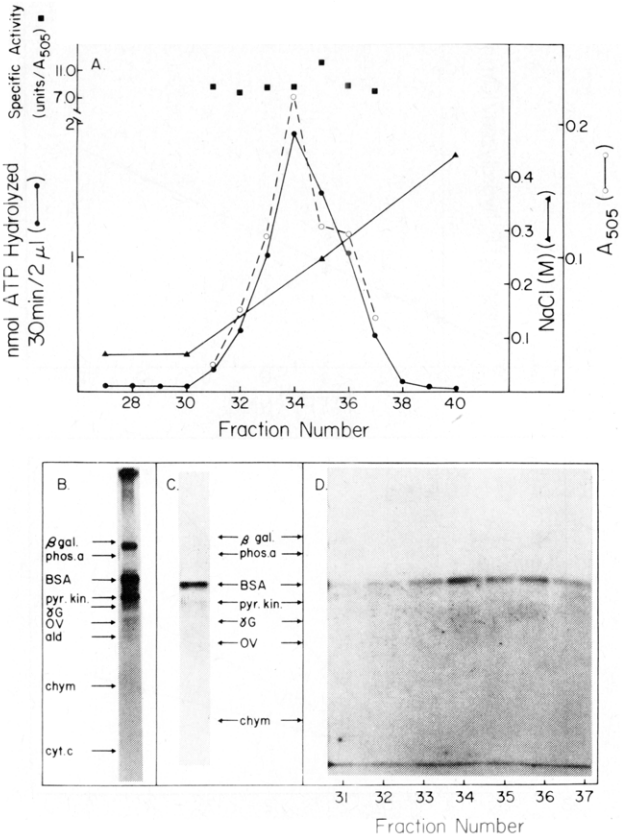

FIGURE 1: Elution profile and sodium dodecyl sulfate–polyacrylamide gel analysis of KB cell DNA-dependent ATPase on agarose–ATP. (A) Fraction VII ATPase, 8200 units, was chromatographed on agarose–ATP (see Table I and the text for details), and 2 μL of each 0.8-mL fraction was assayed for enzyme activity. Each column fraction that encompassed the ATPase peak was labeled with iodo[^{14}C]-acetamide and analyzed on a sodium dodecyl sulfate–8% polyacrylamide slab gel as described under Materials and Methods. The fluorogram of the gel (D) was scanned at 505 nm, and the absorbance of the single protein band has been plotted in arbitrary units in (A). The film was exposed for 950 h. The iodo[^{14}C]acetamide-labeled standard proteins (from the top to the bottom of the gel) are as follows: β-galactosidase, phosphorylase *a*, bovine serum albumin, pyruvate kinase, the heavy chain of bovine γ-globulin, ovalbumin, and chymotrypsinogen. The ATPase migrates slightly slower than serum albumin and is assigned a M_r of 75 000. (Note: bovine serum albumin migrates as a protein of M_r 74 000 when it is treated with iodo-[^{14}C]acetamide and electrophoresed under these conditions.) (B) Sodium dodecyl sulfate–7 to 14% polyacrylamide gradient slab gel analysis of iodo[^{14}C]acetamide-labeled fraction VII ATPase (570 units). The film was exposed for 2000 h. The labeled protein standards included those listed in (D) plus aldolase, the light chain of bovine γ-globulin, and cytochrome *c*. (C) Sodium dodecyl sulfate–8% polyacrylamide gel analysis of iodo[^{14}C]acetamide-labeled fraction VIII ATPase (230 units). The fluorogram of a slab gel lane is shown. Only a single protein band of 75 000 daltons was detected. The film was exposed for 340 h.

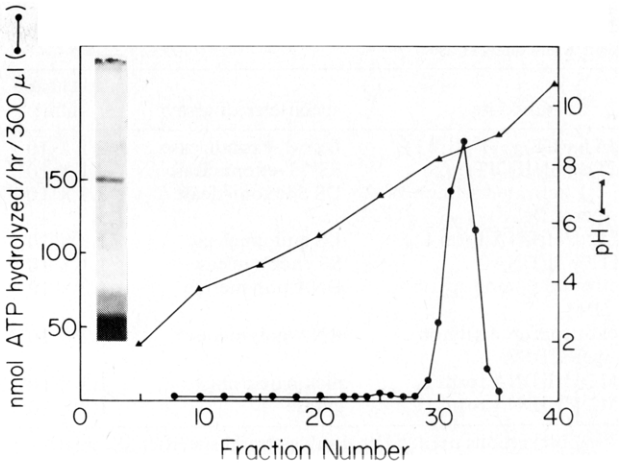

FIGURE 2: Isoelectric focusing of KB cell DNA-dependent ATPase and sodium dodecyl sulfate–polyacrylamide gel analysis of the activity peak. Fraction VIII ATPase, 650 units, was focused in a glycerol gradient as described under Materials and Methods for 4 days at 4 °C. Fractions (300 μL) were collected and assayed for enzyme activity. The pH of every fifth fraction was determined. The peak fractions of ATPase activity were pooled, treated with iodo[^{14}C]acetamide, and analyzed on a denaturing 8% polyacrylamide slab gel as described in Figure 1. The fluorogram of the gel lane is displayed. The film was exposed for 950 h. The dark zone at the bottom of the lane is due to labeled ampholytes that migrate near the dye front.

have thus far been unable to stabilize the activity by adding such ingredients as glycerol, sucrose, dextrose, ethylene glycol, Triton X-100, bovine serum albumin, or lysozyme in concentrations up to 1 mg/mL, lysine, or ATP. Fraction VIII is stored routinely at −70 °C, and the enzyme is stable for at least 4 months at this temperature.

