HUMAN TOPOISOMERASES AND DNA GEOMETRY: PUTTING A POSITIVE TWIST ON ENZYME ACTION

By

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There are several people in this world outside of the laboratory that took on the responsibility of helping to keep me sane throughout the last few years. No one provides love and support like your family, and I am fortunate enough to have a lot of family. To my parents, thank you for providing endless encouragement while I have been in graduate school, and for a quiet country home that I can retreat to when I want to forget that I'm in graduate school. To my sisters, thank you for being a never-ending source of entertainment. You are brilliant, beautiful and hilarious, and I could not imagine my life without you. To Kathy and Bill, thank you for treating me like a daughter. I don't know how I'm going to manage without all of the dinners, shopping trips, and crazy family gatherings! To Shelly and Jamie, thank you for being my other sisters. You are two of the kindest, most considerate people I have ever known, and I will miss the time we have spent together. To Brayden, my amazing little nephew, thank you for constantly reminding me of what is really important in life. You have no idea how much you mean to me. To Bruce and Sherrie, thank you for introducing me to the world of sailing (no matter how reluctant I was), and for being great friends. To all other members of this growing family, thank you for everything! I hope that I have meant as much to your lives as you have to mine!

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amsacrine	4′-(9-acridinylamino)methanesulfon-*m*-anisidide
AML	acute myeloid leukemia
ATP	adenosine triphosphate
CP-115,953	6,8-difluoro-7-(4′-hydroxyphenyl)-1-cyclopropyl-4-quinolone-3-carboxylic acid
CVM-1	chlorella virus Marburg-1
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
etoposide	4′demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucoside)
GyrA	gyrase A
GyrB	gyrase B
hTop2αΔ1175	human topoisomerase IIα with a C-terminal truncation at amino acid 1175
hTop2αCTDβ	human topoisomerase IIα with the C-terminal domain of human topoisomerase IIβ
hTop2βCTDα	human topoisomerase IIβ with the C-terminal domain of human topoisomerase IIα
kDa	kilodaltons
MLL	mixed lineage leukemia
NLS	nuclear localization sequence
PBCV-1	*Paramecium bursaria* chlorella virus-1
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
Tris	tris-(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

DNA Topology

One of the most striking features of DNA is the intertwining of the two complementary strands of the double helix (1). Discovery of this characteristic led to the immediate recognition that biological processes such as replication would be severely affected by the topological state of the genetic material (2).

DNA in all species ranging from bacteria to humans is globally underwound (*i.e.*, negatively supercoiled) (3-6). This underwinding makes it easier to separate complementary DNA strands from one another and greatly facilitates initiation of replication and the assembly of replication forks. Once the fork begins to travel along the DNA template, however, the deleterious effects of topology manifest themselves (Figure 1). Since helicases separate, but do not unwind the two strands of the double helix, fork movement results in acute overwinding (*i.e.*, positive supercoiling) of the DNA ahead of the replication machinery (Figure 1B) (3,5-7). This overwinding has two major consequences. First, it increases the difficulty of separating duplex DNA into individual strands. Therefore, accumulation of positive supercoils presents a formidable block to fork movement (5,7-11). Second, DNA overwinding ahead of the fork leads to a compensatory underwinding behind the replication machinery. If the replisome rotates around the helical axis of the DNA, this underwinding allows some of the torsional stress in the pre-replicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes (Figure 1C) (6,7,11). If these

