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Purification and Characterization of a Type II DNA Topoisomerase from Bovine Calf Thymus*

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We report here the large scale purification of DNA topoisomerase II from calf thymus glands, using the unknotting of naturally knotted P4 phage DNA as an assay for enzymatic activity. Topoisomerase II was purified more than 1300-fold as compared to the whole cell homogenate, with 22% yield. Analysis of the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed two bands of apparent molecular masses of 125 and 140 kDa. Tryptic maps of the two bands indicated that they derive from the same protein. Using these fragments, specific polyclonal antisera to topoisomerase II were raised in rabbits. Immunoblotting of whole cell lysates from various species indicated that topoisomerase II is well conserved among mammals and has a native subunit molecular mass of 180 kDa. Analytical sedimentation and gel filtration were used to determine a sedimentation coefficient of 9.8 S and a Stokes radius of 68 Å. The calculated solution molecular mass of 277 kDa implies a dimer structure in solution. The purified topoisomerase II unknots P4 DNA in an ATP-dependent manner and is highly stimulated in its relaxation activity by ATP. A DNA-stimulated ATPase activity, as has been found with other type II topoisomerases, is associated with the purified enzyme. Approximate kinetic parameters for the ATPase reaction were determined to be: a V_{max} of 0.06 nmol of ATP/(μg of protein) (min) and K_m of 0.2 mM in the absence of DNA, and a V_{max} of 0.2 nmol of ATP/(μg of protein) (min) and K_m of 0.4 mM ATP in the presence of supercoiled plasmid DNA.

DNA topoisomerases are a class of enzymes unique from all others. Unlike most other enzymes which alter the chemical structure of molecules, DNA topoisomerases act by catalyzing the interconversion of three-dimensional conformations of DNA. Two classes of topoisomerases, types I and II, are known to exist in prokaryotes and eukaryotes (1-4).

Type II topoisomerases have been identified and isolated from a wide range of species. The first type II topoisomerase to be isolated was gyrase from *Escherichia coli* by Gellert *et al.* (5). Liu *et al.* (6) isolated a distinct type II topoisomerase from T4-infected cells. Although T4 topoisomerase lacks the supercoiling activity of gyrase, it retains the requirement for ATP for relaxation of supercoiled DNA. Type II topoisomerases isolated from eukaryotic sources have been shown to have enzymatic properties analogous to T4 topoisomerase. Miller *et al.* (7) reported the purification of topoisomerase II from a mammalian source, HeLa cell extracts. The purification of topoisomerase II from *Drosophila* embryos, yeast, and *Xenopus* oocytes has also been reported (8-11).

E. coli DNA topoisomerase II, DNA gyrase, has been shown to be central to the regulation of superhelical density and thereby controls many genetic processes (12). Recent studies have shown that topoisomerase II plays a crucial role in eukaryotic cell growth and division. The topoisomerase II gene (TOP2) of yeast has been cloned and found to be a single copy, essential gene (13). Studies of a ts mutant in the yeast TOP2 gene indicate that topoisomerase II is required for DNA replication (14). Evidence from recent work with *Xenopus* oocyte extracts indicates that a supercoiling activity may also be present *in vivo* in eukaryotes and have a role in the control of transcription (15, 16). Topoisomerase II has been shown to be a component of the high molecular mass complexes (replitase complexes) responsible for the replication of DNA in yeast and mammalian cells (17, 18). A role for topoisomerase II has been also demonstrated in the growth control of cells; epidermal growth factor (EGF[1]) has been shown to stimulate topoisomerase II activity in inducible cells (19). The increase in the level of topoisomerase II activity in rat liver during regeneration after partial hepatectomy further demonstrates the requirement for topoisomerase II in growing cells (20). Other studies have indicated that topoisomerase II may be a potential target for some antitumor compounds (21-23). Since it has been shown that topoisomerase II is such a critical component of eukaryotic cells, it is important to develop a purification of sufficiently large scale to allow for the generation of antibodies and further studies.

A new assay for type II topoisomerase activity has been recently devised (24). We have used tailless bacteriophage P4 to obtain naturally knotted DNA for use as a substrate in an assay for topoisomerase II double-stranded DNA passing activity. It has been suggested that the absence of a tail fiber permits both cohesive ends of the P4 DNA to be present in the phage head, allowing joining of the ends and random knot formation (24). The reaction is not subject to interference from type I topoisomerase activity and is monitored by gel electrophoresis of the reaction products. During the reaction, the heterogeneous population of randomly knotted DNA molecules, running as a fast moving smear during electrophoresis, is converted to unknotted, nicked circular molecules, running as a slower moving single band during electrophoresis. This assay has enabled us to purify milligram quantities of topoisomerase II from calf thymus glands. The amount of enzyme

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[1] The abbreviations used are: EGF, epidermal growth factor, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.

obtained has been sufficient for physical and enzymatic characterization as well as the production of a high titer antiserum against topoisomerase II.

EXPERIMENTAL PROCEDURES

Purification of Topoisomerase II

Preparation of Nuclei—All steps during the purification of topoisomerase II were carried out at 4 °C. Four calf thymus glands, approximately 1 kg wet weight of tissue, were collected on ice and used within 30 min after slaughter. The thymus glands were trimmed of all fat and membranous tissue and minced prior to homogenization. Homogenization was accomplished in a Waring blender, 2 pulses of 30 s each at the highest speed, in a total volume of 1 liter of H buffer. The crude lysate was then passed through 2, 4, and 8 successive layers of cheesecloth to remove unblended material and connective tissue (Fraction I). Nuclei were collected by centrifugation at 8700 × *g* and washed with 1 liter of H buffer.