Structural Characterization. By sodium dodecyl sulfate–polyacrylamide gel electrophoresis, fraction VIII can be shown to contain only a single protein species of 75 000 daltons (Figure 1C). Because the ATPase fails to enter nondenaturing gels, even at 4% acrylamide, two other methods were used to establish the identity of this protein band with the enzyme activity. (1) Each individual fraction of the peak of ATPase activity eluted from the final step of chromatography on agarose–ATP (Figure 1A) was analyzed on a denaturing slab gel (Figure 1D). Only the single protein band of M_r 75 000 was displayed, the quantity of which was directly proportional to the enzyme activity across the elution profile. (2) A similar procedure was used to evaluate the peak of ATPase activity resolved by isoelectric focusing. As is shown in Figure 2, the enzyme focused sharply at a p*I* of 8.5, and denaturing gel analysis of the fractions that comprised the activity peak again revealed only the single 75 000-dalton protein species (Figure 2, insert).

Velocity Sedimentation and Gel Filtration Analysis. The ATPase sediments in 300 mM NaCl at 5.3 S and in the absence of salt at 7.0 S. This result suggests that the enzyme may dimerize at low ionic strength. The Stokes' radius determined by Sephadex G-200 gel filtration in 300 mM NaCl is 3.85 nm. From the methods of Siegel & Monty (1966), and assuming a range of partial specific volumes of 0.71–0.76, one may calculate native molecular weight values for the ATPase activity of 82 000–94 000. This computation, together with the result of the prolonged-exposure fluorogram (Figure 1B) that excludes the presence of stoichiometric quantities of proteins in the size range $5000 \leq M_r \leq 40\,000$ from the enzyme fraction, indicates that the enzyme is comprised of a single 75 000 polypeptide chain.

Absence of Contaminating or Associated Enzymatic Activities. For our ultimate objective of evaluating the possible interaction of the ATPase with DNA polymerases α and β on defined primer–templates, it was essential to document the absence of contaminating or associated enzymatic activities from the preparation. The results of our extensive survey of these activities are summarized in Table II. Both endodeoxyribonuclease (single strand and double strand specific) and exodeoxyribonuclease (3′ → 5′ and 5′ → 3′) activities can be excluded to levels of 10^{-6}–10^{-8} of the ATPase activity. At comparable levels of sensitivity, we have also been able to document the absence of DNA polymerase, RNA polymerase, nicking–closing, and gyrase activities from the fraction VIII ATPase.

Table II: Exclusion of Contaminating Activities from KB Cell ATPase

substrate	specificity of assay	exclusion limit
$(dA)_{\overline{n}}\cdot(dT)_{\overline{200}}$-[^{3}H](dT)$_{\overline{4}}$	DS[a] 3′-exonuclease	9.1×10^{-7}
$(dT)_{\overline{200}}$-[^{3}H](dT)$_{\overline{4}}$	SS[a] 3′-exonuclease	9.1×10^{-7}
5′-^{32}P activated salmon sperm DNA	DS 5′-exonuclease	8.9×10^{-7}
PM2 [^{3}H]DNA form I	DS endonuclease	1.8×10^{-8}
M13 [^{3}H]DNA	SS endonuclease	1.1×10^{-7}
activated salmon sperm DNA	DNA polymerase	4.2×10^{-6}
heat-denatured salmon sperm DNA	RNA polymerase	4.2×10^{-6}
PM2 [^{3}H]DNA form I	nicking-closing	1.3×10^{-6}
PM2 [^{3}H]DNA form IV	gyrase	1.3×10^{-6}

[a] Abbreviations used: DS, double-strand specific; SS, single-strand specific.

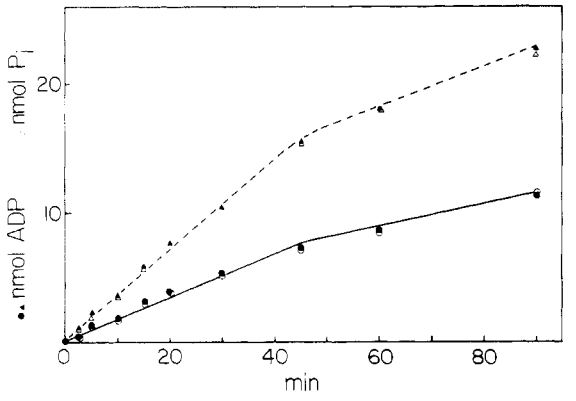

FIGURE 3: Time course and stoichiometry of ATP hydrolysis at two concentrations of enzyme. Reactions were formulated as described under Materials and Methods with 10 units (● and ○) and 20 units (▲ and △) of fraction VIII ATPase. The incubations contained [^{3}H]ATP (50 mCi/mmol) and [γ-^{32}P]ATP (15 mCi/mmol). Aliquots (2 μL) were removed at the times indicated and analyzed on PEI-cellulose plates to follow the generation of [^{3}H]ADP and $^{32}P_i$.

Enzymological Characterization. (1) General Properties of the ATPase Reaction. All of the studies to be presented were carried out with fraction VIII ATPase. The enzyme is a nucleoside triphosphate phosphohydrolase that converts ATP or dATP to the corresponding nucleoside diphosphate and P_i; no generation of nucleoside monophosphate can be detected. The stoichiometry of the reaction is illustrated in Figure 3. The hydrolysis of ATP is linear with time for up to 40 min, and the rate is directly proportional to enzyme concentration. ATP and dATP are the only triphosphates that can be hydrolyzed; the rate of hydrolysis of dATP is 90% of that with ATP. Kinetic analysis of the ATPase reaction is shown in the form of a Lineweaver–Burk plot in Figure 4A, from which the K_m for ATP can be determined to be 0.32 mM. Although none of the other NTPs or dNTPs can be hydrolyzed by the KB enzyme, they all appear to be competitive inhibitors of the reaction with respect to ATP (Table III), with K_i values of 1.2–1.7 mM. Both ADP and AMP are more potent competitive inhibitors, with K_i values (0.35 mM) close to the K_m values for the substrates. Dixon plot analysis of the effect of ADP on the hydrolysis of ATP (Figure 4B) displays simple competitive inhibition kinetics. Adenosine has no effect on the reaction, suggesting that at least one phosphate group is necessary for binding to the enzyme. Although the enzyme appears to have a high degree of specificity for the adenine moiety, the structure of the sugar is less important, since *ara*-ATP is also a potent competitive inhibitor (K_i = 0.34 mM) (Table III).