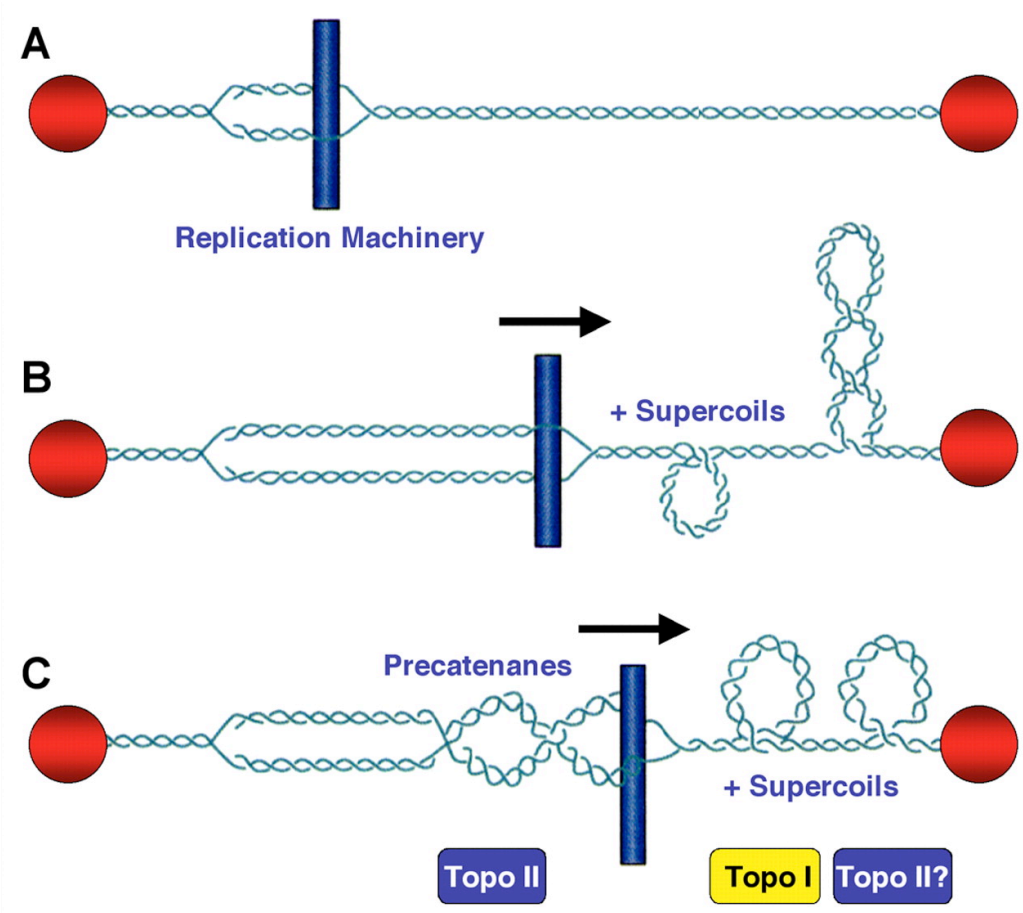

Figure 1. Model for topoisomerase function and topological stress associated with DNA replication [adapted from Wang (3)]. The replication machinery is represented by a rod moving through the double helix. DNA ends are anchored to hypothetical immobile structures existing in the nucleus. *A,* Upon initiation of DNA replication, the two strands of duplex DNA are separated and the replication fork is formed. *B,* Movement of the replication machinery through the immobilized DNA template strands induces acute overwinding (*i.e.*, positive supercoiling) ahead of the fork. *C,* If the replisome rotates around the helical axis of the DNA, compensatory underwinding (*i.e.*, negative supercoiling) behind the replication machinery allows some of the torsional stress in the pre-replicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes. If these precatenanes are not resolved, they ultimately lead to the formation of catenated duplex daughter chromosomes. Topoisomerase I is proposed to work ahead of the replication fork to remove positive DNA supercoils, while topoisomerase II is proposed to work primarily behind the fork to remove precatenanes.

precatenanes are not resolved, they ultimately lead to the formation of catenated duplex daughter chromosomes.

DNA Topoisomerases

The topological state of DNA in the cell is modulated by enzymes known as topoisomerases (5,7,12-16). These ubiquitous enzymes regulate DNA over- and underwinding, and remove knots and tangles from the genetic material by creating transient breaks in the sugar-phosphate backbone of the double-helix (5,7,12-18). Topoisomerases maintain genomic integrity during this process by forming covalent attachments between their active site tyrosyl residues and the terminal DNA phosphates generated during the cleavage reaction (7,12,15,16). This covalent linkage is the hallmark characteristic of all DNA topoisomerases. The actions of these enzymes allow the progression of virtually all metabolic events in the cell.

