Z-DNA Binding and Inhibition by GTP of *Drosophila* Topoisomerase II

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ABSTRACT: A Z-DNA binding protein has been isolated and characterized by biochemical means from *Drosophila melanogaster* tissue culture cells and embryos. This protein shares the following properties with the known, cloned *Drosophila* topoisomerase II: (1) expression of an ATP-dependent relaxation activity on supercoiled DNA; (2) a monomer mass of 165 kDa in SDS denaturing gels; (3) a sedimentation coefficient, $S_{20,w}$, of ~10 S for the active enzyme; (4) cross-reactivity for the respective monoclonal and polyclonal antibodies; (5) generation of covalent enzyme–DNA intermediates at preferred cutting sites in the *Drosophila* HSP70 intergenic spacer region; (6) inhibition of DNA relaxation activity by antitumor drugs, e.g., the etoposide VM26, and by monospecific antibodies raised against the protein; and (7) in vitro phosphorylation by a casein kinase activity. However, we have identified new properties for our topoisomerase II preparation not previously reported for the conventionally isolated enzyme: (1) The enzyme binds to Z-DNA with an affinity 2 orders of magnitude greater than that for B-DNA. (2) The binding to Z-DNA is increased 5–10-fold by GTP or GTP-γ-S. (3) GTP and GTP-γ-S inhibit the catalytic activity of topoisomerase II through a proposed allosteric mechanism. (4) Z-DNA inhibits the relaxation of closed circular supercoiled DNA. (5) The preparation consists of a single polypeptide chain of 165 kDa on denaturing SDS gels with no evidence of proteolytic degradation. We postulate that the Z-DNA binding activity of undegraded topoisomerase II may be important in targeting the enzyme both to structural motifs required for chromatin organization and to sites of local supercoiling. Some of these features arise during processes such as replication and gene expression and may be more frequent during embryogenesis and early development.

The polymorphic nature of DNA secondary structure is now widely accepted. The growing array of identifiable structures includes helical isomers (A, B, C, and Z); topographical variants such as bent or curved DNA (Diekmann & v. Kitzing, 1988); three- and four-stranded structures (Voloshin et al., 1988); bubbles, junctions, and hairpins (Lilley et al., 1987); and parallel-stranded helical conformations (Jovin, 1991). Although the majority of the DNA in chromatin presumably exists as a right-handed B helix, alternative DNA conformations are postulated to be essential in DNA replication, transcription, and recombination. The first DNA crystal structure (Wang et al., 1981) established left-handed Z-DNA as the preferred conformation for alternating purine(G)–pyrimidine(C) tracts at high salt concentrations (Pohl & Jovin, 1972). The central question, then and now, concerns the biological significance of the Z conformation. Extensive physicochemical data have accumulated, establishing the ability of various DNA sequences to form stable left-handed conformations under physiological salt conditions, particulary in negatively supercoiled circular molecules (Jovin et al., 1987; Rich et al., 1984). Direct evidence for the structure in vivo, however, has been hard to obtain because (a) probably less than 1% of natural DNA is capable of adopting the Z conformation (Arndt-Jovin et al., 1985; Trifonov et al., 1985; Wittig et al., 1989) and (b) the transition from B- to Z-DNA is in thermodynamic equilibrium, influenced strongly by divalent cations, polycations, proteins, and the superhelix density of the DNA (Jovin et al., 1987).

Soon after the crystal structure was solved, antibodies to Z-DNA were discovered in the serum of patients suffering from the autoimmune disease lupus erythematosus (Lafer et al., 1983). Additionally, constitutive Z-polynucleotides were found to be good immunogens, resulting in a battery of monoclonal and polyclonal antibodies, some having sequence or base specificity in addition to their global requirement for the Z conformation (Jovin et al., 1983; Stollar, 1986). We and others have probed cytological preparations from numerous sources with these antibodies and have demonstrated *potential* Z-DNA sequences in all species investigated (Arndt-Jovin et al., 1983; Jovin et al., 1983; Lafer et al., 1981; Lancillotti et al., 1985; Malfoy & Leng, 1981; Soyer-Gobillard et al., 1990; Stollar, 1986). Antibodies are selective ligands capable of shifting the B–Z equilibrium in favor of Z-DNA (Jovin et al., 1983; Lafer et al., 1986; Wittig et al., 1989; Zarling et al., 1984) and thus provide potentially ambiguous evidence for the in vivo presence of Z-DNA. In addition, the antibodies access chromatin poorly in the absence of prior fixation, a process involving either removal of proteins or local reversible denaturation, or both (Hill, 1991; Hill & Stollar, 1983; Robert-Nicoud et al., 1984; Wittig et al., 1989); one exception is given by a primitive dinoflagellate genome lacking histones (Soyer-Gobillard et al., 1990). Strong, but indirect, evidence for the in vivo existence of Z-DNA has been provided by specific plasmid constructs that demonstrate changes in transcriptional activity and the inhibition of methylation sites when linked to Z-DNA sequences (Jaworski et al., 1987, 1988; McLean & Wells, 1988; Rahmouni & Wells, 1989). In metabolically active, permeabilized mammalian cells the measured levels of Z-DNA change upon inhibition of transcription, replication, and topoisomerase I (Wittig et al., 1991,

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1992). However, in the absence of cellular proteins that can be demonstrated to generate Z-DNA or interact specifically with Z-DNA, the biological role of this alternative conformation remains obscure (Hill, 1991).

Numerous groups have attempted the isolation of Z-DNA binding proteins from a variety of species and tissues: wheat germ (Lafer et al., 1985), testes (Gut et al., 1987), bacteria (Kitayama et al., 1985; Lafer et al., 1988), mammalian cells (Azorin & Rich, 1985; Fishel et al., 1988; Leith et al., 1988; Moore & Fishel, 1990), yeast (Zhang et al., 1992) and *Drosophila* (Nordheim et al., 1982). All of these proteins have been isolated by selective binding to columns of constitutive Z-DNA prepared from brominated d(G-C) polynucleotides. None of them have been completely purified or shown to have any biological activity dependent upon the Z-DNA or B-DNA binding. Indeed, the validity of the isolation procedure was called into question in two reports. In one case, yolk proteins were isolated from nematodes, chicks, and frogs on brominated d(G-C) columns (Krishna et al., 1988), although they are not associated in the embryo with DNA. More recently, presumed Z-DNA binding proteins isolated from testes (Gut et al., 1987) were found to prefer B-DNA when tested with nonbrominated Z-DNA substrates (Rohner et al., 1990). The Rec 1 protein from *Ustilago* (Kmiec & Holloman, 1986) may have a legitimate Z-DNA interaction associated with functional activity, and several of the other Z-DNA binding proteins mentioned above are still under investigation.

We have undertaken to establish the role of Z-DNA during cellular processes involving rapid replication cycles and abrupt changes in gene expression such as embryogenesis. We reported previously that DNA becomes less accessible to Z-DNA antibodies at the mid-blastoderm transition stage in the embryonic development of both insects and amphibians, compared to times prior to or following this period (Jovin & Arndt-Jovin, 1988). Salt extraction suggested that more proteins were bound to the potential Z-DNA tracts during this developmental stage, a time when massive changes in zygotic gene activity and a switch in the control of DNA synthesis occur. We set about to isolate by purely biochemical means Z-DNA binding proteins from *Drosophila* embryos and from tissue culture cell nuclei. Our criterion for such proteins was a substantial (>100-fold) differential affinity for Z-DNA over B-DNA in a blotting assay. In this paper we show the successful isolation and purification of a Z-DNA binding protein to homogeneity. We describe its DNA binding characteristics determined by filter binding and gel retardation assays. The protein is a very active topoisomerase II and is identical to intact, full-length *Drosophila* topoisomerase II. We have recently reported that the enzyme also preferentially binds supercoiled minicircles containing a Z-DNA insert (Glikin et al., 1991). Finally, GTP is demonstrated to be both an inhibitor of enzymatic activity and an effector for DNA binding, increasing the binding affinity for Z-DNA.