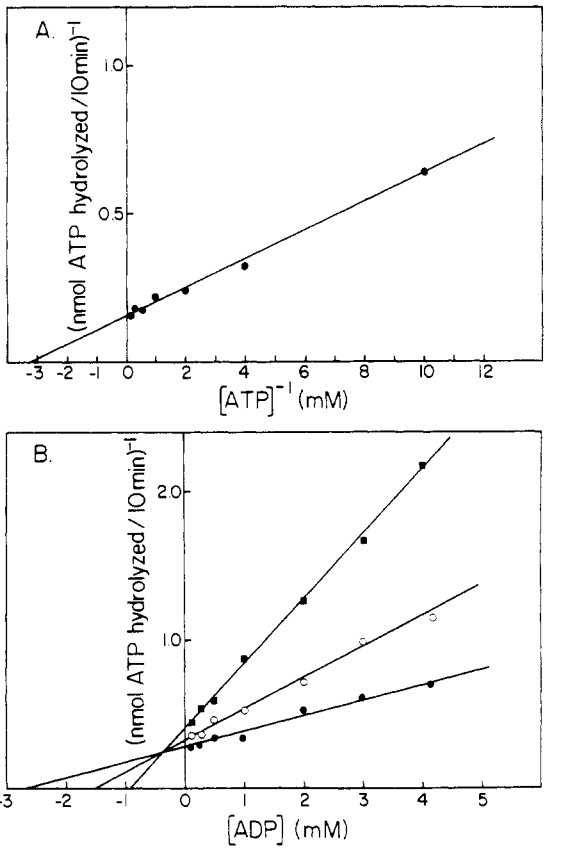

FIGURE 4: Hydrolysis of ATP and competitive inhibition by ADP. (A) Lineweaver–Burk plot of ATP hydrolysis. Reactions were formulated as described under Materials and Methods with 35 units of ATPase fraction VIII and ATP concentrations that ranged from 0.05 to 4.0 mM. The K_m for ATP is 0.32 mM. (B) Dixon plot analysis of the inhibition of ATP hydrolysis by ADP. Reactions were formulated as described above with 25 units of fraction VIII ATPase and with ATP at 0.50, 1.0, and 2.0 mM. The concentration of ADP varied from 0.125 to 4.15 mM. The K_i for ADP was determined to be 0.35 mM.

Table III: K_m and K_i Values for Nucleotides

nucleotide	K_m (mM)	K_i (mM)
ATP	0.32	
dATP	0.33	
GTP		1.22
CTP		1.57
UTP		1.49
dGTP		1.28
dCTP		1.67
TTP		1.52
ADP		0.35
AMP		0.37
ara-ATP		0.34

The ATPase requires a divalent cation for activity. In the presence of a constant concentration of 2 mM ATP, Mg^{2+} is the preferred cation at an optimal level of 2 mM; Mn^{2+}, at an optimal level of 1 mM, and Ca^{2+}, at an optimal level of 2 mM, support 70 and 30%, respectively, of the maximal activity obtained with Mg^{2+}.

The enzyme has a broad pH optimum of 6.8–8.8. It is moderately sensitive to sulfhydryl antagonists; thus, 5 mM *N*-ethylmaleimide decreases the activity by 40% (Table IV) (note that this experiment was performed in the presence of 2 mM 2-mercaptoethanol due to the high concentration of this compound in the enzyme buffer and our inability to dialyze fraction VIII). The ATPase is resistant to salt (NaCl or KCl) up to concentrations of 200 mM. Interestingly, KPO_4 up to

Table IV: Effect of Added Reagents on the ATPase Reaction

addition	concn (mM)	% act.[a]
NaCl	200	100
	300	60
	400	22
KCl	200	100
	300	52
	400	18
KPO_4	50	100
$NaPP_i$	10	93
	20	57
	30	24
2-mercaptoethanol	5	85
	20	100
NEM[b]	5	60
	20	32
nalidixic acid	0.090	100
novobiocin	0.075	100

[a] 100% activity is defined as that observed under standard assay conditions. [b] In the presence of 2 mM 2-mercaptoethanol; NEM, *N*-ethylmaleimide.

50 mM has no effect on the enzyme activity, while 20 mM $NaPP_i$ decreases the activity by ~40%. Neither nalidixic acid (up to 90 μM) nor novobiocin (up to 15 μM) has any detectable effect on the ATPase reaction. At these concentrations, both of these compounds will completely inhibit the activity of the *E. coli* gyrase (Higgins et al., 1978).

We have failed to detect any evidence for a phosphorylated enzyme intermediate in the ATP hydrolysis reaction. Thus, we could not detect any acid-precipitable ^{32}P after incubation of the enzyme with [γ-^{32}P]ATP, either at pH 7.5 or at pH 5.0 (Luduena & Sussman, 1976; Milstein, 1964) in the presence or absence of DNA. (The latter result also indicates that the enzyme does not possess a polynucleotide kinase activity that can be detected with single-stranded DNA.) Similarly, we have been unable to observe ^{32}P exchange following incubation of the enzyme with [γ-^{32}P]ATP and [^3H]ADP, under standard assay conditions in the presence or absence of DNA.