Lysis of Nuclei and Polymin P Precipitation—Washed nuclei were resuspended and lysed in 1 liter of NW buffer (see "Buffers" for description of buffers) with 5 mM EGTA by blending, 2 pulses of 15 s each at the highest speed (Fraction II). After lysis was complete, as monitored by phase contrast microscopy, Polymin P (10% pH 7.8) was added slowly while stirring until a final Polymin P concentration of 0.35% was reached. The precipitate formed was collected by centrifugation at 8700 × *g* for 10 min. The pellet was resuspended by blending in PR buffer, and the volume was adjusted to 1.5 liters. The salt concentration of the mixture was raised by the slow addition of 5 M NaCl until a final concentration of 0.55 M was achieved and extraction of protein from the chromatin-Polymin P complex was allowed to continue for 30 min. Following the salt extraction, in order to reprecipitate nucleic acids, additional Polymin P (approximately twice the volume initially added) was added until small clumps of precipitate formed. After stirring for 15 min, the precipitate was then removed by centrifugation as before, and the supernatant was passed through coarse filter paper (Fraction III).

Ammonium Sulfate Precipitation—To the filtered extract, 0.4 g of ammonium sulfate/ml of extract was added slowly with constant stirring. After 30 min of additional stirring, the precipitate was collected by centrifugation at 17,700 × *g* for 20 min. The precipitate was resuspended in 250 ml of PR buffer and dialyzed for 8 h against four 2-liter changes of PR buffer. The salt concentration after dialysis corresponded to an ammonium sulfate concentration of 250 mM, by conductivity measurements (Fraction IV). A precipitate which formed during dialysis was removed from the sample by centrifugation at 48,400 × *g* for 30 min (Fraction V).

BioRex 70 Column Chromatography—The clarified dialysate from above was loaded onto a 4.5 × 27-cm BioRex 70 ion exchange column equilibrated with 100 mM potassium phosphate in BC buffer. The column was washed with 100 mM potassium phosphate in BC buffer until the A_{280} of the flow-through diminished to baseline. A 2-liter linear gradient of 100 to 700 mM potassium phosphate in BC buffer was used to develop the column. The peak of topoisomerase II activity eluted between 450 to 570 mM potassium phosphate. The pooled fractions were diluted 2.5-fold with BC buffer to lower the salt concentration for hydroxylapatite chromatography (Fraction VI).

Hydroxylapatite Chromatography—The diluted BioRex 70 pool was loaded on a 2.5 × 22-cm hydroxylapatite column equilibrated with 200 mM potassium phosphate in HC buffer. After the sample was loaded, the column was washed with 200 mM potassium phosphate in HC buffer until A_{280} of the eluate returned to baseline. The column was then developed with a 1.5-liter linear salt gradient of 200 to 700 mM potassium phosphate in HC buffer. The peak of topoisomerase II activity eluted between 380 and 460 mM potassium phosphate. EGTA was added to the pooled fractions to 0.5 mM final concentration, and the pool was diluted 2.5-fold with BA buffer, in preparation for blue agarose chromatography (Fraction VII).

Blue Agarose Chromatography—The diluted hydroxylapatite pool was loaded on a 1 × 10-cm blue agarose column equilibrated with 200 mM NaCl in BA buffer. The column was washed with 2 column volumes of 200 mM NaCl in BA buffer, and developed with a 100-ml linear salt gradient of 200 mM to 2.5 M NaCl in BA buffer. A small amount, approximately 5%, of proteolyzed topoisomerase II (125 kDa) eluted from 1.5 to 2.0 M NaCl. The column was further washed with 2 column volumes of 4 M NaCl in BA buffer to remove any contaminating proteins, and the homogeneous topoisomerase II was eluted with 2 column volumes of 2.5 M NaCl, 1% Triton-X 100 in BA buffer (Fraction VIII).

Concentration and Dialysis—The pooled enzyme fraction was concentrated by loading on a 1 × 5-cm hydroxylapatite column, equilibrated with 200 mM potassium phosphate in HC buffer by ascending flow, followed by step elution with 700 mM potassium phosphate in HC buffer by descending flow. The fractions with the highest adsorbance (A_{280}) were pooled to form the main pool (Fraction IX), and trailing fractions with significant adsorbance were also pooled to form the trailing pool. The activities and SDS-gel pattern of both pools were identical, except that the concentration of the trailing pool was one-fourth that of the main pool. Both pools were then dialyzed against storage buffer and stored at −20 °C. Topoisomerase II stored under these conditions has been stable for longer than two years.

Assays

P4 Unknotting Assay—Naturally knotted P4 phage DNA was used to assay for the strand passing activity of topoisomerase II. P4 knotted DNA (0.3 μg) was incubated in a total volume of 20 μl of 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml of bovine serum albumin, 1 mM ATP with 1 μl of the sample to be assayed for 30 min at 37 °C. The reaction was stopped by the addition of 5 μl of 5% SDS, 50 mM EDTA, 20% Ficoll, 0.05 mg/ml of bromphenol blue and the products of the reaction were analyzed on a 0.7% agarose gel in TBE buffer (89 mM Tris borate, pH 8.2, 2 mM EDTA) gel, electrophoresed at 5 V/cm for 4 h for assays of column fractions and 2 V/cm for 16 h for analytical assays. After electrophoresis, gels were stained with ethidium bromide and photographed with ultraviolet (300-nm) illumination. One unit of topoisomerase II activity is defined as the greatest dilution of enzyme that can unknot 0.3 μg of P4 knotted DNA to completion under standard reaction conditions. The usual method of quantitation of the assay is by serial dilution of the sample prior to assay followed by visual interpolation of the results.

DNA Relaxation Assay—DNA relaxation activity was assayed in a manner identical to that for unknotting except supercoiled pKE1 plasmid (pBR322 with an approximately 500-base pair *Micrococcus luteus* DNA insert) was used as the substrate DNA, and ATP was omitted from the reaction. One unit of relaxation activity is defined as the greatest dilution of enzyme that can relax 0.3 μg of supercoiled plasmid DNA to completion under standard reaction conditions.