EXPERIMENTAL PROCEDURES

Synthesis of [dG-m^{5}C] Polynucleotides. Alternating dG-dC/dm^{5}C polynucleotides with specific ratios of dm^{5}C to dC were synthesized from a poly[d(I-C)] template with a non-recombinant DNA polymerase from *Micrococcus luteus* (2.7.7.7) (Pharmacia) at 1 unit/100-μL reaction volume. The template concentration was 10 μM. Deoxynucleoside triphosphates (Pharmacia) were used at 0.3 mM, and the synthesis was allowed to proceed until at least 30% of the substrates were incorporated into polynucleotide. The ratio of dm^{5}C to dC incorporation and total synthesis were monitored by incorporation of [^{3}H]dCMP and [^{32}P]dGMP. The absorbance ratios at the descriptive wavelengths for the B- and the Z-polynucleotide were B-[^{32}P]poly[d(G-m^{5}C/C)], A_{280}/A_{260} = 0.53 and A_{295}/A_{260} = 0.18; and Z-[^{32}P]poly[d(G-m^{5}C/C)], A_{280}/A_{260} = 0.68 and A_{295}/A_{260} = 0.35. The corresponding values for the fully methylated polynucleotide are given in Job et al. (1988). The conformational states were also confirmed by CD spectroscopy.

The probes were synthesized at a ^{32}P specific activity of 1 μCi/nmol for Z-DNA blotting and at 0.1 μCi/nmol for B-DNA blotting and filter binding. For blotting with the Z-probes, 1 nmol of ^{32}P-labeled polynucleotide was diluted in 10 mL of binding buffer at 50 °C for 10 min and incubated at 37 °C with the DPT–cellulose-bound protein fractions in the presence of 150–200 nmol of nonradioactive calf thymus DNA. For blotting B probes, 5–10 nmol of ^{32}P-labeled polynucleotide was diluted without carrier in the binding buffer at 37 °C and applied to a duplicate set of fractions bound to DPT–cellulose.

Other Polynucleotides. Poly[d(G-br^{5}C)] was synthesized using poly[d(I-C)] as template and dGTP and dbr^{5}CTP (Pharmacia) as substrates, according to the procedure described above. All other polynucleotides were purchased from Pharmacia.

End-Labeling of DNA. DNA fragments were purified by electroblotting according to Sander and Hsieh (1983). DNA fragments were radioactively labeled at the 3′ end by the Klenow fragment of *E. coli* DNA polymerase I for 1 h at 14 °C (Maniatis et al., 1982).

Preparation and Activation of APT/DPT–Cellulose. APT–cellulose was prepared as described by Maniatis et al. (1982) from Whatman 3MM paper and was stored in the APT form at −20 °C in sealed bags containing N_2. It did not deteriorate in storage for more than a year. Activation was performed by incubation of the paper in cold 1.2 N HCl containing 0.3 mg/mL $NaNO_2$ for 30 min followed by four 5-min water washes and two 10-min washes with 50 mM potassium phosphate (pH 6.8). The paper was drained of excess buffer and spotted on a wet filter paper with aliquots of the column elution fractions. After 15 min to allow the protein to bind at room temperature, the paper was blocked with 5% milk powder or 1% glycine and 1% BSA in 75 mM NaCl and 20 mM Tris-HCl, pH 7.5. The blocked paper was washed in binding buffer and incubated with the radioactive probes in 20 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.02% BSA, 0.02% PVP and 0.02% Ficoll for the B-polynucleotide probes and in the same buffer containing 10 mM $MgCl_2$ and a 200-fold excess of nonlabeled calf thymus DNA for the Z-polynucleotide probes for a minimum of 4 h before being washed with 75 mM NaCl and 20 mM Tris-HCl, pH 7.5, for 1 h, dried, and developed for radioautography.

Tissue Culture. Schneider's *Drosophila* S3 cell line derived from embryos (the kind gift of A. Dübendorfer) was grown in spinner flasks to a density below 1.8 × 10^6 cells/mL at 25 °C in tissue culture humidified incubators with 4% CO_2 in M3 medium (Shields & Sang, 1977) prepared from analytical grade chemicals and Millipore Super-Q purified water containing 10% heat-inactivated fetal calf serum.

Preparation of S3 Nuclear Extract. All procedures after harvesting the cells were carried out at 4 °C. The 1 × 10^{10} tissue culture cells were harvested by centrifugation at 2500*g* and washed in cold phosphate-buffered saline. The cell pellet (usually 6–10 g) was resuspended in 20 mL of 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM spermidine, 0.3 mM spermine, 1 mM DTT, 0.2 mM PMSF, and 0.25 M sucrose. An equal volume of buffer containing 0.4% nonionic

detergent NP40 was mixed with the cell suspension, and the nuclei were released from the swollen cells by gentle Dounce homogenization using a tight-fitting pestle. The suspended nuclei were centrifuged at 4000*g*, resuspended in the 0.25 M sucrose buffer containing 1 mM EDTA and 0.1 mM EGTA, layered over 2 volumes of a buffer with the same ionic constitution containing 1 M sucrose, and centrifuged at 10 000 rpm for 20 min in a swinging-bucket rotor (JS 13, Beckman or SW 41, Beckman). The nuclear pellet was washed once with 0.25 M sucrose buffer, pelleted at 10 000 rpm, resuspended in 4 mL of the same buffer, and extracted by addition of 0.1 volume of cold saturated $(NH_4)_2SO_4$ at 4 °C for 40–60 min. The nuclear extract was separated from the DNA and nuclear debris by centrifugation at 100 000 rpm for 10 min (TL-100 rotor, Beckman) and could be stored frozen at –80 °C prior to protein purification.

Purification of Z-DNA-Binding Topoisomerase II. The $(NH_4)_2SO_4$ nuclear extract was dialyzed against 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.3 mM DTT, and 10% glycerol, cleared of precipitated proteins by centrifugation at 100 000 rpm (TL-100, Beckman) for 15 min, and processed through the following chromatography steps.

(*a*) *Heparin–Agarose.* A 1.5 × 10.5 cm column of heparin–agarose type I (Sigma) was equilibrated with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% glycerol. The dialyzed nuclear extract was loaded onto the column and eluted with an 80-mL linear gradient from 0.15 to 1.5 M NaCl. The Z-DNA binding and topoisomerase II activity eluted between 0.9 and 1.1 M salt, and the topoisomerase I activity eluted between 1.0 and 1.2 M salt.

(*b*) *MonoQ FPLC.* The active fractions from the heparin–agarose column were diluted to 150 mM NaCl concentration and applied to a MonoQ 5/5 column (Pharmacia) equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.1 mM DTT and eluted with a linear gradient of 150–450 mM NaCl. Topoisomerase I activity eluted at 220 mM, and the topoisomerase II and Z-DNA binding activities eluted at 350 mM NaCl.

(*c*) *Superose FPLC.* The Z-DNA binding topoisomerase II active fractions from the MonoQ column were concentrated to 0.5 mL either by binding to a small hydroxylapatite column (Bio-Rad) and eluting with a 0.4 M NaCl salt step or by centrifugation in Centricon-10 or -30 (Amicon) tubes. These fractions were chromatographed on a Superose-12 (Pharmacia) sizing column in 50 mM sodium phosphate, pH 6.8, 0.5 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT. The active fractions were made 50% in glycerol and stored at –20 °C.

Topoisomerase Relaxation Assays. Topoisomerase assays were as described (Hsieh & Brutlag, 1980) with NaCl concentration varied as indicated. The standard reaction mixture contained 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 50 μg/mL BSA, 1 mM ATP, and 0.15 μg of supercoiled plasmid or *Crithidia* catenated DNA. Reactions were performed routinely by addition of 1 μL of enzyme to 10 μL of reaction mixture and incubation for 30 min at 30 °C, unless otherwise indicated. One unit of activity is defined as the amount of enzyme necessary to completely relax or decatenate the DNA in 30 min under the conditions described above. Enzyme dilutions were made in 10 mM sodium phosphate, pH 7.1, 50 mM NaCl, 0.1 mM EDTA, 0.5 mg/mL BSA, 10% glycerol, and 0.2 mM DTT. The reactions were terminated by addition of 10% of the reaction volume of stop buffer: 1% SDS, 100 mM EDTA, 4% sucrose, 0.1% bromphenol blue, and 150 μg/mL proteinase K followed by digestion at 50 °C for 15 min.

Catenated DNA was prepared from *Crithidia lucidiae* by lysis in 3% sarkosyl with 1 mg/mL pronase followed by RNase treatment and isolation of the networks by centrifugation onto a CsCl step gradient (ρ = 1.370 and 1.404) at 20 000 rpm for 10 min.

Agarose Gel Electrophoresis. Relaxation assays were analyzed on 0.7% agarose gels in Tris–borate–EDTA (89 nM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.3) buffer. Gels were stained with ethidium bromide or blotted with ^{32}P-labeled plasmid fragments.