Topoisomerase I

There are two classes of topoisomerases that are distinguished by their catalytic mechanisms. Type I enzymes act by generating a transient single-stranded break in the double helix, followed by either a single-stranded DNA passage event or controlled rotation about the break (14,19,20). As a result, type I topoisomerases are able to alleviate torsional stress (*i.e.*, remove superhelical twists) in duplex DNA. These enzymes are further divided into two sub-classes. Type IA topoisomerases form a 5′-phosphotyrosyl linkage to the cleaved DNA strand, while type IB topoisomerases form a 3′-phosphotyrosyl linkage

(14,19,20). Human topoisomerase I belongs to the type IB subclass and will be further discussed in the following sections.

Human Topoisomerase I Domain Structure

Human topoisomerase I consists of a single polypeptide of 765 amino acids (~91 kDa) and functions as a monomer (14,19,20). The enzyme is divided into four domains based on sequence homology between proteins of various species and sensitivity to proteolysis (Figure 2). The N-terminal domain (amino acids 1-214) is highly variable and dispensable for activity in vitro. It contains nuclear localization sequences and sites for interaction with other proteins (20-22). The core domain of topoisomerase I (amino acids 215-635) is highly conserved and contains residues for enzyme catalysis, as well as DNA substrate recognition and binding (14,20,23,24). A dispensable linker domain (amino acids 636-712) is positioned between the core region and C-terminal domain of the enzyme. This linker domain is poorly conserved and interacts with the DNA substrate (20,25). Finally, the C-terminal domain of topoisomerase I (amino acids 713-765) is conserved and contains the active site tyrosine required for DNA cleavage and religation (14,20,23,24).

Human Topoisomerase I Catalytic Cycle

Human topoisomerase I activity occurs independently of ATP or a divalent cation (though the presence of a divalent cation stimulates enzyme activity) (14,19). The catalytic cycle of topoisomerase I can be divided into five discrete steps (5,14,19): (1) site-specific, noncovalent binding of a DNA substrate; (2) generation of a single-stranded DNA break and covalent linkage to the 3′

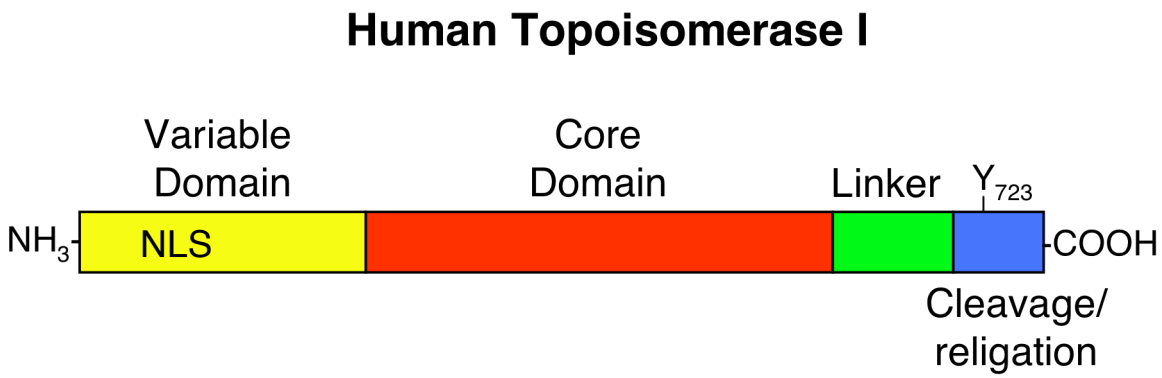

Figure 2. Domain structure of human topoisomerase I. Domain organization of human topoisomerase I: N-terminal domain (yellow), core domain (red), linker (green), and C-terminal domain (blue). The N-terminal domain is variable and contains nuclear localization sequences and sites for protein interactions. The conserved core domain contains residues required for catalysis and substrate recognition. The linker region is poorly conserved and dispensable for catalysis. Finally, the conserved C-terminal domain contains the active site tyrosine (Y273) required for DNA cleavage/religation.

terminus of the break, creating the "cleavage complex"; (3) controlled rotation of the double-helix around the single-stranded DNA break; (4) religation of the cleaved DNA strand, regenerating the active site tyrosine residue and re-establishing noncovalent binding; (5) and enzyme dissociation or initiation of a new round of catalysis.