Charcoal ATPase Assay—To assay the ATPase activity associated with topoisomerase II activity, a charcoal adsorption assay was used. Diluted enzyme (200 ng/assay) was incubated in 20 μl of the assay buffer from above with various dilutions of unlabeled ATP and a constant amount of [γ-^{32}P]ATP for 5 min at 37 °C. The reaction was stopped on ice by the addition of 0.5 ml of 0.1 M HCl and 0.2 ml of 5 mg/ml BSA, 0.025 M $Na_4P_2O_7$, 0.025 M potassium phosphate, pH 7.0. To each reaction, 0.2 ml of a 1:4 acid washed charcoal slurry was added and allowed to mix for several minutes. The reaction was then centrifuged for 5 min at 3000 × *g* and 0.8 ml of the supernatant was transferred to a fresh tube. To this tube, an additional 0.8 ml of charcoal suspension was added, mixed, and centrifuged. A 0.8-ml aliquot of the resulting supernatant was then counted in 2 ml of Liquiscent (National Diagnostics).

Immunoblotting—Transfer of protein from SDS gels to nitrocellulose membrane was accomplished in a Bio-Rad Transfor cell at 10 V/cm for 8 h at 4 °C in Tris-glycine (25 mM Tris, 192 mM glycine, pH 8.2) buffer containing 0.2% SDS and 20% methanol. After transfer, blots were soaked in 1% gelatin in PTX (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, 1 mM EGTA, 1 mM NaN_3) for 1 h and then incubated with 1:500 dilution of topoisomerase II-specific antiserum in PTX overnight at room temperature. Following incubation, blots were washed 4 times in PTX and once in urea wash (2 M urea, 1% Triton X-100, 100 mM glycine) and 2 additional times in PTX. Blots were then incubated with ^{125}I-labeled Protein A, 5 × 10^5 cpm/ml in PTX, for 1 h and washed as before. The blots were dried and visualized by autoradiography.

Protein Assays—Samples were assayed for protein using either the dye binding assay developed by Bradford (25) or by fluorescamine fluorescence assay (26).

Generation of Antisera—Antisera to topoisomerase II was generated in female New Zealand White rabbits. Animals were injected with 100 μg of purified topoisomerase II, either as undenatured protein or as a SDS-PAGE band, mixed with Freund's complete adjuvant and then boosted at three-month intervals with antigen in Freund's incomplete adjuvant. Serum was collected at 7 and 14 days

after boosting and used as serum or an IgG fraction purified by ammonium sulfate precipitation and DEAE-cellulose column chromatography (27).

Tryptic Maps—Topoisomerase II was labeled with ^{125}I using the chloramine-T method of Greenwood *et al.* (28). After removal of free label by gel filtration, protein was precipitated with 12.5% trichloroacetic acid, 0.5 mg/ml deoxycholate. Trichloroacetic acid pellet was resuspended in SDS-PAGE loading buffer, neutralized with 2 M Tris base, and electrophoresed on a 5% SDS-polyacrylamide gel. After staining with Coomassie Blue, the stained 125- and 140-kDa bands were cut out and tryptic mapping was performed as described by Gomer and Lazaridies (29).

SDS-PAGE—SDS-polyacrylamide gel electrophoresis was preformed as described by Laemmli (30).

Buffers—The composition of the buffers used is as follows. H buffer is 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. NW buffer is 5 mM potassium phosphate, pH 7.5, 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. PR buffer is 20 mM potassium phosphate, pH 7.0, 10% glycerol, 0.5 mM EGTA, 10 mM $NaHSO_3$, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. BC buffer is the indicated concentration of potassium phosphate, pH 7.0, in 10% glycerol, 0.5 mM EGTA, 10 mM $NaHSO_3$, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. HC buffer is the indicated concentration of potassium phosphate, pH 7.0, in 10% glycerol, 10 mM $NaHSO_3$, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. BA buffer is the indicated concentration of NaCl in 20 mM potassium phosphate, pH 7.0, 10% glycerol, 0.5 mM EGTA, 10 mM $NaHSO_3$, 10 mM 2-mercaptoethanol, 0.5 mM PMSF. Storage buffer is 30 mM potassium phosphate, pH 7.0, 50% glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA.

Materials—Knotted P4 phage DNA was prepared as described by Liu *et al.* (24). Plasmid DNA was prepared as described by Liu and Miller (39). Calf thymus glands were obtained fresh from a local slaughter house. BioRex 70, hydroxylapatite (Bio-Gel HT), Coomassie Brilliant Blue (G-250 and R-250), and gelatin were from Bio-Rad. Tris (Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) grade), blue agarose resin, 2-mercaptoethanol, Triton X-100, fluorescamine, bovine serum albumin, and SDS-PAGE gel marker proteins were from Sigma. Polymin P, dithiothreitol, and ultrapure glycerol were from Bethesda Research Laboratories. Ultrapure ammonium sulfate and sucrose were from Schwarz/Mann. Protein A, Ficoll, Sephacryl S-300, and sucrose sedimentation marker proteins were from Pharmacia. $Na^{125}I$ and [γ-^{32}P]ATP were from Amersham. Freund's adjuvant was from GIBCO. Rabbits were from Bunnyville Farms, PA. DEAE-cellulose (DE52) was from Whatman. Nitrocellulose membrane (BA85) was from Schleicher & Schuell. Dialysis tubing (12,000 M_r cutoff) was from Spectrum Medical Industries. Cellulose thin layer plates were from EM Reagents. PMSF and acrylamide were from Eastman Kodak. All other chemicals were reagent grade or higher.