SDS–PAGE and Immunoblotting. Gradient (5–20%) or nongradient (7%) polyacrylamide gel electrophoresis was performed as described by O'Farrell et al. (1977). For immunoblots the separated protein bands were electrophoretically transferred from the gel to polyvinylidene difluoride membranes (Immobilon, Millipore) according to the manufacturer's instructions, blocked with a 5% solution of dried milk powder in saline buffer, washed with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), and incubated with the appropriate antibodies at a concentration of ~1 μg/mL. The immunoreactive bands were visualized after reaction with peroxidase-coupled second antibody and diaminobenzidene substrate.

Filter Binding Assays. Polynucleotides were either synthesized as described above or purchased from Pharmacia and radioactively labeled by nick translation. They were sonicated to approximately the same average chain length. Cellulose nitrate 0.45-μm pore 25-mm circular filters (Millipore, type HAWP) were washed twice with boiling water and equilibrated in wash buffer. The Z conformation of [^{32}P]-poly[d(G-m^{5}C/C] was induced by incubating the DNA at 50 °C for 10 min in binding buffer containing 7 mM $MgCl_2$ and by maintaining the DNA at 37 °C during the binding assay. Unless otherwise noted, each assay point consisted of 15 ng of polynucleotide in 2 mL of binding buffer (75 mM NaCl, 25 mM Hepes, pH 7.5, 50% glycerol, 7 mM $MgCl_2$, and 50 μg/mL BSA) incubated with the desired protein concentration for 10 min at 37 °C. The Z conformation of [^{32}P]poly[d(G-m^{5}C)] was induced in the same buffer containing 1.5 mM $MgCl_2$. [^{32}P]Poly[d(G-C)] in buffer containing 1.5 mM $MgCl_2$ or [^{32}P]poly[d(G-m^{5}C/C] in buffer containing 1.5 mM $MgCl_2$ or 1 mM EDTA was used as the B-DNA ligand. Binding buffers for these polynucleotides were as above with appropriate $MgCl_2$ concentrations. The filters were washed once with 0.5 mL of wash buffer, and the protein–DNA complex was trapped by filtering the mixture at a constant rate of 3.4 mL/min followed by 0.5 mL of wash buffer containing no glycerol. Competitors and effectors were added to the binding buffer as indicated in the Results section and the figure captions. A monoclonal antibody, 23B6, specific for Z-DNA (Glikin et al., 1991) was always used as a control to calculate the amount of Z-DNA present in the polynucleotide mixture under the particular salt conditions.

Topoisomerase II Cleavage of the HSP70 Heat Shock DNA. One-half microgram of DNA from *Eco*RI-cut plasmid 122 DNA (Goldschmidt-Clermont, 1980) was incubated in 10 mM Tris, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 1.25 mM ATP, and 50 μg/mL BSA for 5 min at 30 °C with purified Z-DNA binding topoisomerase II. The reaction was terminated by SDS (1% final concentration) followed by the addition of EDTA and proteinase K to 20 mM and 50 μg/mL, respectively. After a 1-h incubation at 50 °C, the DNA fragments were separated by electrophoresis in 0.7% agarose gel, transferred to a nitrocellulose membrane, and hybridized with the nick-translated 122 DNA. In control experiments the topoisomerase II cleavage reaction was

quenched with EDTA (20 mM final concentration) before addition of the enzyme followed by the addition of SDS and proteinase K. The effect of VM26 on the topoisomerase II induced DNA cleavage was studied by supplementing the cleavage buffer described above with 1.5, 5, 10, and 20 μg/mL VM26. A 5 mg/mL VM26 stock solution was prepared in acetone. VM26 was the kind gift of the Bristol-Myers Corporation.

Dephosphorylation of Calf Thymus and Drosophila Topoisomerase II with Calf Intestinal Phosphatase. Agarose-bound calf intestinal phosphatase (Sigma) was washed several times with topoisomerase reaction buffer and finally resuspended at 1.5 units/20 μL of reaction buffer. Either *Drosophila* Z-DNA binding topoisomerase II (ca. 50 ng) or calf thymus topoisomerase II (70 ng) was added to the agarose-bound CIP and incubated with occasional shaking at 30 °C for 30 min. Aliquots of Sephadex G25 (Pharmacia) were washed and incubated with the two different topoisomerase II preparations under identical conditions as described above and served as controls. The enzyme solutions were recovered by centrifugation of the matrices, and aliquots were tested for relaxation by addition of supercoiled plasmid and ATP. The CIP–agarose pellets from the dephosphorylaton reactions were tested for activity by 4-nitrophenyl phosphate hydrolysis.

RESULTS

Synthesis of a Polynucleotide Capable of Existing in Either a Z-DNA or a B-DNA Conformation. As a probe for the DNA binding capacity of the eluted protein fractions, we synthesized a polynucleotide that could exist in either the Z or the B conformation as a function of the magnesium ion concentration. Probing with the alternate forms of a given polynucleotide ensures that the helical conformation of the backbone and not the sites of chemical modification are recognized, thereby diminishing the problems associated with the heterogeneous, constitutive Z-DNA, chemically brominated poly[d(G-C)]. We achieved this goal by synthesizing copolymers of dG and different ratios of dC and dm^5C. Varying the composition allowed us to establish a transition Mg^{2+} concentration between the value for poly[d(G-C)] of 0.7 M (Pohl & Jovin, 1972) and that for poly[d(G-m^5C)] of ≤1 mM (Behe & Felsenfeld, 1981). A polynucleotide with 75% of the dC groups substituted by dm^5C is designated throughout this paper as poly[d(G-m^5C/C)] and was found to maintain a stable Z conformation at 37 °C in the presence of 7 mM $MgCl_2$ as determined by CD and absorption spectroscopy, whereas below 2 mM $MgCl_2$ the B conformation was adopted. The absorbance ratios A_{280}/A_{260} and A_{295}/A_{260} for the two forms of the polynucleotide are convenient measures of the cation-induced transition. The same DNA labeled with ^{32}P could be used to determine both B- and Z-DNA binding activities of each protein fraction.

Isolation of a Z-DNA Binding Protein by FPLC Chromatography. Nuclei were isolated either from *Drosophila* embryos or from Schneider's S3 *Drosophila* tissue culture cells grown in spinner culture. DNA binding proteins were extracted from the chromatin at salt concentrations below that which elutes core histones and were separated by column chromatography. The identification of Z-DNA binding proteins and the optimization of the purification protocol were determined by assaying the chromatographic fractions for DNA binding. An aliquot of each protein fraction was attached to activated APT paper (2-aminophenyl thioether–cellulose) that was subsequently blocked and then blotted with [^{32}P]poly[d(G-m^5C/C)] in the Z conformation, in the presence of excess B-DNA (150–200 fold excess of calf thymus DNA),

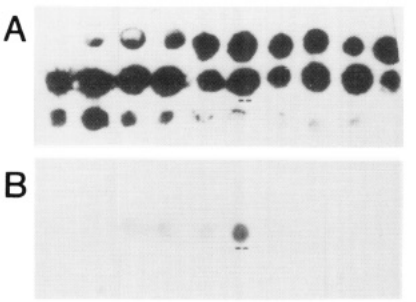

FIGURE 1: Differential DNA binding affinity of MonoQ column fractions. Ten microliters of column eluant fractions was bound to APT–cellulose and blotted with [^{32}P]poly[d(G-m^5C/C)] in the B-conformation (A) or the Z-conformation (B) (in buffer containing 10 mM $MgCl_2$). Successive fractions are displayed from left to right and from top to bottom. A dotted line appears under the Z-DNA binding protein fraction in both blots. For reaction details, see text.