Human Topoisomerase I Physiological Roles and Chemotherapeutic Target

Human topoisomerase I plays important roles in resolving torsional stress associated with the progression of DNA replication and transcription (5,13,19,20), and may have some involvement in maintaining genomic stability and chromosome condensation (13,19,20). There also is evidence suggesting a role for topoisomerase I in transcription initiation independent of the catalytic activity of the enzyme (13,19). Though both yeast and mammalian cells lacking topoisomerase I are viable, the enzyme is required for embryogenesis in higher organisms, suggesting an additional essential role in early development (13,19).

In addition to the physiological role of topoisomerase I in cells, this enzyme is a target for an emerging class of anticancer agents derived from the compound, camptothecin (Figure 3). Camptothecin is a naturally occurring compound found in the bark of the Chinese tree *Camptotheca acuminata* (17,19,26-28). Topotecan and CPT-11 (irinotecan) are derivatives of camptothecin and are U.S. FDA approved for ovarian and colon cancer (29). The most important aspect of these topoisomerase I-targeted drugs is that they act on a wide spectrum of cancers that currently have few, if any, other treatment options. Camptothecin was used in work described in chapter IV of this dissertation.

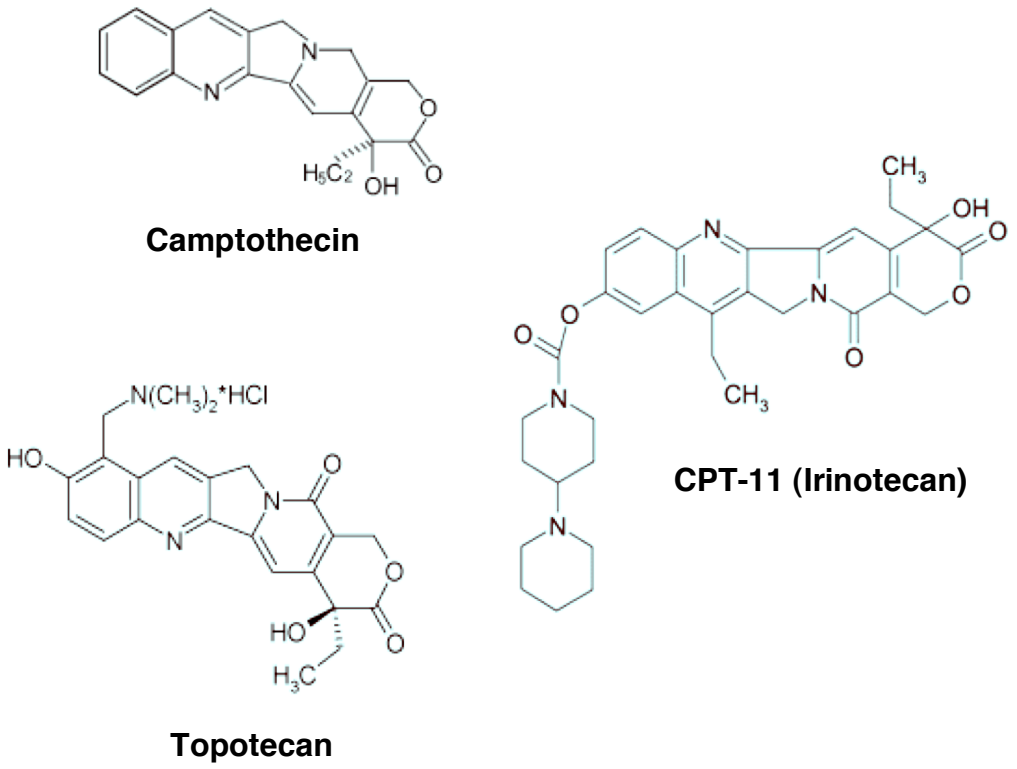

Figure 3. Topoisomerase I-targeting agents. Camptothecin, topotecan, and cpt-11 (irinotecan) are drugs that target topoisomerase I. Camptothecin is a naturally occurring compound found in the bark of the Chinese tree *Camptotheca acuminata*. Topotecan and CPT-11 are derivatives of camptothecin. The most important aspect of these drugs is that they act on a wide spectrum of cancers that currently have few, if any, treatment options.