RESULTS

Early Fractionation Steps—The protocol devised for the early fractionation steps in the purification of topoisomerase II is shown in Fig. 1. Calf thymus glands were chosen as the starting material for the purification. It was found that unlike other tissues tested, there was a high level of topoisomerase II activity present in thymus extracts without any pretreatment of the animal (20). The thymus tissue is also uniform in composition and does not contain significant amounts of connective tissue. This allowed for the easy isolation of a nuclear fraction by homogenization in an iso-osmolar sucrose-containing buffer (H buffer) and low speed centrifugation. The pellet obtained was highly enriched for nuclei as judged by phase contrast microscopy. The nuclei were then washed with H buffer to remove cytosolic proteins trapped in the pellet, and the washed nuclei were resuspended into a low salt, EGTA-containing lysis buffer (NW buffer). After several minutes of incubation at 4 °C, complete lysis of nuclear membranes occurred as monitored by phase contrast microscopy. A final concentration of 0.35% Polymin P was used to precipitate the nucleic acids and associated proteins. The Polymin P-chromatin precipitate was collected by low speed centrifugation and extracted with a final concentration of 0.55 M NaCl. This concentration of NaCl may be insufficient to release all of the topoisomerase II from the pellet, but it was found that higher salt concentrations began to release histones and other chromosomal proteins from the DNA. The release of these proteins causes the hydration of the DNA to increase and made the preparation difficult to pellet. The salt-extracted proteins were separated from the bulk of the nucleic acid by the further addition of Polymin P and recentrifugation. The proteins in the salt-extracted supernatant were then precipitated with ammonium sulfate. It was found that 0.4 g/ml of ammonium sulfate (62% saturation) completely precipitated the topoisomerase II activity, but did not precipitate many low molecular mass nuclear proteins and the proteolyzed forms of topoisomerase I. The precipitate was collected by centrifugation, dialyzed against low salt buffer (NW buffer), and recentrifuged to remove a precipitate formed during dialysis. The assay for topoisomerase II activity was interfered with by high salt and residual Polymin P (data not shown), so the values reported in Table I for the BioRex 70 load (Fraction III) are artificially low.

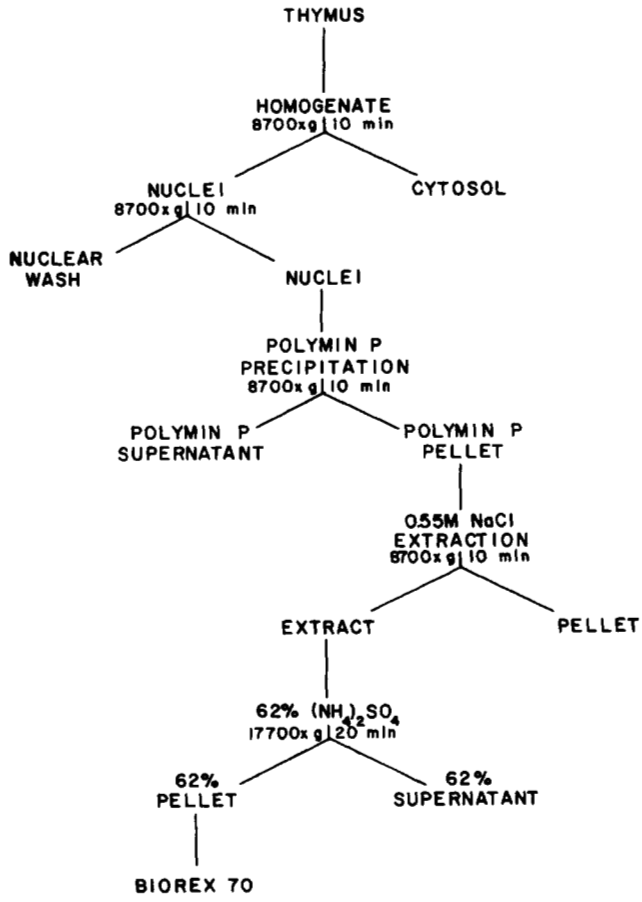

FIG. 1. **Early purification steps.** Relative centrifugation forces and times were as indicated.

Column Chromatography—For the first column chromatographic step in the purification of topoisomerase II, BioRex-70 was chosen because of its high protein binding capacity and its stability during changes in ionic strength. It was discovered that topoisomerase II bound tightly to the weak anionic exchange resin and eluted only at high salt concentrations (Fig. 2). This step gave a significant purification, removing nearly all the topoisomerase I activity. Hydroxylapatite, because of its low protein binding capacity, was used

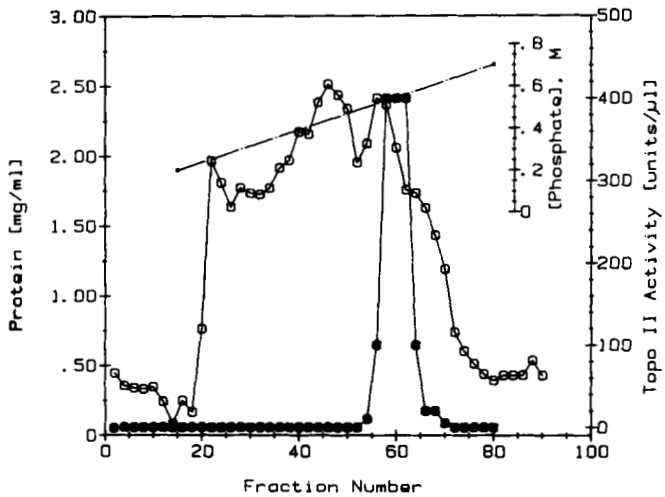

FIG. 2. **BioRex-70 column chromatography.** Protein concentration as determined by the Bradford dye assay is shown in *open circles*; topoisomerase (*topo*) II activity, determined by serial dilution and assay with P4 knotted DNA, is shown in *closed circles*; and potassium phosphate gradient is shown by the *broken line*.

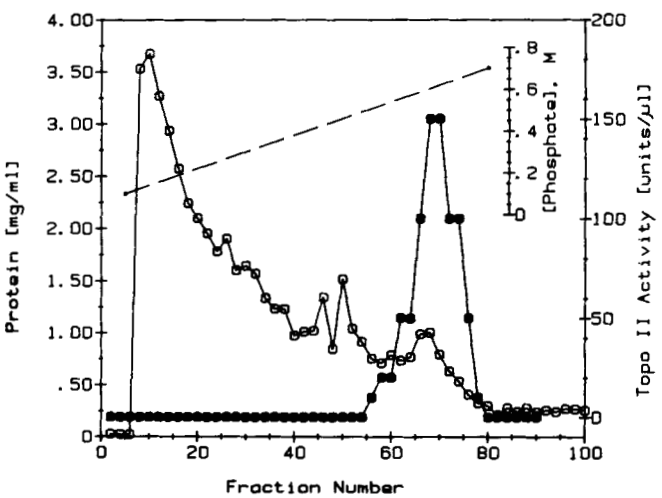

FIG. 3. **Hydroxylapatite column chromatography.** Protein concentration as determined by the Bradford dye assay is shown in *open circles*; topoisomerase (*topo*) II activity, determined by serial dilution and assay with P4 knotted DNA, is shown in *closed circles*; and potassium phosphate gradient is shown by the *broken line*.