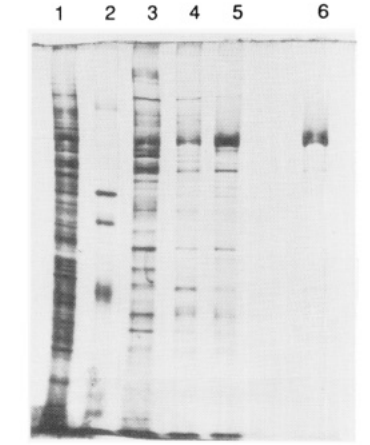

FIGURE 2: SDS gel electrophoresis of Z-DNA binding topoisomerase II fractions; 7% polyacrylamide–SDS denaturing and reducing gel of Z-DNA binding topoisomerase fractions, silver stained. Lane 1, nuclear extract fraction after 150 mM salt dialysis. Lane 2, MW standards: 29, 45, 66, 97.4, 116, and 205 kDa. Lane 3: MonoQ fraction containing topoisomerase II activity, weak Z-DNA binding, 165- and 155-kDa immunoreactive bands (see lane 1, Figure 4). Lanes 4 and 5: most active topoisomerase II fractions from MonoQ chromatography, peak of Z-DNA binding activity, single immunoreactive band at 165 kDa (see lanes 2 and 3, Figure 4). Lane 6: topoisomerase and Z-DNA binding fraction from Superose-12 chromatography, immunoreactive 165-kDa band (see lanes 4 and 5, Figure 4).

or in the B conformation as is seen for fractions of a MonoQ (Pharmacia) column elution in Figure 1. The B-DNA binding reflects primarily the protein concentration in the fractions as shown by protein staining or absorbance.

The purification protocol is described in Experimental Procedures. SDS–PAGE was used to monitor the protein species in the elution profiles and to determine homogeneity of the preparation. The final fraction was judged to have a purity of >95% and a monomer mass of 165 kDa (Figure 2). Glycerol gradient centrifugation of the purified protein demonstrated that the active protein is probably a dimer with an $S_{20,w}$ of ~10 S. The enzyme was stable for greater than 6 months when stored in 50% glycerol at –20 °C.

The Z-DNA Binding Protein is Intact, Active Topoisomerase II. Z-DNA binding blots (described above) and topoisomerase relaxation assays using supercoiled plasmid DNA or a decatenation assay with kinetoplast DNA from *Crithidia lucidae* were performed on the fractions eluted from hepain–agarose (Sigma), MonoQ, and Superose-12 FPLC columns (Pharmacia) and a 15–40% glycerol gradient. The ATP-dependent topoisomerase II activity copurified with the Z-DNA binding activity in all cases (Figure 3). In addition, the final protein had a topoisomerase specific activity of at

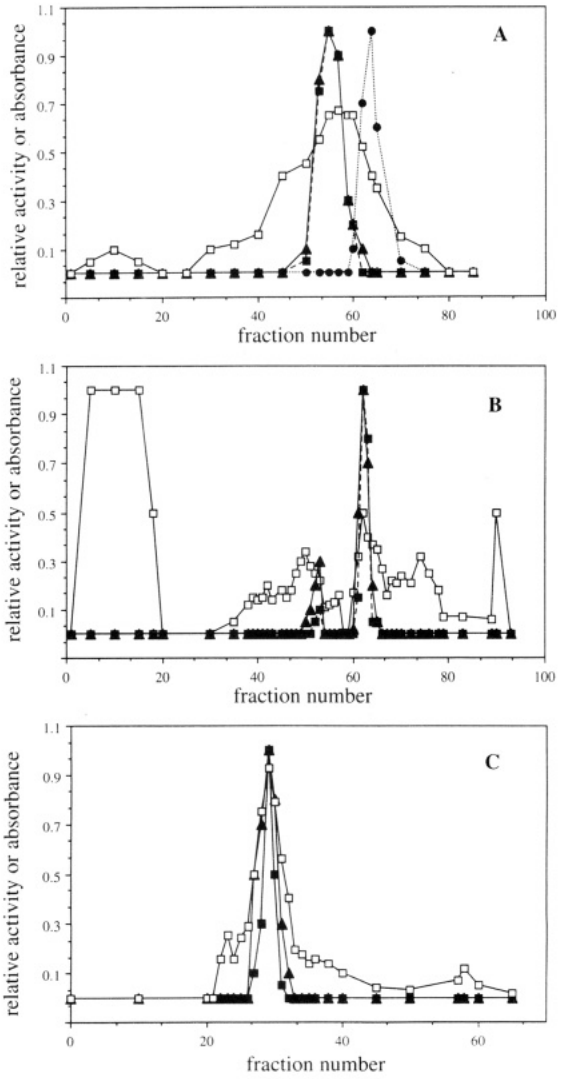

FIGURE 3: Elution profiles from column chromatography of Z-DNA binding activity. The relative absorbance at 280 nm (□) is plotted against the fraction numbers which were assayed for enhanced Z-DNA binding (■), topoisomerase II (▲), and topoisomerase I (●) activities. (A) Heparin–agarose elution. (B) MonoQ FPLC elution. (C) Superose-12 FPLC elution.

least 2–5 × 10^6 units/mg, comparable to preparations of the enzyme described in the literature isolated by conventional means.

A number of other assays were performed to determine the nature of the topoisomerase II activity of the Z-DNA binding protein. The optimal $MgCl_2$ and KCl concentrations for supercoiled plasmid relaxation or decatenation of *Crithidia* networks were the same as that described for topoisomerase II by Osheroff et al. (1983). The protein was found to be capable of relaxing both positive and negative supercoils, and the analysis of relaxation of an isolated topoisomer indicated that the linking number was changed by 2 in each step.

Three polyclonal rabbit IgG fractions and four monoclonal antibodies raised against the purified Z-DNA binding protein cross-reacted with topoisomerase II prepared by the method of Shelton et al. (1983). Conversely, six monoclonal antibodies raised against *Drosophila* topoisimerase II (Hadlaczky et al., 1988) reacted equally well with the Z-DNA binding topoisomerase II and the known topoisomerase II. The polyclonal antibodies W4 and H2 at IgG concentrations less than 10 μg/mL specifically recognized topoisomerase II on Western blots, on dot blots, and in the nuclear chromatin of cytological preparations of *Drosophila* cells and embryros. They showed no species cross-reactivity to mammalian topoisomerase II.

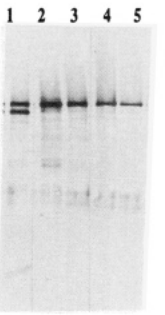

FIGURE 4: Western immunoblots of MonoQ and Superose column fractions with topoisomerase II activity. Lane 1, MonoQ column fraction (similar protein content as lane 3, Figure 2); lanes 2 and 3, MonoQ fractions (same protein content as lanes 4 and 5, Figure 2); lanes 4 and 5, two concentrations of a Superose column fraction (same protein content as lane 6, Figure 2).

IgG fractions of the polyclonal antibodies inhibited topoisomerase relaxation of supercoiled DNA, whereas nonspecific rabbit IgG at 10 times higher concentration had no effect. Monospecific IgG fractions were prepared by low-pH elution from a solid-phase membrane-bound topoisomerase II and showed the same inhibition properties in both in vitro and in vivo assays (Buchenau et al., 1993).

We observed a second, less acidic fraction of topoisomerase II activity eluting from some MonoQ columns containing approximately equal quantities of a 165-kDa band an a 155-kDa band recognized in Western blots of SDS gels using polyclonal IgG W4 (Figure 4). The earlier elution position from the column suggests either that this fraction contained a heterodimer composed of one degraded and one intact chain or that both chains may have been less acidic due to proteolytic and/or phosphatase action. The fraction also showed a much reduced Z-DNA binding activity, indicating that degraded topoisomerase II loses its differential DNA recognition.

Z-DNA Binding Topoisomerase II Cleavage of HSP70 Heat Shock DNA. The Z-DNA binding topoisomerase II purified from S3 tissue culture cells as described above produced preferential cleavages within the HSP70 heat shock region. A DNA cleavage assay was performed with the protein on the *Eco*RI fragment of plasmid 122 containing the *Drosophila* HSP70 gene region in the presence and absence of the drug VM26 (Bristol-Meyers) (Goldschmidt-Clermont, 1980). The DNA was separated on agarose gels, transferred to nitrocellulose filters, and subsequently probed with a ^{32}P-labeled plasmid. The cleavage sites were found to be in the intergene spacer sequences separating the two identical HSP70 genes, in good agreement with data obtained previously with topoisomerase II prepared from *Drosophila* embryos by conventional techniques (Udvardy et al., 1985).

The cleavage data are shown in Figure 5. In the presence of a low concentration of VM26 (1.5 μg/mL), lane 2, there was a 2–3-fold increase in the intensity of the cleavage sites located in the intergene spacer, without any change in the specificity of DNA–topoisomerase II interaction (compare with lane 1). Higher concentrations of VM26 greatly stimulated the cleavage of DNA at secondary sites (lanes 3–5), as found previously using topoisomerase II prepared from *Drosophila* embryos (Udvardy et al., 1986).