Topoisomerase II

Type II topoisomerases act by generating a transient double-stranded DNA break, followed by a double-stranded DNA passage event (14-16,30). Consequently, these enzymes are able to remove superhelical twists from DNA and resolve knotted or tangled duplex molecules. Type II topoisomerases are required for recombination, chromosome segregation, and proper chromosome condensation and decondensation (5,7,12-16).

Topoisomerase II Isoforms

Whereas lower eukaryotes such as yeast and *Drosophila* encode only a single type II topoisomerase (31,32), vertebrate species express two discrete forms of the enzyme, topoisomerase IIα and IIβ (14,18,33,34). These enzymes display a high degree of amino acid sequence identity (~70%) and similar enzymological characteristics. However, they differ in their protomer molecular masses (170 vs. 180 kDa, respectively) and are encoded by separate genes (14,18,35). Either topoisomerase IIα or β can complement the loss of topoisomerase II in yeast (36-38), but the two enzymes have distinct patterns of expression and physiological functions in vertebrate cells.

Topoisomerase IIα is upregulated dramatically during cell proliferation and is tightly associated with mitotic chromosomes (13,39-42). In contrast, expression of the β isoform is independent of proliferative status and the enzyme dissociates from chromosomes during mitosis (13,35,39,43). Thus, topoisomerase IIα is believed to be the isoform that functions in growth-dependent processes, such as DNA replication and chromosome segregation (7,13). While cells can survive the

absence of topoisomerase IIβ, this isoform cannot compensate for the loss of topoisomerase IIα (35,44,45).

Topoisomerase II Domain Structure

The primary structures of topoisomerase IIα and β are very similar and can be divided into three domains based on sequence homology with the bacterial type II enzyme, DNA gyrase (5,14,15,18,35,46) (Figure 4).

N-terminal domain: The N-terminal domain (first ~670 amino acids) of topoisomerase II is homologous to the B-subunit of DNA gyrase (GyrB). This portion of the enzyme contains the site of ATP binding and hydrolysis (14,18,47). Crystal structures of this domain recently were solved for yeast topoisomerase II (48) and human topoisomerase IIα (49).

Central domain: The central domain (amino acids ~671-1200) of topoisomerase II is homologous to the A-subunit of DNA gyrase (GyrA) (14,18,50). This portion of the enzyme contains the active site tyrosine (amino acid 805 for topoisomerase IIα and 821 for topoisomerase IIβ) required for DNA cleavage and religation. A crystal structure for this domain in the absence of a DNA substrate was solved for yeast topoisomerase II (51).

C-terminal domain: The C-terminal domain (amino acids ~1201-1521 for topoisomerase IIα and ~1201-1621 for topoisomerase IIβ) is highly variable among species and between the two human isoforms. While it is dispensable for catalytic activity *in vitro*, this domain contains both nuclear localization sequences (52-58) and sites of phosphorylation (52,59-61).

While crystal structures have been solved for both the N-terminal and central domains of a eukaryotic topoisomerase II, a structure for the C-terminal