(2) Characterization of the DNA Cofactor Requirement. The relative efficiency and some of the kinetic parameters of a variety of nucleic acids in supporting the ATPase reaction are presented in Table V. ATP hydrolysis is entirely dependent on the presence of a single-stranded heteropolymeric or homopolymeric polydeoxynucleotide. Thus, denatured calf thymus DNA, closed circular single-stranded M13 DNA, poly(dT), poly(dA), and poly(dC), all at a constant concentration of 100 μM (nucleotide), were all equally effective in supporting the ATPase activity, and they all yielded similar kinetic values of apparent K_m [1.1–1.5 μM (nucleotide)] and V_{max} (700–800 pmol of ADP per min). The relatively high efficacy of activated salmon sperm DNA was presumably a reflection of the high concentration of single-stranded gaps (Fisher et al., 1979) in this population of DNA molecules. The identical efficiency of M13 DNA and single-stranded linear molecules suggested that free termini were not significantly involved in the interaction of the enzyme with single-stranded polydeoxynucleotides, and this interpretation was strengthened by the observation that single-stranded DNA fragments ~400 nucleotides long that bore either 3′-hydroxyl or 3′-phosphoryl termini (see Materials and Methods) were indistinguishable in their ability to support ATP hydrolysis (data not shown). Finally, the comparable efficiency of $(dT)_{\overline{200}}$ and $(dT)_{\overline{16}}$, and the complete inactivity of $(dT)_{\overline{4}}$, indicated that there was a minimum length of oligodeoxynucleotide that was necessary to satisfy the cofactor requirement.

Table V: Nucleic Acid Specificity

nucleic acid	[^3H]ADP formed[a] (% of control)	K_m[b]	V_{max}[b] (pmol of ADP per min)
denatured calf thymus DNA	(100)	1.2 μM	714
none	1		
M13	116	1.5 μM	828
$(dT)_{\overline{4}}$	0.8		
$(dT)_{\overline{16}}$	103	1.4 μM	735
$(dT)_{\overline{200}}$	106	1.1 μM	757
poly(dA)	102		
poly(dC)	96		
poly(dG)	23		
poly(dA-dT)	22		
poly(rA)	5		
yeast sRNA	3		
activated salmon sperm DNA	88		
PM2 form I	23		
PM2 form II (singly nicked)	22	4 nM (nicks)	157
PM2 form IV	5		
blunt-end DNA fragment	23	5 nM (sites)[c]	164

[a] Each nucleic acid was present at a concentration of 100 μM (nucleotide) in the standard ATPase reaction mixture with 40 units of fraction VIII ATPase. [b] The kinetic parameters were determined from Lineweaver–Burk plots. Values of K_m are expressed as μM of nucleotide, unless otherwise indicated. [c] Analysis of the blunt-ended 1950 base-pair fragment of mouse mtDNA (see Materials and Methods) by alkaline agarose gel electrophoresis demonstrated the presence of an average of 1.2 nicks per duplex fragment. Computation of K_m values for this cofactor was performed under two different assumptions. (1) Only the nicks were utilized; i.e., there were 1.2 "sites" per fragment; K_m = 16 nM. (2) Both nicks and blunt ends were used with equal affinity; i.e., there were 3.2 "sites" per fragment; K_m = 5 nM. Since the value of K_m calculated under the first assumption is about four-fold higher than that determined with singly nicked PM2 DNA, we believe that the second assumption is more attractive and that the result suggests, but does not prove, that the ATPase can utilize blunt-end termini.

The data in Table V further discriminate two additional classes of polynucleotide molecules with respect to their capacity to support the ATPase activity. Thus, polyribonucleotides and yeast sRNA were essentially inactive, demonstrating the substantial specificity of the enzyme for deoxypolymers, as was PM2 form IV DNA, indicating that the ATPase was incapable of interacting with intact duplex DNA. A second group of molecules, of diverse composition, demonstrated an intermediate capacity to satisfy the cofactor requirement, and for some of this group, the explanation for this behavior is most probably due to particular structural properties of the polymers themselves. Thus, poly(dG) is known to have considerable secondary structure in solution, while, conversely, poly(dA-dT) and supercoiled PM2 form I DNA molecules would be expected to possess a significant degree of single-stranded structure under these incubation conditions.

Of greater interest were the results obtained with singly nicked PM2 form II DNA and blunt-ended duplex DNA molecules generated by *Hae*III restriction endonuclease digestion. Both classes of molecules supported ATP hydrolysis with a V_{max} ~20% of that obtained with single-stranded polymers, and given the inability of the enzyme to utilize intact, fully duplex DNA (PM2 form IV), these results suggested that the ATPase might be capable of recognizing both internal