as the second chromatography step. It acted to resolve topoisomerase II from proteins complexed with it (Fig. 3). Topoisomerase II binds tightly to hydroxylapatite and elutes at a salt concentration much higher than most other proteins. This step resulted in a 7-fold increase in purity, yielding nearly homogeneous topoisomerase II (Table I). Previous experiments with hydrophobic chromatography had shown that topoisomerase II is highly hydrophobic after hydroxylapatite chromatography (data not shown). To take full advantage of these observations, blue agarose chromatography was employed. It is probable that topoisomerase II binds to the blue dye molecule by a combination of hydrophobic and hydrophilic interactions. The enzyme binds in low salt, indicating some possible hydrophilic interactions, but does not elute in high salt alone, indicating some hydrophobic interactions. The optimal elution conditions required both high concentrations of salt (2.5 M NaCl) and detergent (1% Triton X-100). This purification step removed proteins weakly associated with topoisomerase II.

Final Enzyme Pool—The pooled topoisomerase II activity obtained after blue agarose chromatography was further concentrated and the detergent was removed by adsorption to a 1-ml hydroxylapatite column, followed by elution in the opposite direction with a 0.7 M potassium phosphate step. The eluted material was pooled and dialyzed into storage buffer. Topoisomerase II has been found to be stable at −20 °C in storage buffer for over two years.

Physical Characterization—To determine the reduced molecular mass, and to assess the purity of the final topoisomerase II pool, SDS-PAGE was performed (Fig. 4). The doublet of bands at the 125–140-kDa region of the gel has been characteristic of all calf thymus topoisomerase II preparations. Because higher molecular mass (170–180 kDa) topoisomerase II had been found in HeLa cells (7) and *Drosophila* embryos (8, 9), it was suspected that these two bands represented proteolytic products of a single higher molecular mass species. Three lines of evidence support this conclusion. 1) Phosphate transfer experiments, similar to those described in Rowe *et al.* (31), demonstrated that both bands could accept phosphate label from DNA (data not shown). 2) Immunoblots of whole calf thymus homogenate (Fig. 5*a*, *lane A*) and tissue culture cells from various mammalian sources (Fig. 5 *b*) with topoisomerase II-specific antisera all show a single high molecular mass species (180 kDa). 3) Tryptic maps of both bands show no significant differences (Fig. 8). Purification in the presence of protease inhibitors such as PMSF, trasylol, or sodium bisulfite has not yielded a higher molecular mass form of the enzyme. The unreduced solution molecular mass of the purified topoisomerase II was determined by employing sucrose gradient sedimentation (Fig. 6) to determine the sedimentation coefficient, and Sephacryl S-300 gel permeation chromatography (Fig. 7) to determine the Stokes radius. The sedimentation coefficient determined was 9.8 S and the Stokes radius 68 Å. A solution molecular mass of 277 kDa and a frictional ratio (f/f_o) of 1.5 was calculated by the method of Siegel and Monty (32) from these values, assuming a partial specific volume for the protein of 0.725 cm^3/g. This molecular mass implies that the molecule is a globular dimer in solution.

Enzymatic Characterization—To determine the enzymatic properties of the purified enzyme, several reactions were examined. The unknotting of knotted P4 phage DNA is shown in *panels A* and *B* of Fig. 9. As the reaction procedes, the faster migrating smear of multiply knotted DNA is converted to the slower moving heavy band of unknotted circular molecules. A comparison of *panel A*, with 1 mM ATP in the reaction mixture, and *panel B*, without ATP in the reaction mixture, shows that the unknotting reaction is highly ATP-dependent. *Panels C* and *D* show the relaxation of supercoiled plasmid DNA. A similar comparison between *panel C*, with 1 mM ATP, and *panel D*, without ATP, shows that there is some relaxation reaction in the absence of ATP, but it is highly stimulated by the presence of ATP.

The stimulation of the ATPase activity of the purified topoisomerase II by DNA was examined. Results from preliminary kinetic studies indicated that the presence of 115 µg/ml of supercoiled plasmid DNA causes a stimulation of the ATP hydrolysis activity of topoisomerase II. Double reciprical plots of data points ranging from 0.01 to 0.05 mM ATP (Fig. 10) are linear. An approximate K_m of 0.2 mM ATP and a V_{max} of 0.06 nmol of ATP/(µg of protein) (min) were determined without DNA present, and an approximate K_m of 0.2 mM ATP and a V_{max} of 0.4 nmol of ATP/(µg of protein) (min) were determined with DNA present. Reaction velocity measurements made at ATP concentrations between 0.1 and 1.0

TABLE I

Purification table

Enzyme activities were determined by the P4 unknotting assay, and protein concentrations were determined by fluorescamine fluorescence assay.

Fraction	Step	Volume	Total units[a]	Total protein[b]	Purification	Yield
		ml			*-fold*	%
I	Crude homogenate	1560	126	18,000	1	100
II	Lysed nuclei	1410	114	15,000	1	90
III	Polymin P extract	1100	89	ND[c]		
IV	Ammonium sulfate precipitation	520	41	2,100	2	33
V	BioRex 70 Load	500	42	2,000	2	33
VI	BioRex 70 Pool	500	120	190	110	96
VII	Hydroxylapatite pool	300	72	15	710	58
VIII	Blue agarose pool	27	54	5	1,100	43
IX	Main pool	1.4	27.2	3	1,300	22

[a] Units of enzyme activity ($\times 10^{-6}$) as determined by P4 unknotting assay.
[b] Milligrams of protein as determined by fluorescamine assay.
[c] ND, not determined.