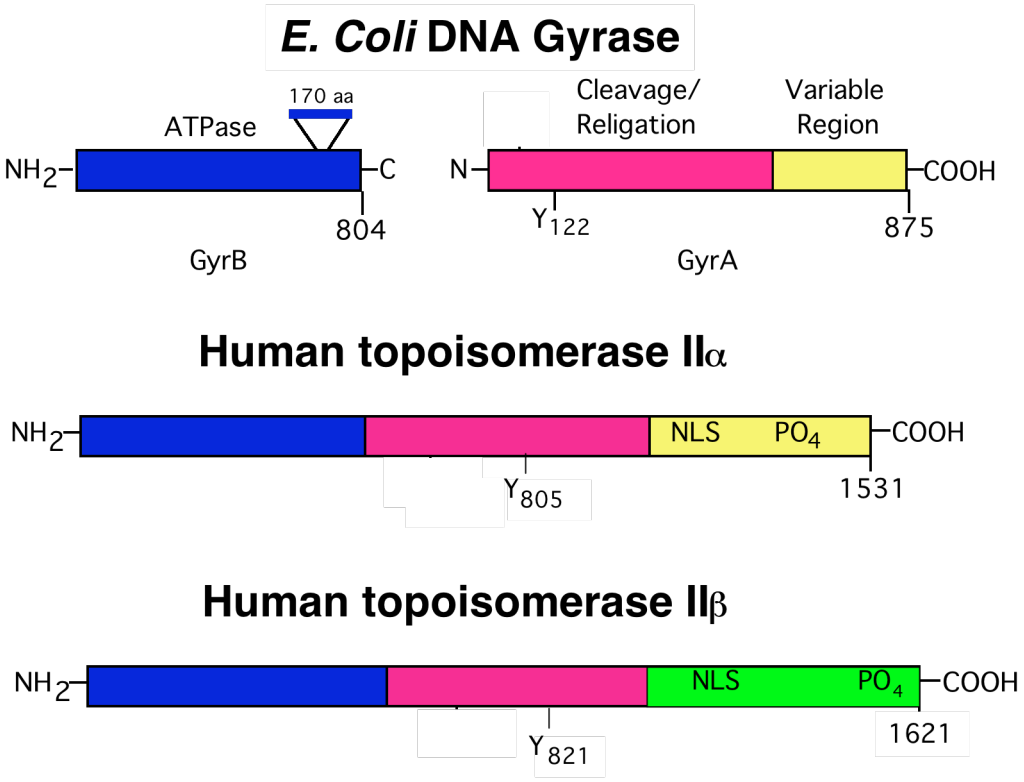

Figure 4. Domain structure of type II topoisomerases. The domain structures of *E. coli* DNA gyrase, human topoisomerase IIα and human topoisomerase IIβ are shown. Both human isoforms contain domains similar to the GyrB homology domain (blue), which is the ATPase region of the enzyme. They also encode regions homologous to the GyrA cleavage/religation domain (pink). This domain contains the active site tyrosine residue for topoisomerase II. The C-terminal domain (yellow or green) of the type II enzyme is highly variable between different species of topoisomerase II and between the two human isoforms.

domain of the enzyme has yet to be solved. However, within the last year, several groups have provided structural information on the C-terminal domains of the two bacterial type II enzymes. These groups revealed that the C-terminal domains of DNA gyrase (GyrA) and topoisomerase IV (ParC) adopt a unique fold called a β-pinwheel (Figure 5, *left panel*) (62-64). The outer rim of this fold was observed to be positively charged (Figure 5, *right panel*) and is thought to both bind and bend DNA substrates.

Further evidence suggests that the C-terminal domains of DNA gyrase and topoisomerase IV have a profound effect on enzyme activity. Truncation of the C-terminal domains of both enzymes weakens their catalytic activity (64). In addition, the C-terminal domains of DNA gyrase and topoisomerase IV appear to be responsible for the preference of each enzyme for a specific DNA substrate (62-65). For example, deletion of the C-terminal domain of *Escherichia coli* topoisomerase IV abolishes the ability of the enzyme to preferentially relax positively supercoiled DNA substrates, though it does not abolish enzyme activity altogether (64).

While there currently are no structural data for the C-terminal domain of a eukaryotic type II enzyme, recent enzymological data suggests that the C-terminal domain of human topoisomerase IIα also impacts enzyme activity. This idea will be discussed further in chapter V of this dissertation.