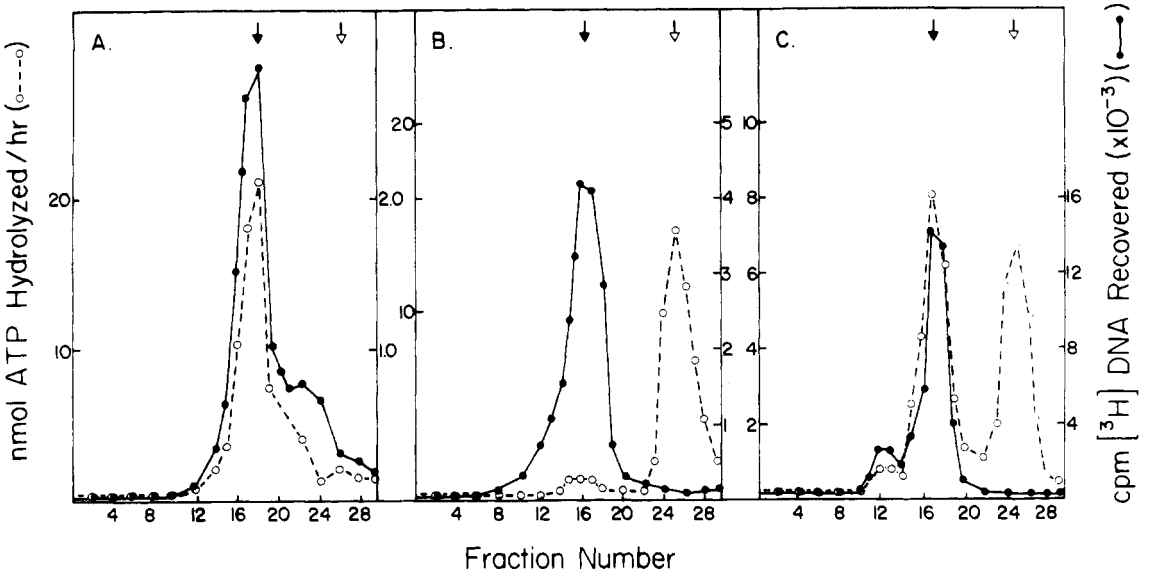

FIGURE 5: Glycerol gradient sedimentation analysis of the binding of the KB cell ATPase to DNA. (A) Cosedimentation with M13 [^{3}H]DNA. Linear 20–40% glycerol gradients were formulated as described under Materials and Methods and contained in addition 1 mM $MgCl_2$, 0.1 M NaCl, and ±1 mM ATP. The samples to be sedimented contained 100 units of fraction VIII ATPase, 8.6 μg of M13 [^{3}H]DNA, 1 mM $MgCl_2$, and ±1 mM ATP in a volume of 100 μL and were incubated for 2 min at 37 °C prior to loading on the gradient. Centrifugation was in the SW 60 Ti rotor at 4 °C and 45 000 rpm for 6 h. Parallel gradients were run identically with either DNA or ATPase alone. Fractions (130 μL) were collected from the bottom of the tubes, 5 μL was assayed for ATPase activity, and 100 μL was precipitated with 15% trichloroacetic acid on GF/C filters to locate the [^{3}H]DNA. Recovery of loaded enzyme activity in this and the subsequent panels varied from 60 to 80%. In all panels, the position of the DNA sedimented alone is indicated by the *closed arrow*; the position of the ATPase alone is indicated by the *open arrow*. (B) Cosedimentation of ATPase with PM2 form IV [^{3}H]DNA. Gradients were formulated as in (A) and loaded with 70 units of fraction VIII ATPase and 7.8 μg of DNA. Sedimentation was in the SW 60 Ti rotor at 4 °C for 12 h at 38 000 rpm. (C) Cosedimentation of ATPase with singly nicked PM2 form II [^{3}H]DNA. Gradients were formulated as in (A) and loaded with 70 units of ATPase and 7.8 μg of DNA. Sedimentation conditions were exactly as in (B). The more rapidly sedimenting minor peak of DNA is most likely the small fraction of intact form I DNA that was present in the form II DNA preparation. Note: each cosedimentation experiment was performed both in the presence and in the absence of 1 mM ATP without effect on the resulting sedimentation profiles.

(nicks) and flush-end (duplex) termini. Assuming that this interpretation was correct, the apparent K_m of the enzyme for these termini could be shown to be 4–5 nM. Moreover, with the nicked duplex DNA cofactors, the ATPase did not appear to discriminate between 3′-hydroxyl and 3′-phosphoryl termini. Thus, PM2 form II DNA molecules that contained an average of 10 nicks per molecule were prepared either with pancreatic DNase I (to generate 3′-hydroxyl termini) or with micrococcal nuclease (to generate 3′-phosphoryl termini), as described under Materials and Methods. The two preparations of nicked duplex DNA molecules were essentially indistinguishable in their ability to support the ATPase reaction.

Direct examination of the binding interaction between the ATPase and its polynucleotide cofactors by velocity gradient sedimentation (Figure 5) demonstrated the binding of the enzyme to closed circular single-stranded (M13) DNA (Figure 5A) but not to intact duplex (PM2 form IV) DNA (Figure 5B) and proved (Figure 5C) that the ATPase can bind to singly nicked circular duplex DNA molecules with sufficient affinity to be detectable by this technique.

(3) Evidence for Catalytic Interaction of the ATPase with Human DNA Polymerases α and β. In view of the catalytic properties of the KB cell ATPase that have been described above, and particularly considering both the complete absence of a variety of contaminating or associated activities from this enzyme and the ability of the ATPase to utilize nicked duplex DNA cofactors, we have carried out some initial experiments to try to evaluate the possibility that this enzyme might play a role in KB cell DNA replication. Our strategies have been closely modeled on those that have proved successful in prokaryotic studies. Efforts to demonstrate that the KB enzyme possesses either DNA unwinding activity or primase activity by the direct assays described under Materials and Methods have to date been unsuccessful. However, with respect to the latter function, it must be recalled that of the set of prokaryotic DNA-dependent ATPases of interest, a clear demonstration of primase activity has thus far been accomplished only with the phage T7 gene 4 protein (Scherzinger et al., 1977; Romano & Richardson, 1979a,b). Moreover, the negative result of the unwinding assay is also not conclusive because (1) only a single duplex polynucleotide substrate has been used, and a positive assay would have required the unwinding of both DNA–DNA and RNA–DNA chains, (2) due to the extremely small quantities of enzyme protein that are currently available, the assay was performed at a molar ratio of enzyme to DNA that is well below the levels required for the demonstration of unwinding activity by the well-characterized prokaryotic DNA helicases (Kuhn et al., 1979), and (3) of the several prokaryotic DNA-dependent ATPases that have been implicated in strand-displacement DNA synthesis, only one, the *E. coli rep* protein, has been shown by direct assay to catalyze DNA unwinding (Scott et al., 1977; Yarranton & Gefter, 1979).