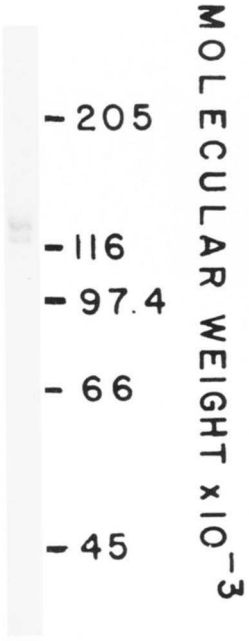

FIG. 4. **SDS-gel electrophoresis of final enzyme pool.** 4 μg of the final enzyme pool after concentration and dialysis was electrophoresed on a 7.5% SDS-polyacrylamide gel at 4-mA constant current for 18 h. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Molecular mass markers are shown in kilodaltons.

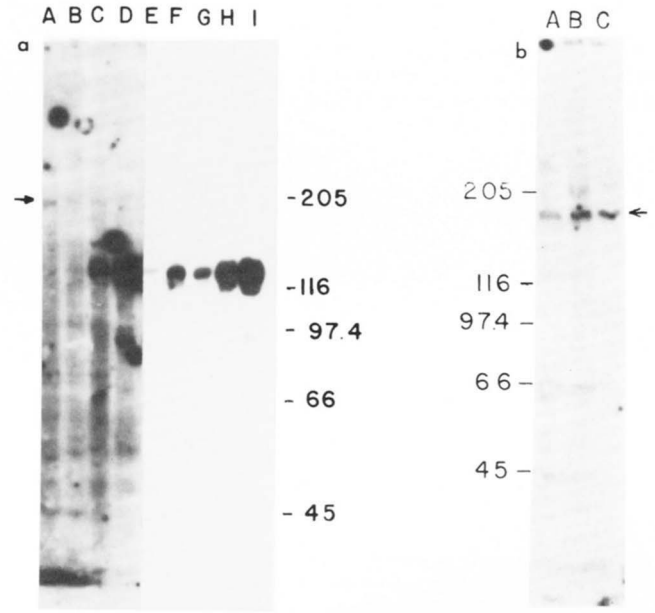

FIG. 5. **Immunoblots of intermediate and final enzyme pools and three cell lines.** *Panel A*, samples from intermediate pools corresponding to 35 μg (*lanes A–F*), 10 μg (*lane G*), or 2 μg (*lanes H* and *I*) of protein were electrophoresed as described in Fig. 4. Transfer to nitrocellulose membrane and immunoblotting were as described under "Experimental Procedures." The samples in *lanes A–F* correspond to fractions I through IX, respectively. The autoradiograph of *lanes A–D* was exposed for a 20-fold longer period than for *lanes E–I*. *Panel b*, 5×10^4 cells/lane were lysed in 2 × SDS-PAGE sample buffer and electrophoresed, transferred to nitrocellulose membrane, and immunoblotted as described above. The samples were as follows: *lane A*, HeLa (human); *lane B*, BSC-1 (monkey); and *lane C*, Chinese hamster ovary. The molecular mass markers are shown in kilodaltons and the high molecular mass form of topoisomerase II is marked with an *arrow*.

mM indicated a signfiicant activation of the ATPase activity at high concentrations of ATP (data not shown).

DISCUSSION

The purification of type II topoisomerase has been previously undertaken from a wide variety of biological sources, *i.e.* bacteria, *E. coli* and *M. luteus*; bacteriophage, T4; yeast, saccharomyces; insects, *Drosophila*; amphibians, *Xenopus*; and mammals, HeLa cells. Such a broad spectrum of expression argues for the importance of topoisomerase II in normal cell physiology. Genetic and biochemical studies in both bacteria and yeast have revealed that topoisomerase II is an essential protein for cell growth.

The purification of topoisomerase II reported here is significantly different from the previously reported procedures in that it is the first large scale purification of topoisomerase II from a mammalian source. The previous purification of topoisomerase II from HeLa cells, reported by Miller *et al.* (7), did not yield sufficient amounts of either protein or enzymatic activity for extensive analytical study. The method presented here for the purification of topoisomerase II is also unique in that it utilizes the unknotting of naturally knotted P4 phage DNA as an assay for topoisomerase II enzymatic activity. This assay is a significant improvement over previous assays which monitored ATP-dependent relaxation of supercoiled plasmid DNA, catenation of plasmid DNA, or decatenation of kinetoplast DNA networks from trypanosomes. Unlike those assays, the P4 unknotting assay has been shown to

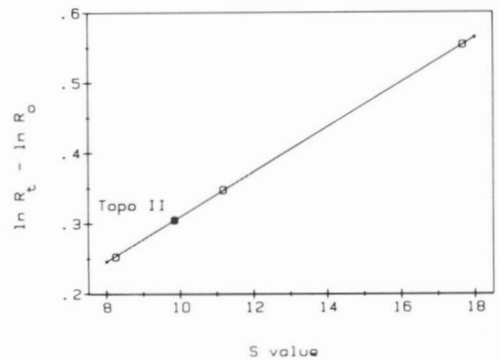

FIG. 6. **Analytical sucrose sedimentation.** Purified topoisomerase (*topo*) II was diluted and centrifuged on a 5–20% sucrose gradient in a Beckman SW50.1 rotor for 12 h at 37,500 rpm (132,000 × *g*) at 4 °C. Marker proteins were centrifuged on an identical gradient in the same centrifuge run. Gradients were fractionated and the position of topoisomerase II was determined by P4 assay. The positions of marker proteins were determined by protein assay followed by SDS-PAGE to confirm the identities of the peaks. The marker proteins and their $S_{w,20}$ values were ferritin (17.7 S), catalase (11.2 S), and aldolase (8.3 S). The position of topoisomerase II was as marked.