Topoisomerase II Catalytic Cycle

Human topoisomerase IIα and β function as homodimers, and their catalytic activities are dependent on the presence of a divalent cation (such as magnesium) and ATP (15,18,46,66). Their catalytic cycles are virtually identical

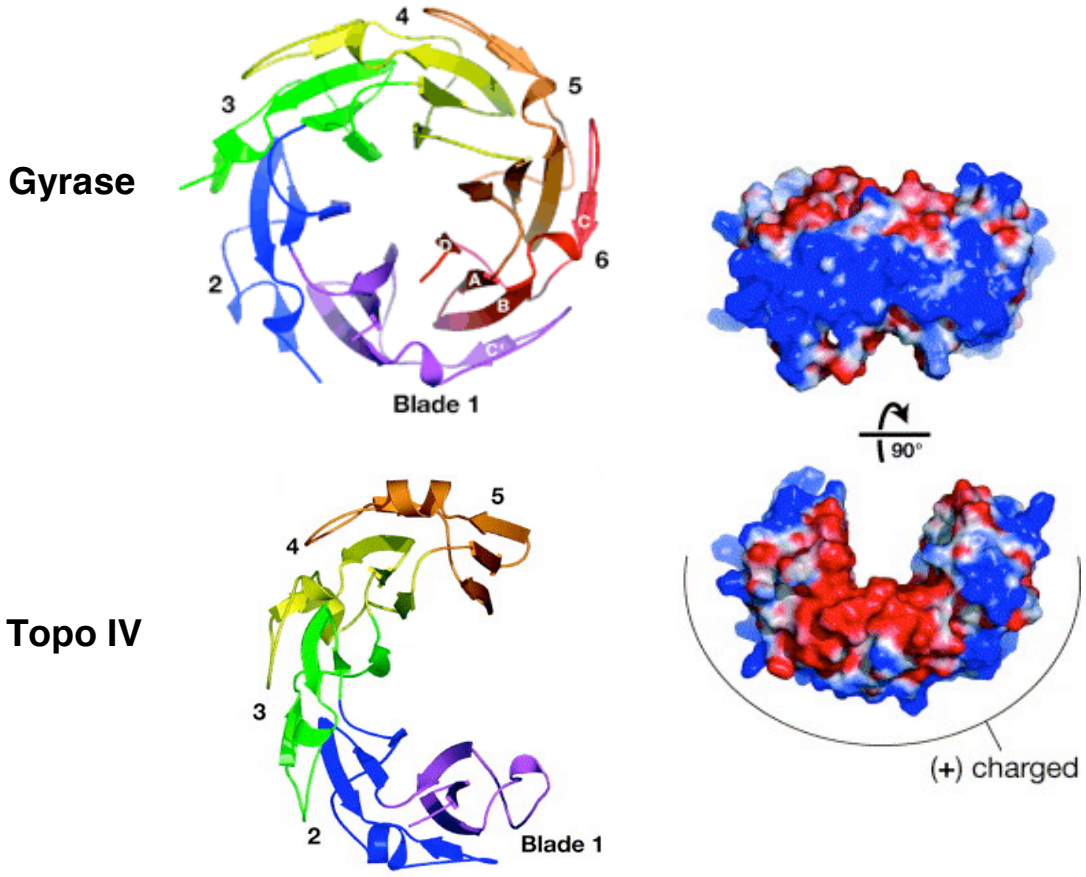

Figure 5. C-terminal domain structure of bacterial type II topoisomerases. The C-terminal domain structures for the two bacterial type II topoisomerases, DNA gyrase (*top*) and topoisomerase IV (*bottom*) are shown (*left panel*). The overall structure is termed a β-pinwheel and is made up of several blades of β sheet DNA strands. Blades 1-6 for DNA gyrase are purple, blue, green, yellow, orange, and red, respectively (*top, left panel*). Blades 1-5 for topoisomerase IV are purple, blue, green, yellow, and orange, respectively (*bottom, left panel*). The electrostatic surface of the side (top, right panel) and top (bottom, right panel) views of the C-terminal domain of topoisomerase IV also is shown. The curved, positively charged outer surface likely comprises the DNA binding surface. These images were adapted from Corbett *et al*. and Corbett *et al*. (62,64).

and can be divided into six discrete steps. One round of catalysis by topoisomerase II is depicted in Figure 6.