Phosphorylation of Z-DNA Binding Topoisomerase II. The purified Z-DNA binding topoisomerase II showed no autophosphorylation activity but could be phosphorylated in vitro by a casein kinase-like activity derived from a homogenate from the S3 cells using the protocol of Ackerman et al. (1988). The reaction was inhibited by heparin and GTP. Treatment of the protein with agarose-bound calf intestinal alkaline phosphatase at pH 7.9 resulted in a protein with unaltered

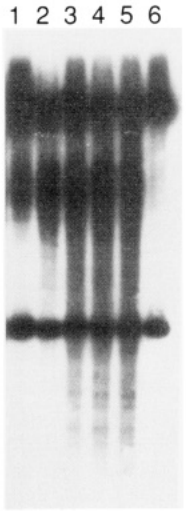

FIGURE 5: Topoisomerase II cleavage enhancement by VM26 of the *Eco*RI fragment of plasmid 122 containing the *Drosophila* HSP70 gene region. Lane 1, no drug. Lanes 2–5, 1.5, 5, 10, and 20 μg/mL VM26, respectively. Lane 6, reaction quenched by 20 mM EDTA before enzyme addition.

topoisomerase activity. The agarose-bound phosphatase was tested in a colorimetric assay and found to be fully active after exposure to the protein. These results are in disagreement with those of Saijo et al. (1990), who demonstrated a loss of activity of the mouse topoisomerase II using a similar dephosphorylation protocol.

Characterization of the Z-DNA Binding Properties of the Protein. Filter binding assays were performed with the protein and B- and Z-[^{32}P]poly[d(G-m^{5}C/C)], Z-[^{32}P]poly[d(G-m^{5}C)], or B-[^{32}P]poly[d(G-C)] in the presence of various salt, ions, effectors, and competitor DNAs. Figure 6A shows the binding of 15 ng of DNA with increasing topoisomerase II protein concentrations. The mean length of the linear Z-DNA was 800 bp, indicating that 1–2 protein molecules were bound to a single polynucleotide at saturation. The differential affinity for B- and Z-polynucleotide is shown in Figure 6B, using calf thymus DNA as a competitor or a labeled B-polynucleotide for direct binding. The binding to Z-DNA was salt dependent and virtually disappeared at 400 mM NaCl (Figure 6C). If, however, the protein was allowed to bind for 5 min to the Z-polynucleotides prior to the addition of salt, a large fraction of the complex was retained (Figure 6C). DTT had no effect on the affinity of the topoisomerase II for DNA.

At a protein concentration which was below saturation of the DNA binding, addition of 1 mM GTP caused an increase in the affinity of the protein for Z-DNA as shown in Figure 7. Data for several different concentrations of the enzyme demonstrated that GTP increased the binding by a factor of >5. dGTP had a smaller effect, and ATP, dATP, CTP, dCTP, UTP, and dTTP had no effect. A 20-fold excess of single-stranded polydeoxynucleotides showed no competition for the Z-DNA binding.

The Effect of Competitive Linear Z-DNA on Topoisomerase II Relaxation of Supercoiled Plasmids. Relaxation assays of supercoiled plasmid (350 ng) were performed in the presence of increasing concentrations (0–30 ng) of linear poly[d(G-C)] (B-DNA) or poly[d(G-br^{5}C)] (Z-DNA). The inhibition was monitored by agarose gel electrophoresis and analysis of the intensities of the supercoiled and relaxed plasmid bands after digitization of the photographs (Figure 8). Increasing concentrations of the Z-DNA decreased the relaxation, whereas the same concentration of B-DNA had no effect on the assay. The maximum concentration of the

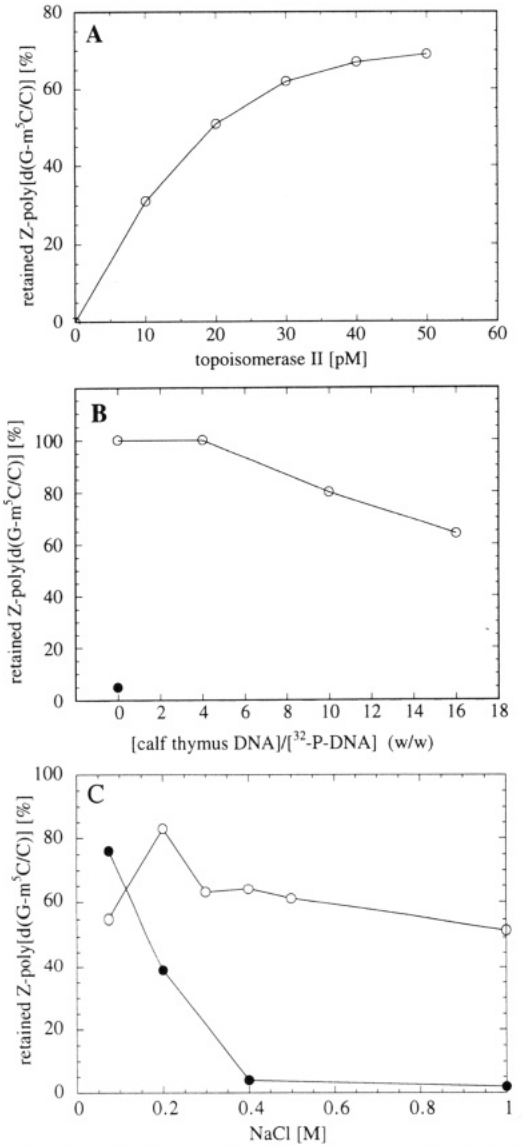

FIGURE 6: Filter binding studies of topoisomerase II. (A) Titration of Z-polynucleotide by increasing concentrations of topoisomerase II. A constant 15 ng of Z-[^{32}P]poly[d(G-m^{5}C/C)] in 2 mL of binding buffer was titrated with increasing amounts of topoisomerase II. The average polynucleotide length was 800 bp. (B) Comparative binding of topoisomerase II to Z- and B-DNA. The curve (○) shows the relative retention of 15 ng of Z-[^{32}P]poly[d-(G-m^{5}C/C)] by topoisomerase II in the presence of increasing concentrations of nonradioactive carrier calf thymus DNA. The single point (●) shows the retention of 15 ng of B-[^{32}P]poly[d-(G-m^{5}C/C)] (in buffer containing 1.5 mM $MgCl_2$) by the same amount of topoisomerase II. (C) The effect of increasing salt on the filter retention. The enzyme and Z-DNA either were incubated in binding buffer containing increasing concentrations of NaCl for 15 min at 37 °C and then filtered (●) or were first incubated for 5 min in 75 mM NaCl binding buffer at 37 °C before addition of NaCl. After an additional 15 min of incubation the solutions were filtered (○).

competitor linear DNA was less than 10% that of the supercoiled plasmid in these assays, demonstrating the increased affinity of the enzyme for Z-DNA.

Nucleoside Triphosphate (GTP and GTP-γ-S) Inhibition of Supercoiled Plasmid Relaxation by Z-DNA Binding Topoisomerase II. Deoxyadenosine triphosphate was able to substitute for ATP in the supercoil relaxation assay, as has been previously described (Osheroff et al., 1983). The enhancement of Z-DNA binding evoked by GTP (Figure 7) led us to investigate further the effect of additional (non-ATP) nucleoside triphosphates on the relaxation assay. In most experiments, the enzyme, supercoiled DNA, and test nucleoside triphosphate were present in standard reaction

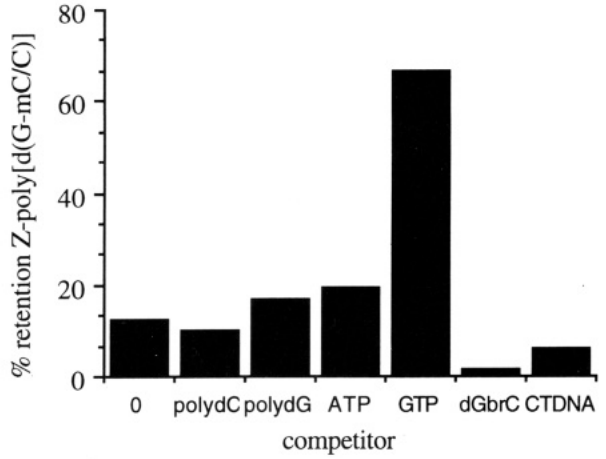

FIGURE 7: Effect of polynucleotides and nucleotide triphosphates on the binding of Z-polynucleotide by topoisomerase II. Constant amounts of Z-[^{32}P]poly[d-(G-m^{5}C/C)], 15 ng, and topoisomerase II, 7 ng, were incubated for 10 min at 37 °C alone (0) or in the presence of 1 mM nucleotide (ATP, GTP), 500 ng of single-stranded competitor polynucleotide (polydC, polydG) or calf thymus DNA (CTDNA), or 150 ng of Z-poly[d(G-br^{5}C)] (dGbrC) and then assayed for filter retention of the radioactive Z-polynucleotide. The bar graph displays the percent retention of the DNA in the presence of the different competitors.