We therefore performed some preliminary experiments (in collaboration with Dr. T. S.-F. Wang) to determine whether the KB cell ATPase could be shown to interact with human DNA polymerases α or β in a manner consistent with the facilitation of strand-displacement synthesis. The rationale for these experiments was based on our previous studies (Wang & Korn, 1980) in which we had demonstrated with our near homogeneous polymerase preparations that DNA polymerase α was essentially unreactive with nicked duplex PM2 DNA substrates, while DNA polymerase β performed a limited synthetic reaction with these substrates to the extent of incorporating an average of 15 nucleotides at each nick. The results of these experiments are presented in Figure 6. The primer–template used was multiply nicked KB cell DNA,

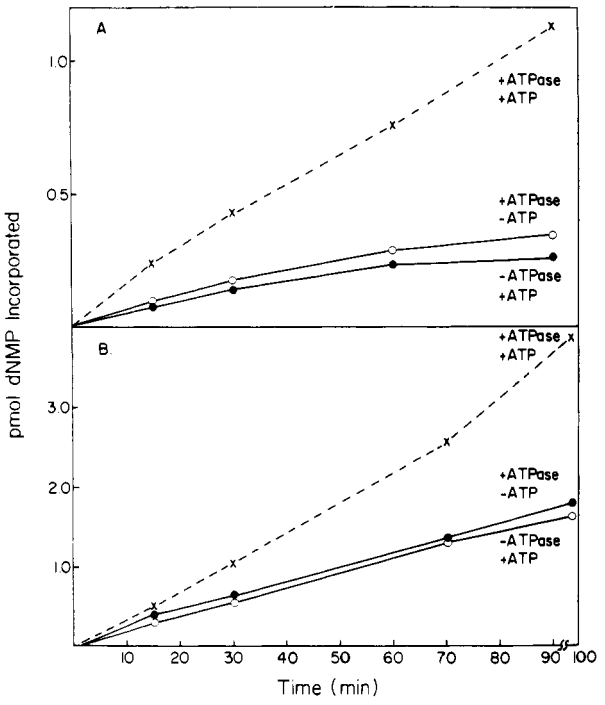

FIGURE 6: (A) Stimulation of incorporation by human DNA polymerase α on multiply nicked KB DNA in the presence of the KB cell ATPase. Reactions (100 μL) contained 40 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 200 μg/mL bovine serum albumin, 40 μM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (5720 cpm/pmol), 4 mM $MgCl_2$, 50 μM (nucleotide) multiply nicked (Wang & Korn, 1980) KB [^{3}H]DNA, 1.0 unit of fraction VIII (Fisher & Korn, 1977) KB DNA polymerase α, $\pm$2 mM ATP, and $\pm$10 units of fraction VIII ATPase. Incubation was at 35 °C, and aliquots (20 μL) were removed at the indicated times and assayed for [^{32}P]dTMP incorporation as in the standard polymerase assay (Fisher & Korn, 1977). (B) Stimulation of incorporation by human DNA polymerase β on multiply nicked KB DNA in the presence of the KB cell ATPase. Reactions (100 μL) contained 50 mM Tris-HCl, pH 8.9, 300 μg/mL bovine serum albumin, 25 μM each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (7100 cpm/pmol), 2 mM $MnCl_2$, 25 μM (nucleotide) multiply nicked KB [^{3}H]DNA, 0.13 unit of the isoelectric focused fraction (Wang et al., 1977) of human hepatic DNA polymerase β, $\pm$2 mM ATP, and $\pm$10 units of fraction VIII ATPase. Incubation was at 35 °C, and aliquots (20 μL) were removed at the indicated times and processed for [^{32}P]dTMP incorporation as in (A).

prepared with pancreatic DNase I and containing an average of approximately one strand interruption per 830 nucleotides (Wang & Korn, 1980). It is apparent that in the presence of the ATPase, there is a three- to fivefold stimulation of the rate of dNMP incorporation catalyzed by DNA polymerase α (Figure 6A) or β (Figure 6B), and this effect is totally dependent on the presence of ATP. [Note that "native" KB cell DNA is itself a very poor, but usable, substrate for these polymerases (Fisher et al., 1979).] Results qualitatively similar to those in Figure 6A have also been obtained with DNA polymerase α and multiply nicked PM2 DNA, and the polymerization product has been proven by agarose gel electrophoresis to be covalently linked to the appropriate nicked circular form II and linear form III PM2 DNA molecules.

Discussion

We have purified to near homogeneity the single species of DNA-dependent ATPase activity that we have identified in extracts of KB cell nuclei. The enzyme is comprised of a single polypeptide chain of 75 000 daltons, and this size is in excellent agreement with the minimal molecular weight of the ATPase protomer (82 000–94 000) estimated from velocity gradient sedimentation and gel filtration analyses at high ionic strength. Although we have no evidence for the presence of more than a single protein subunit in this enzyme, the fact that the sedimentation value of the ATPase activity increases at low ionic strength from 5.3 to 7.0 S raises the possibility that the enzyme may function as a homodimer under the conditions of the standard assay. The purified enzyme, which is devoid of contaminating or associated deoxyribonuclease, polymerase, or topoisomerase activities, demonstrates an absolute substrate specificity for ATP and dATP, but all of the other NTPs and dNTPs, as well as ADP and AMP (other NDPs and NMPs were not tested), are effective competitive inhibitors of the enzyme with respect to ATP.

The KB cell enzyme has an absolute requirement for a divalent cation ($Mg^{2+} > Mn^{2+} > Ca^{2+}$) and a polydeoxynucleotide cofactor. We have examined the latter requirement both kinetically and by direct DNA binding studies in some detail and have demonstrated that the ATPase is unable to interact with RNA, polyribonucleotides, or intact duplex DNA but that it does bind with moderate to high affinity to single-stranded DNA and deoxyhomopolymers and to duplex DNA molecules that present either internal nicks or (probably) blunt ends. The binding interaction of the ATPase with single-stranded polydeoxynucleotides is indifferent to the presence or structure of free-chain termini, whereas the ability of the enzyme to bind to duplex DNA appears to be absolutely dependent on the presence of termini. Although a detailed analysis of the binding reaction at nicks and blunt ends remains for the future, we may note that the equivalent cofactor capacity of multiply nicked PM2 DNA molecules prepared with either pancreatic DNase I or micrococcal nuclease suggests that the ATPase does not discriminate between 3′-hydroxyl and 3′-phosphoryl functions at nick sites. It is also of interest that the V_{max} of the ATPase reaction with nicked or blunt-ended duplex DNA is identical and is only ~20% of the V_{max} measured with single-stranded polymers. This observation may be a reflection of a catalytically limiting rate of duplex unwinding with the former class of cofactors (for which we have no direct evidence at this time), a hypothesis that would be entirely consistent with the alteration of specific catalytic properties by different DNA cofactor molecules that has been demonstrated for the *E. coli rep* protein (Kornberg et al., 1978) and suggested for the phage T4 gene 44/62 and 45 complex (Piperno & Alberts, 1978).