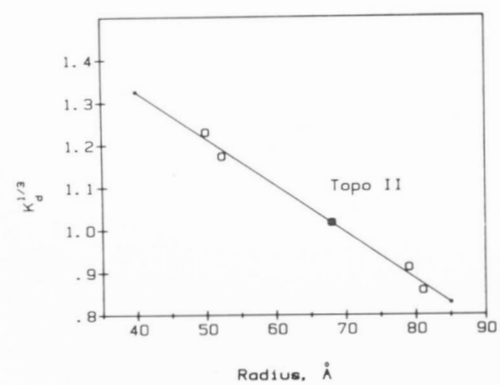

FIG. 7. **Analytical gel filtration chromatography.** Purified topoisomerase (*topo*) II was mixed with marker proteins and chromatographed on a Sephacryl S-300 column (50 × 1.5 cm) in 150 mM NaCl in BA buffer. The void volume of the column was taken to be the elution volume of dextran blue 2000 ($M_r \sim 2{,}000{,}000$). The elution positions of the marker proteins were determined by protein assay followed by SDS-PAGE to confirm identities of the peaks, and the elution position of topoisomerase II was determined by P4 assay. The marker proteins and their Stokes radii were thyroglobulin (81 Å), ferritin (79 Å), catalase (52.3 Å), and aldolase (50 Å). The position of topoisomerase II was as marked.

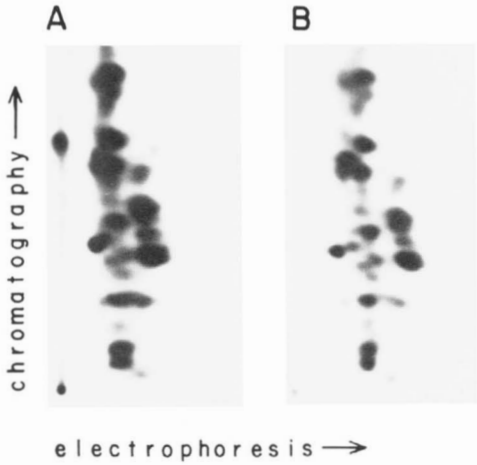

FIG. 8. **Two-dimensional tryptic maps.** Two-dimensional tryptic maps of the two bands in the final pool of topoisomerase II were performed as described under "Experimental Procedures." *Panel A* shows the map of the upper band and *panel B* shows the map of the lower band.

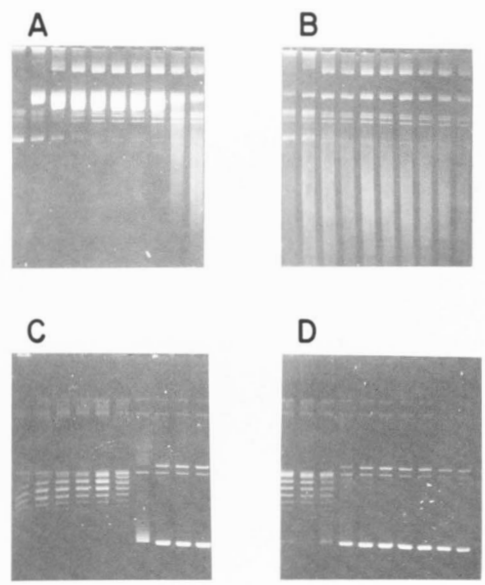

FIG. 9. **Assay for unknotting and relaxation activity.** The amount of unknotting and relaxation activity in the purified topoisomerase II was determined using serial 1:3 dilutions of enzyme (Fraction IX, 2 mg/ml) with either 0.3 μg of knotted P4 DNA or 0.3 μg of supercoiled plasmid (pKE1) DNA as a substrate in the presence or absence of ATP. *Panel A,* knotted P4 DNA, 1 mM ATP. *Panel B,* knotted P4 DNA, no ATP. *Panel C,* supercoiled plasmid DNA, 1 mM ATP. *Panel D,* supercoiled plasmid DNA, no ATP. The *leftmost lane* in *each panel* represents 1 μl of undiluted enzyme/assay.

be free from interference by other enzymatic activities, such as topoisomerase I, and DNA binding proteins (24). This allows the quantitative analysis of topoisomerase II activity at all stages of the purification, even whole cell lysates, by the P4 unknotting reaction.

Several problems were encountered during the course of a number of purification attempts, some of which gave some insights into the physical nature of topoisomerase II. Proteolysis was a recurrent problem in early attempts. Topoisomerase II purified from various eukaryotic species has been shown to consist of a high molecular mass species and a number of proteolytic products. The topoisomerase II that we have purified consists almost entirely of intermediate molecular mass forms (125 to 140 kDa). The "native" molecular mass of the enzyme in the crude lysate as determined by immunoblotting of unfractionated homogenate is approximately 180 kDa. The complete conversion from the 180 kDa form to the intermediate molecular mass species occurred between the isolation of the nuclei and the salt extraction of topoisomerase II from precipitated chromatin. It is possible that the low salt conditions during nuclear lysis and the precipitation of chromatin induced complete proteolysis of the high molecular mass form of topoisomerase II to a stable intermediate molecular mass form, or that the high molecular mass form is insoluble under the conditions used, and only the proteolyzed form is extractable from the Polymin P pellet with high salt. The second hypothesis is consistent with the observation that topoisomerase II has been localized in the nuclear matrix[2] and the metaphase chromosome scaffold.[3]

[2] B. D. Halligan, D. Small, B. Vogelstein, T.-S. Hsieh, and L. F. Liu, unpublished results.

[3] Earnshaw, W. C., Halligan, B. D., Cooke, C. A., and Liu, L. F., submitted for publication to *J. Cell. Biol.*

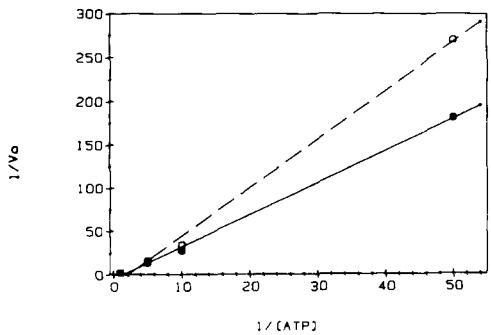

FIG. 10. **Double reciprocal plot of ATPase kinetics.** Kinetic parameters of the ATPase activity of purified topoisomerase II were determined in the presence (*solid squares*) or absence (*open squares*) of 115 μg/ml of supercoiled plasmid DNA.