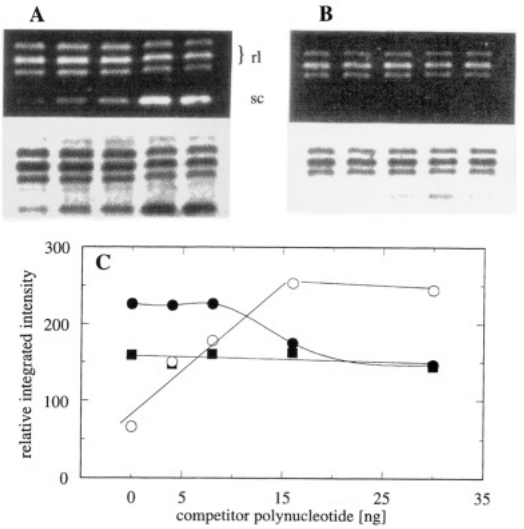

FIGURE 8: Effect of competitive linear Z-DNA on topoisomerase II relaxation of supercoiled plasmids. Relaxation assays of supercoiled pUC18 were performed in the presence of increasing concentrations of linear Z-DNA poly[d(G-br^{5}C)] (A) or B-DNA poly[d(G-C)] (B). Topoisomerase II was mixed in reaction buffer with the DNA substrates and incubated at 11 °C for 15 min. Relaxation was initiated by addition of 1 mM ATP and shifting of the reaction mixture to 28 °C for 20 min. The reactions were terminated by addition of stop buffer. Each reaction contained 350 ng of supercoiled plasmid DNA and from 0 to 30 ng of linear B- or Z-polynucleotide. The top panels are the digitized photographs of the ethidium bromide stained agarose gel electrophoresis lanes, and the middle panels are the respective logarithmic intensities. The relative intensities of the plasmid DNA bands versus the concentration of competitor polynucleotide are plotted in panel C. The three slowly migrating bands were integrated together for determining the relaxed plasmid (rl), and the single, fast migrating band was integrated for the supercoiled plasmid (sc). Data for competition with Z-DNA poly[dG-br^{5}C], panel A: relaxed plasmid concentration (●), supercoiled plasmid (○). Data for competition with poly[d(G-C)], panel B: relaxed plasmid concentration (■).

buffer, and the relaxation reactions were initiated by addition of 1 mM ATP. UTP had no effect on the enzyme at concentrations up to 10 mM, whereas >4 mM ATP inhibited the enzyme, in agreement with the data of Osheroff et al. (1983). This inhibition was reversible upon simple dilution of the enzyme–ATP solution to ATP concentrations below 3

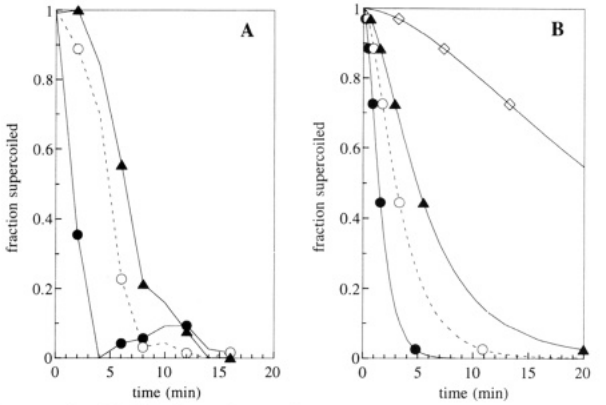

FIGURE 9: Inhibition of topoisomerase II supercoiled plasmid relaxation by GTP-γ-S. Enzyme, pUC18, 1 mM ATP, and 0 (●), 100 (○), or 200 μM (▲) GTP-γ-S were incubated at 30 °C in reaction buffer. Each reaction point contained 0.3 μg of supercoiled plasmid and 2 ng of enzyme. Aliquots of each reaction mixture were stopped at 0, 2, 4, 6, 8, 10, 12, 14, and 16 min, and the extent of supercoil relaxation was assayed by gel electrophoresis. (A) Fraction of supercoiled substrate as a function of time and inhibitor concentration. For each time point, the faster migrating band corresponding to the supercoiled substrate was integrated and normalized to the total integrated density. (B) Simulation of inhibition mechanism of topoisomerase II by GTP and GTP-γ-S. Conditions: concentrations (μM) of GTP-γ-S, symbols as in (A) with additional 500 μM points (□); 0.2 nM supercoiled plasmid; 0.05 nM topoisomerase II (approximately the conditions of the experiment in panels A and B); and constants K_m = 0.02 nM (supercoiled plasmid), k_{ass} = 10 nM^{-1} s^{-1}, k_{rel} = 8 × 10^{-3} s^{-1}, $K_{GTP\text{-}\gamma\text{-}S}$ = 0.1 mM, k_{inact} = 8 × 10^{-4} s^{-1}. See text for details.

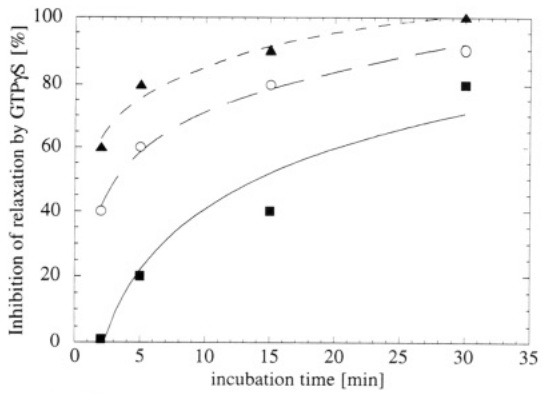

FIGURE 10: Temperature dependence of GTP-γ-S inhibition of topoisomerase II. Enzyme, DNA substrate, and GTP-γ-S were incubated at 4 (■), 15 (□) and 22 °C (▲). At the indicated times 1 mM ATP was added, and the reactions continued at 30 °C. Reactions were terminated after 30 min and the percent of supercoiled DNA remaining was used as a measure of the percent inhibition of relaxation.

mM. We confirmed that >0.5 mM ITP is inhibitory but could not demonstrate inhibition by CTP up to 10 mM, in contradiction to the results of Osheroff et al. (1983). The major finding was a pronounced inhibition of the enzyme by GTP at concentrations > 1.5 mM and by dGTP at >2 mM. The nonhydrolyzable GTP analog GTP-γ-S was even more potent, causing complete inhibition at micromolar concentrations. Figure 9A depicts the inhibition of DNA relaxation by 0.1 and 0.2 mM GTP-γ-S.

Since both GTP and GTP-γ-S were more effective inhibitors if incubated with the enzyme and the supercoiled DNA substrate prior to the addition of ATP, we reasoned that a conformational change of the enzyme might be involved in the mechanism. Therefore, we studied the time and temperature dependence of inhibition of the enzyme by GTP-γ-S; the results are shown in Figure 10. (Similar data were obtained with GTP.) Enzyme, DNA, and GTP-γ-S in reaction buffer were incubated at 4, 15, or 22 °C. At the indicated

times, ATP was added and the reaction mixture was shifted to 30 °C for 30 min. The reactions were stopped and processed as usual. Control reactions without GTP-γ-S were run in parallel and showed that the enzyme was fully active after all incubation conditions except 30 min at 22 °C. The enzyme could be inhibited by incubation with the nucleoside triphosphate in reaction buffer without the DNA substrate, indicating that the postulated conformational change does not require DNA binding.

In another reaction, the enzyme, 0.1 mM GTP-γ-S, and 1 mM ATP, or enzyme and GTP-γ-S alone, were incubated for 5 min at 22 °C. A relaxation assay was initiated by addition of substrate or substrate and 1 mM ATP, respectively, and incubation at 30 °C. Aliquots were taken at 2, 4, 8, 16, and 32 min. The kinetics of inhibition were the same whether ATP was present at 22 °C or not, indicating that GTP-γ-S is a noncompetitive inhibitor. In addition, we found that GTP-γ-S inhibits the ATPase activity of the enzyme.