In contrast to the abundant body of information that is now available regarding a variety of prokaryotic DNA-dependent ATPases of diverse functions, there have been only a few reports to date describing some of the structural and enzymatic properties of DNA-dependent ATPases from eukaryotic sources. Hotta & Stern (1978) have isolated a DNA-dependent ATPase activity from meiotic cells of *Lilium* that had an estimated M_r of 130 000, was inactive with RNA or synthetic polydeoxynucleotides, and appeared to possess a modest capacity to unwind DNA–DNA, but not RNA–DNA, hybrid molecules that could be demonstrated at high protein/DNA ratios. To the best of our knowledge, this is the only report that has yet described a DNA unwinding activity from any eukaryotic organism. Hachmann & Lezius (1976) have described an ATPase activity from mouse myeloma that had a sedimentation coefficient of 5.5 S, an estimated M_r of ~100 000, and a p*I* of 6.5. The myeloma enzyme required a single-stranded polydeoxynucleotide cofactor and could hydrolyze both NTPs and dNTPs. Two nucleic acid dependent NTP phosphohydrolases (I and II) have been extensively purified from vaccinia virus cores (propagated on HeLa cells) by Paoletti et al. (1974) and Paoletti & Moss (1974). One

of these enzymes, phosphohydrolase I, was very similar to the KB cell ATPase in its absolute substrate specificity for ATP and dATP and in its structure, which was shown to be comprised of a single polypeptide that comigrated with bovine serum albumin on denaturing polyacrylamide gels. However, the vaccina enzyme differed in some of its catalytic properties from the KB cell ATPase in that it was not inhibited by ADP or AMP and it was unable to use single-stranded homodeoxypolymers as cofactors. It should be noted that Paoletti et al. had no evidence to indicate whether either or both of the phosphohydrolases were virus specific or of host cell origin. Cobianchi et al. (1978) have presented some initial data describing a DNA-dependent ATPase activity from a heteroploid human cell line (EUE). The activity had an estimated M_r of 110000, an apparent substrate specificity for ATP and dATP, and a requirement for single-stranded DNA. Cobianchi et al. also presented some very preliminary evidence suggesting that their ATPase activity could stimulate a partially purified fraction of HeLa cell DNA polymerase α on several model primer–templates. Chang et al. (1979) have reported in abstract their extensive purification of two immunologically related but chromatographically separable DNA-dependent ATPases from yeast that each has a M_r of ~67000 and can hydrolyze both NTPs and dNTPs. During the preparation of this manuscript, Assairi & Johnston (1979) described their isolation and partial characterization of a DNA-dependent ATPase activity from whole calf thymus homogenates. This activity, which exhibited a variable degree of chromatographic heterogeneity, had a sedimentation coefficient of 4.4 S at high or low ionic strength and showed an absolute substrate specificity for ATP and dATP. In contrast to our findings with the KB cell ATPase, the cofactor requirement of the calf thymus activity was satisfied by single-stranded DNA and poly(dT) but not by the other single-stranded synthetic deoxyhomopolymers; the calf thymus enzyme could utilize poly(dA-dT) as well as poly(dT), but it did not accept Ca^{2+} in place of Mg^{2+} or Mn^{2+}, and it showed a greater sensitivity to salt inhibition (100% inhibition at 200 mM Na^+ or K^+). Finally, the most purified fraction of the calf thymus ATPase contained two major polypeptides of M_r 55000 and 60000, as well as a minor species of M_r 28000, but it was not evident which, if any, of those protein bands corresponded to the catalytic activity.

Given the considerable diversity in specific enzymatic properties that has been recorded for the several prokaryotic DNA-dependent ATPases that have been shown to possess helicase activity or to be implicated in DNA replication, an attempt to compare the KB ATPase, on the basis of such properties, with any of the DNA-dependent ATPase activities that have been described thus far is premature. We may reasonably conclude, however, that in terms of the structural and catalytic properties described in this report, the KB enzyme can be readily distinguished from all of the eukaryotic DNA-dependent ATPases that have been reported to date.

The in vivo functions of the KB cell ATPase are presently unknown. However, we believe that our preliminary demonstration of an ATP-dependent stimulation by this ATPase of the synthetic reaction of human DNA polymerases α and β on nickel duplex DNA primer–templates is encouraging and supports the speculation that this enzyme may play a role in KB cell DNA replication.

Acknowledgments

We thank Adriana Johnson for expert technical assistance with cell cultures and Drs. P. A. Fisher and T. S.-F. Wang for their constructive suggestions and criticism. We are grateful to James Battey for providing us with a sample of plasmid pMS 4375 and guiding us in the preparation of the blunt-end DNA fragment.

References

Abdel-Monem, M., & Hoffmann-Berling, H. (1976) *Eur. J. Biochem. 65*, 431–440.

Abdel-Monem, M., Durwald, H., & Hoffmann-Berling, H. (1976) *Eur. J. Biochem. 65*, 441–449.

Abdel-Monem, M., Chanal, M.-C., & Hoffmann-Berling, H. (1977a) *Eur. J. Biochem. 79*, 33–38.

Abdel-Monem, M., Durwald, H., & Hoffmann-Berling, H. (1977b) *Eur. J. Biochem. 79*, 39–45.

Alberts, B. M., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.

Arai, K.-I., & Kornberg, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4308–4312.

Assairi, L. M., & Johnston, I. R. (1979) *Eur. J. Biochem. 99*, 71–79.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Chang, L. M. S., Plevani, P., & Badaracco, G. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 2907.