Both of these structures are characterized by insolubility. The doublet of bands on SDS-PAGE has been observed in all calf thymus topoisomerase II purifications (Fig. 4). Tryptic peptide mapping of the two bands (Fig. 8) give nearly identical results, implying that both are derived from the same higher molecular mass form of topoisomerase II. The solution molecular mass calculated for the purified topoisomerase II, 277 kDa, indicates that the enzyme exists in a dimeric form in solution.

Another difficulty encountered in many purification attempts was the complete loss of activity, possibly due to oxidation of the enzyme. It was found that if care was not taken to prevent oxidation of the 2-mercaptoethanol added to the buffers, the strand passing activity of the enzyme was rapidly lost (data not shown). The loss of strand passing activity was accompanied by an increase in DNA cleavage activity. The cleavage of double- and single-stranded DNA by the purified enzyme has been characterized both in the presence and absence of anti-tumor compounds (21–23). It was found that the double-stranded DNA cleavage reaction was stoichiometric, producing two DNA fragments with topoisomerase II molecules linked to their 5′ ends. The DNA cleavage sites were found to be nonrandom, with a 4-base pair stagger between the cut sites on the opposite strands. It is possible that the cleavage activity detected in the absence of drugs is due to a subpopulation of the enzyme damaged by oxidation or proteolysis.

The hydrophobic nature of topoisomerase II also impeded purification. A good indication of the hydrophobicity of topoisomerase II is its tight binding to hydrophobic chromatography resins, such as phenyl-Sepharose. It was found that topoisomerase II remained tightly bond to moderately hydrophobic matrices even in the presence of high levels of detergent or ethylene glycol (data not shown). The hydrophobic nature of the enzyme also presented a problem in that the enzyme was not soluble at high concentrations. The high degree of hydrophobicity exhibited by calf thymus topoisomerase II may have biological relevance. The subunits of T4 topoisomerase II have been identified as membrane-bound proteins (33, 34), and the mammalian enzyme may also be membrane-associated.

A high molecular mass complex containing topoisomerase II was observed in early purification attempts, and in later attempts before the hydroxylapatite chromatography step. This complex was found to elute in the void volume when chromatographed on Sephacryl S-300, which implied a molecular mass of greater than one million daltons. Sucrose density gradient centrifugation of the complex determined the S value of the complex to be approximately 30 S. Both DNA ligase and α-DNA polymerase activities were also identified in this high molecular mass complex. The relationship of this complex to the replitase complex identified by Pardee (18) is not known.

The purification of homogeneous topoisomerase II allowed for the characterization of its enzymatic properties. As shown in Fig. 9, the unknotting of knotted P4 phage DNA occurs in a highly ATP-dependent manner. Relaxation of supercoiled plasmid DNA was also assayed and is shown in Fig. 9, *panels C* and *D*. There is a significant level of ATP-independent relaxation activity, but it is not clear if this activity is due to contaminating topoisomerase I or a stoichiometric relaxation reaction by a subpopulation of topoisomerase II molecules. The final dilution which shows ATP-independent relaxation (*panel D, lane 3*) contains approximately 10 topoisomerase II dimers/plasmid DNA molecule.

As expected from studies of other type II topoisomerases, a DNA-stimulated ATPase was detected in the final purified fraction. The V_{max} and K_m determined at low ATP concentrations for the ATPase reaction of calf thymus topoisomerase II are comparable to those determined for gyrase (35, 36). The degree of DNA stimulation of the ATPase reaction is less than that observed for *Drosophila* topoisomerase II (37). The difference in the degree of DNA stimulation may be due either to the higher concentrations of DNA used to stimulate the *Drosophila* enzyme or a less tightly coupled reaction by the calf thymus enzyme. The lack of strict coupling of ATP hydrolysis to DNA strand passing may reflect either damage to the enzyme, by proteolysis, oxidation, or other causes, or a difference in the reaction mechanism. We have also observed an ATP-dependent stimulation of the DNA cleavage reaction (data not shown). A full understanding of the kinetic behavior of topoisomerase II and the coupling of ATP hydrolysis to the energetically favorable relaxation or unknotting reactions await further study.

The relatively large scale of this purification has yielded enough homogeneous enzyme to allow for the generation of specific high titer antisera to topoisomerase II in rabbits. Both the purified enzyme and isolated bands from SDS-PAGE have been used to immunize animals successfully. The antisera produced recognize a single band on immunoblots of whole cells from a wide range of mammalian species (Fig. 5*b*) and are capable of neutralizing topoisomerase II enzymatic activity *in vitro* (data not shown). We have used this antibody to demonstrate the presence of topoisomerase II in both nuclear matrix fractions[2] and metaphase chromosome scaffolds.[3] This localization implies that topoisomerase II may play a role in the regulation of the topological state of chromosomal DNA loops.

The most puzzling aspect of all the purified eukaryotic topoisomerase II preparations is the lack of a DNA supercoiling activity. Although the eukaryotic enzyme is thought to have a reaction mechanism similar to that of DNA gyrase, it has never been shown to introduce either negative or positive supercoils. Unlike DNA gyrase, which has been shown to wrap DNA around its surface and protect approximately 140 base pairs (38), neither wrapping nor protection of DNA has been observed with the calf thymus enzyme (data not shown). It is not clear if this lack of an apparently essential enzyme activity is due to damage to the enzyme during purification, loss of a subunit required for supercoiling, or a strict DNA site specificity for the supercoiling reaction. Recent studies in *Xenopus* oocytes (15, 16) and SV40 minichromosomes (40) have indicated that topoisomerase II may possess supercoiling functions *in vivo*. Since assays for supercoiling activity in crude extracts are interfered with by high levels of relaxation

activity due to the presence of topoisomerase I, further studies are required to establish if eukaryotic topoisomerase II indeed has a supercoiling function.

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