We have found GTP to be an inhibitor of (a) *Drosophila* topoisomerase II purchased from United States Biochemicals, (b) a preparation of calf thymus enzyme kindly supplied by C. Austin, and (c) the human 170-kDa and 180-kDa isoenzymes (D. J. Arndt-Jovin and T. Bechert, unpublished results), suggesting that it may be a general effector of eukaryotic class 2 topoisomerases.

DISCUSSION

Topoisomerase II Is a Z-DNA Binding Protein. In the search for proteins with binding specificity for Z-DNA, we sought to avoid the problems inherent to affinity chromatography with constitutive Z-DNA (Krishna et al., 1988; Rohner et al., 1990). A protein with a high preference for Z-DNA was purified to homogeneity by purely biochemical means. The purification and DNA preference were monitored by blotting the protein fractions and column eluants with ^{32}P-labeled Z-polynucleotides in the presence of 150–200-fold excess of unlabeled B-DNA. As a probe, we synthesized a polynucleotide of mixed composition, poly[d(G-m^{5}C/C)], that can be shifted from the B to the Z conformation by changes in the Mg^{2+} concentration. Nonspecific interactions of the protein with base substituents are thereby avoided. However, the same protein is detected using ^{32}P-labeled Z-poly[d(G-br^{5}C)] or the Z form of poly[d(G-m^{5}C)] in the presence of excess unlabeled B-DNA (data not shown). The purified Z-DNA binding activity is shown in this paper to be undegraded topoisomerase II, identical to that described and cloned by Wyckoff et al. (1989). The ATP-dependent topoisomerase and Z-DNA binding activities copurify through three columns (Figure 3) and a glycerol gradient. The final specific activity of the purified fractions and the Z-DNA binding stoichiometry rule out the possibility that some minor contaminant in the final protein fraction could be responsible for the Z-DNA binding. It should be noted that most preparations used in the initial characterizations of the enzyme (Hsieh, 1983; Hsieh & Brutlag, 1980; Miller et al., 1981; Sander & Hsieh, 1983; Shelton et al., 1983) were proteolytically degraded. We find that fractions containing topoisomerase II activity but with degraded polypeptide chains lose their affinity for Z-DNA (Figure 3 and data not shown). By use of *Drosophila* tissue culture cell nuclei instead of embryos or whole cell extracts, proteolytic degradation during isolation of topoisomerase II is reduced.

Udvardy et al. (1985) observed a positive correlation between topoisomerase II and micrococcal nuclease cleavage sites in the HSP70 and histone gene segments. Recently, Reitman and Felsenfeld (1990) reported a similar correlation of topoisomerase II cleavage and DNase I cleavage sites in the chick globin gene complex. These studies suggest that the secondary structure of the DNA substrate may be of greater significance to topoisomerase II binding than the base sequence per se. In fact, the DNA consensus cleavage sequences for *Drosophila* (Sander & Hsieh, 1985) and for mammalian topoisomerase II (Spitzner & Muller, 1988) are degenerate and show considerable homology with simple alternating purine–pyrimidine sequences. Two groups have detected strong cleavage in blocks of simple repeats of this type (Lee et al. 1989; Spitzner et al., 1990). Some of these repeats are the preferred sites of B–Z DNA conformation transitions (Jovin et al., 1987; Rich et al., 1984) and have been found to be hotspots for recombination as well (Blaho & Wells, 1989; Freund et al., 1989). In the isolation of a mammalian recombination activity, Fishel and co-workers identified mammalian topoisomerase II associated in a large multienzyme complex bound to their Z-DNA affinity column (R. A. Fishel, private communication).

One problem in interpreting the existing cleavage and sequence affinity data for topoisomerase II in light of our results showing preferential binding to Z-DNA is that sequence mapping has been performed on linearized DNAs and often in the presence of drugs. The latter are effective in trapping the 5′-phosphoryl protein adduct but modulate the cleavage activity (see Figure 5) and, presumably, the affinity of the enzyme for DNA. Pommier and co-workers (1989) showed that the DNA affinity of the mammalian topoisomerase II is highest for supercoiled, intermediate for relaxed, and lowest for linear DNA of the same sequence, and it is enhanced by polyamines. In addition, strong cleavages are found on bent DNA sequences despite only moderate matches to the consensus sequence (Spitzner et al., 1990). Similar results were recently reported by Howard et al. 1991) from an electron microscope study of *Drosophila* topoisomerase II binding to SV40 and kinetoplast DNA. Thus, a high affinity for unusual structural elements in chromatin that may or may not correlate with a propensity for cleavage could have been easily overlooked in previous studies. Our results suggest that Z-DNA sequences constitute some of these high-affinity sites and that they are preferred to supercoiled substrates by at least an order of magnitude (Figure 8). In support of this conclusion, we have shown recently (Glikin et al., 1991) that *Drosophila* topoisomerase II binds with a 1–2 orders of magnitude greater affinity to supercoiled minicircles containing a short Z-DNA insert than to those without the insert. Whether the enzyme is capable of cleavage and religation of the Z-DNA is not yet clear.

Topoisomerase II Is under Allosteric Control by GTP. Our findings that GTP and GTP-γ-S increased the affinity of the enzyme toward Z-DNA (Figure 7) led us to investigate the effect of various triphosphates on the relaxation activity of the enzyme. We found CTP and UTP to be completely ineffective at inhibiting the enzyme, whereas GTP, dGTP, GTP-γ-S, ATP-γ-S, and ITP as well as high concentrations of ATP were inhibitory. ATP-γ-S, ITP, and GTP-γ-S inhibited the relaxation of negatively supercoiled DNA at submillimolar concentrations in the presence of 1 mM ATP, whereas 1.5 mM GTP was required in order to produce the same effect. These findings are not in agreement with the data of Osheroff et al. (1983), who reported no inhibition by up to 3 mM GTP but strong inhibition by CTP.

The inhibition of the relaxation of supercoiled DNA by GTP-γ-S is complex (Figure 9A). The time and temperature dependence of inhibition (Figure 10) suggests that a conformational change in the enzyme is involved, which under

appropriate conditions can be rate-limiting. This hypothesis is supported by the fact that inhibition was observed when we incubated the enzyme with the guanine nucleotide alone and then initiated the reaction by the simultaneous addition of DNA and ATP.

From these results and the filter binding data, we postulate that GTP and GTP-γ-S induce a conformational change in the enzyme that decreases the affinity for B-DNA and, concomitantly, increases the affinity for Z-DNA. In the absence of ATP, without which catalytic turnover is not possible, topoisomerase II is irreversibly inactivated by GTP and GTP-γ-S at a rate which is reduced by the presence of the DNA substrate. The simplest kinetic scheme which rationalizes all the experimental observations is one which incorporates the following features (and kinetic constants): (1) reversible binding [k_{ass}, k_{dis}] of the enzyme to the supercoiled substrates and stepwise catalytic relaxation [k_{rel}; $K_m = (k_{dis} + k_{rel})/k_{ass}$]; (2) specifiable different affinity for the relaxed product; (3) binding of free enzyme to GTP or GTP-γ-S ($K_{GTP,GTP\text{-}\gamma\text{-}S}$), followed by a first-order irreversible inactivation (k_{inact}). Assuming the steady-state approximation for all bound enzyme species, one can solve this scheme analytically and predict the time course of the reaction for any set of initial conditions (concentrations, rate and equilibrium constants). As the simplest representation of the experimental situation, we assume a single intermediate state between the supercoiled and relaxed DNA, which defines the demarcation between the topoisomer species scored in the gels as supercoiled and those which are resolved in the ladder of more relaxed molecules. Although such a scheme incorporates a number of constants that are not independently determinable in practical experiments, only certain combinations lead to simulations which predict the time course and concentration dependencies of the experiments in Figure 9A. An example is depicted in Figure 9B. It can be seen that one can reproduce the inhibition by the effector ligand (reflected in the $t_{1/2}$ for relaxation), the prolongation of the initial lag phase (reflecting progression through the species scored in the single band of higher order topoisomers), and the ultimate complete suppression of relaxation at high ligand concentrations. This model corresponds to a classical allosteric mechanism (Monod et al., 1965) in that conformational states are coupled to homotropic (effector binding) and heterotropic (substrate binding) ligand interactions. Pronounced allosteric effects of nucleotide cofactors on the DNA binding of other proteins have been reported, most recently in the case of DNA helicase (Wong & Lohman, 1992).