Cobianchi, F., Riva, S., Mastromei, G., Spadari, S., Pedrali-Noy, G., & Falaschi, A. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 639–647.

Eichler, D. C., Wang, T. S.-F., Clayton, D. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 7888–7893.

Espejo, R. T., Canelo, E. S., & Sinsheimer, R. T. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1164–1168.

Fisher, P. A. (1979) Ph.D. Thesis, Stanford University.

Fisher, P. A., & Korn, D. (1977) *J. Biol. Chem. 252*, 6528–6535.

Fisher, P. A., & Korn, D. (1979a) *J. Biol. Chem. 254*, 11033–11039.

Fisher, P. A., & Korn, D. (1979b) *J. Biol. Chem. 254*, 11040–11046.

Fisher, P. A., Wang, T. S.-F., & Korn, D. (1979) *J. Biol. Chem. 254*, 6128–6135.

Gellert, M., Mizuuchi, K., O'Dea, M. H., & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3872–3876.

Greenfield, L., Simpson, L., & Kaplan, D. (1975) *Biochim. Biophys. Acta 407*, 365–375.

Hachmann, H. J., & Lezius, A. G. (1976) *Eur. J. Biochem. 61*, 325–330.

Higgins, N. P., Peebles, C. L., Sugino, A., & Cozzarelli, N. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1773–1777.

Hotta, Y., & Stern, H. (1978) *Biochemistry 17*, 1872–1880.

Jovin, T. M., Englund, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.

Keller, W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4876–4880.

Kolodner, R., & Richardson, C. C. (1978) *J. Biol. Chem. 253*, 574–584.

Kolodner, R., Masamune, Y., LeClerc, J. E., & Richardson, C. C. (1978) *J. Biol. Chem. 253*, 566–573.

Korn, D., Fisher, P. A., Battey, J., & Wang, T. S.-F. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 613–624.

Kornberg, A., Scott, J. F., & Bertsch, L. L. (1978) *J. Biol. Chem. 253*, 3298–3304.

Krell, H., Durwald, H., & Hoffmann-Berling, H. (1979) *Eur. J. Biochem. 93*, 387–395.
Kuhn, B., Abdel-Monem, M., Krell, H., & Hoffmann-Berling, H. (1979) *J. Biol. Chem. 254*, 11 343–11 350.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Luduena, M. A., & Sussman, H. H. (1976) *J. Biol. Chem. 251*, 2620–2628.
Marco, R., Jazwinski, S. M., & Kornberg, A. (1974) *Virology 62*, 209–223.
Marians, K. J., Ikeda, J.-E., Schlagman, S., & Hurwitz, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1965–1968.
McDonnell, M. W., Simon, M. N., & Studier, R. W. (1977) *J. Mol. Biol. 110*, 119–146.
McMacken, R., Ueda, K., & Kornberg, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4190–4194.
Milstein, C. (1964) *Biochem. J. 92*, 410–421.
Morris, C. F., Moran, L. A., & Alberts, B. M. (1979) *J. Biol. Chem. 254*, 6797–6802.
Nossal, N. G. (1979) *J. Biol. Chem. 254*, 6026–6031.
Nossal, N. G., & Peterlin, B. M. (1979) *J. Biol. Chem. 254*, 6032–6037.
Paoletti, E., & Moss, B. (1974) *J. Biol. Chem. 249*, 3281–3286.
Paoletti, E., Rosemond-Hornbeak, R., & Moss, B. (1974) *J. Biol. Chem. 249*, 3273–3280.
Piperno, J. R., & Alberts, B. M. (1978) *J. Biol. Chem. 253*, 5174–5179.
Piperno, J. R., Kallen, R. G., & Alberts, B. M. (1978) *J. Biol. Chem. 253*, 5180–5185.
Purkey, R. M., & Ebisuzaki, K. (1977) *Eur. J. Biochem. 75*, 303–310.
Radloff, R., Bauer, W., & Vinograd, J. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1514–1521.
Reha-Krantz, L. J., & Hurwitz, J. (1978a) *J. Biol. Chem. 253*, 4043–4050.
Reha-Krantz, L. J., & Hurwitz, J. (1978b) *J. Biol. Chem. 253*, 4051–4057.
Richet, E., & Kohiyama, M. (1976) *J. Biol. Chem. 251*, 808–812.
Richet, E., & Kohiyama, M. (1978) *J. Biol. Chem. 253*, 7490–7495.
Romano, L. J., & Richardson, C. C. (1979a) *J. Biol. Chem. 254*, 10 476–10 482.
Romano, L. J., & Richardson, C. C. (1979b) *J. Biol. Chem. 254*, 10 483–10 489.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.
Scherzinger, E., Lanka, E., Morelli, G., Seiffert, D., & Yuki, A. (1977) *Eur. J. Biochem. 72*, 543–558.
Scott, J. F., & Kornberg, A. (1978) *J. Biol. Chem. 253*, 3292–3297.
Scott, J. F., Eisenberg, S., Bertsch, L. L., & Kornberg, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 193–197.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.
Studier, F. W. (1965) *J. Mol. Biol. 11*, 373–390.
Vosberg, H. P., & Vinograd, J. (1976) *Biochem. Biophys. Res. Commun. 68*, 456–464.
Wang, T. S.-F., & Korn, D. (1980) *Biochemistry 19*, 1782–1790.
Wang, T. S.-F., Sedwick, W. D., & Korn, D. (1974) *J. Biol. Chem. 249*, 841–850.
Wang, T. S.-F., Eichler, D. C., & Korn, D. (1977) *Biochemistry 16*, 4927–4934.
Weiss, B., Live, T. R., & Richardson, C. C. (1968) *J. Biol. Chem. 243*, 4530–4542.
Wickner, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3511–3515.
Wickner, S., & Hurwitz, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3342–3346.
Yarranton, G. T., & Gefter, M. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1658–1662.