The model would also help explain the apparent discrepancies between our data on GTP inhibition of the enzymatic activity and the data of Osheroff et al. (1983). The latter studied the initial rates of enzymatic activity, i.e., conditions which may have obscured the inhibition since GTP is not a simple competitive inhibitor. That GTP may be a general effector of topoisomerase II is implied by recent experiments in which we demonstrated that GTP inhibits the calf thymus enzyme and both inhibits the human topoisomerase II enzymes as well as increases their binding affinity for Z-DNA (T. Bechert, S. Diekmann, and D. J. Arndt-Jovin, manuscript in preparation). We have not yet established whether there is more than one triphosphate binding site per enzyme monomer.

Our filter binding experiments, demonstrating that the salt sensitivity of the enzyme is drastically reduced after DNA complex formation even in the absence of nucleoside triphosphate, support the hypothesis of a conformational change in the enzyme upon DNA binding. Other data from the literature are consistent with this hypothesis. Increased salt resistance of the enzyme bound to B-DNA was observed in the presence of nonhydrolyzable derivatives of ATP (Osheroff, 1986) and in the binding studies of Pommier et al. (1989). Using very high concentrations of enzyme, some groups have found that strand passage can occur without binding of nucleotide (Minford et al., 1986). However, these results could be explained by assuming a binding of residual nucleotide to less pure enzyme preparations. There is general agreement that binding of ATP, or of an ATP analogue, is necessary for strand passage of the enzyme and that ATP hydrolysis is required for enzyme turnover (Osheroff et al., 1983). We found that GTP-γ-S inhibits the ATPase activity of the enzyme.

DNA Recognition Specificity and Biological Functions of Topoisomerase II. In our study of the binding and cleavage of minicircles containing Z-DNA inserts (Glikin et al., 1991) we could not detect a specific cleavage in the Z-DNA sequence. It is conceivable that binding selectivity and efficiency of cleavage are not synonomous for this enzyme. Chung and Muller (1991) have recently demonstrated that avian topoisomerase II forms DNA–enzyme networks with oligo(dG), and we observed high molecular weight DNA–enzyme complexes formed with the minicircles (Glikin et al., 1991). Chung and Muller also demonstrated that the oligo(dG) binding inhibits relaxation of supercoiled plasmid DNA. In the present study, the competition of supercoil relaxation by linear Z-DNA was effective even at concentrations more than 10-fold lower than the plasmid concentration (Figure 8). These data are in contrast to reports by Osheroff (1986), who found a 4–8-fold decrease in the binding of linear or relaxed B-DNA compared with supercoiled DNA. We conclude that high-affinity DNA binding may involve unusual DNA structures, irrespective of scission, and may reflect a role for the protein in mediating higher order chromatin structure.

Although topoisomerase II is clearly required in chromatid separation at mitosis (Buchenau et al., 1993; Holm et al., 1985; Uemura et al., 1987; Uemura & Yanagida, 1986), no other essential roles have been elucidated. Recent in vitro studies of SV40 chromosome replication (Ishimi et al., 1992) imply that the enzyme may be required as a swivelase in late replication. Topoisomerase II has been isolated as part of the "scaffold", or nuclear matrix complex (Berrios et al., 1985; Earnshaw & Heck, 1985), and may play a structural role in DNA condensation (Adachi et al., 1989, 1991). The possibility that the enzyme can bind more than one DNA helix is suggested by the formation of the higher order complexes cited above (Chung & Muller, 1991; Glikin et al., 1991) and recent electron microscopy pictures (Corbett et al., 1992; Howard & Griffith, 1991). The former group even propose a two-site model for topoisomerase II interaction with DNA, albeit solely in consideration of cleavage and strand passage rather than condensation of DNA per se. We need only to invoke an inhibition of the cleavage reaction by an ATP analogue (e.g., GTP), and the two-site cleavage model could easily be transformed into a model for DNA condensation.

Wang and co-workers (1990) recently reviewed the role of topoisomerases in recombinations and suggested that both positive and negative control may be exerted by topoisomerase II in this capacity. Kmiec and Holloman (1986) have shown that the Z conformation of DNA may be an intermediate in the recombination of DNA catalyzed by the *Ustilago* enzyme Rec1, a finding in accordance with an old suggestion by Pohl (1967) that left-handed DNA might serve to facilitate recombination. The indirect evidence of Fishel and co-workers that Z-DNA binds recombination enzyme complexes (Fishel et al., 1988; Moore & Fishel, 1990) and that these complexes

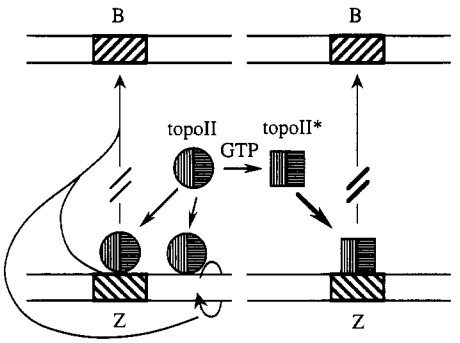

FIGURE 11: Model for topoisomerase II binding to, and functional modulation of, DNA. Left panel: In the presence of ATP, topoisomerase II binds to Z- or B-DNA in constrained segments of chromatin. By strand scission the DNA is relaxed to all-B form. Right panel: In the presence of GTP, topoisomerase II undergoes a conformational change, denoted by topoII*, that increases its preferential binding to Z-DNA.

include topoisomerase II implicates the enzyme further. A direct role of topoisomerase II binding to Z-DNA sequences and in decreasing their concentration by relaxation would serve to suppress any Z-DNA mediated recombination.

Our results show clearly that topoisomerase II has a preferential binding to Z-DNA and that GTP can further enhance that affinity. This binding constitutes an attractive mechanism for directing topoisomerase II to negatively supercoiled regions of chromatin and, thereby, relaxing the DNA in vivo. Transcription within constrained chromatin loops per se could generate negative supercoiling behind the RNA polymerase complex as suggested by Liu and collaborators (Liu & Wang, 1987; Wu and Liu, 1991), thus also generating targets for the enzyme. The formation of Z-DNA sequences has been shown to inhibit or retard transcription in a number of in vitro studies (Butzow et al., 1984; Durand et al., 1983; Job et al., 1988; Peck & Wang, 1985; van de Sande et al., 1982), and a targeted relaxation by topoisomerase II might facilitate transcription.

Since the concentration of *Drosophila* topoisomerase II is itself modulated through the cell cycle and throughout embryogenesis (Fairman & Brutlag, 1988; Nolan et al., 1991), the differential affinity of the enzyme for various DNA structures would be paramount in determining its distribution and action. A recent study of topoisomerase II from chick oviduct suggests that the stability and location of the enzyme are dependent upon the phosphorylation and poly(ADP) ribosylation state (Schröder et al., 1989). The influence of these modifications on the interactions of the *Drosophila* enzyme with DNA is presently under investigation. The phosphorylation states of DNA binding proteins may constitute general, albeit complex, signals for modulating localization and affinity, as discussed in a recent review of H1 phosphorylation and chromatin condensation/decondensation by Roth and Allis (1992).

The two isozymes of mammalian topoisomerase II have been shown to be differentially regulated (Drake et al., 1989) and to have differential nuclear localization (Negri et al., 1992). We have recently determined that both of the human enzymes show high-affinity binding to left-handed Z-DNA (T. Bechert, S. Dieckmann, and D. J. Arndt-Jovin, manuscript in preparation).

We propose that the general mechanism of DNA recognition by topoisomerase II is facilitated by unusual DNA secondary structure rather than by sequence alone. In this paper we have shown that one of these high-affinity recognition conformations is Z-DNA. The finding that topoisomerase II is a Z-DNA binding protein, coupled to the fact that primary DNA sequences can exist in a number of polymorphic DNA conformations and that topoisomerase II is one of the mediators of DNA conformation, leads us to propose schematically in Figure 11 a mechanism by which Z-DNA and GTP might effect a modulation of the enzyme binding and activity on supercoiled domains within chromatin. In this model certain sequences are stabilized in the Z conformation by binding of the enzyme in the presence of GTP, whereas the action of the enzyme–ATP in or near the Z-DNA regions would tend to destabilize the Z-DNA by topological relaxation.

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