Step 1: Topoisomerase II preferentially interacts with DNA crossovers (67-69). To initiate catalysis, the enzyme noncovalently binds two segments of DNA termed the G-segment (the double helix that is cleaved by the enzyme and is opened as a "gate") and the T-segment (the double helix that is "transported" through the open DNA gate) (30). There is no cofactor requirement for this initial binding (70).

Step 2: In the presence of a divalent cation, a pre-strand passage cleavage/religation equilibrium is established (70-73). While magnesium is thought to be the physiologically relevant divalent cation for this process, others such as calcium can substitute in vitro (73,74).

The two monomers of topoisomerase II each cleave one strand in the G-segment, resulting in a transient double-stranded break in the DNA. Sites of cleavage within the homodimer are four bases apart, generating a 4-base 5′ overhang on either side of the double-stranded break (71,72). In order to maintain genomic integrity, the active site tyrosyl residue of each monomer forms a covalent attachment to the new 5′ termini of the cleaved DNA strands (50,71,72,75). This transient intermediate is called the "cleavage complex". While essential for maintaining the integrity of the genetic material, high concentrations of these complexes are potentially harmful for cells (15,16,76-78).

Unlike a traditional ligase, which requires base pairing to correctly position DNA ends for rejoining (79,80), topoisomerase II can religate cleaved DNA strands in the absence of base pairing interactions (81). Therefore, it is believed that topoisomerase II relies on covalent protein-DNA interactions to correctly

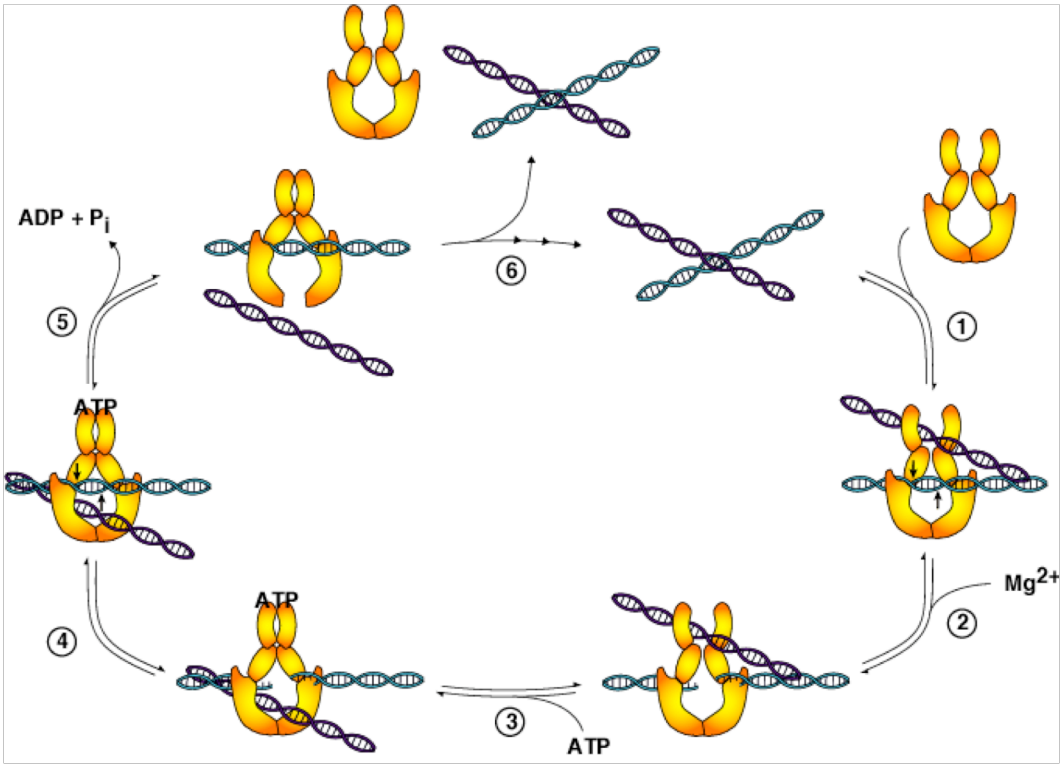

Figure 6. The catalytic cycle of topoisomerase II. The catalytic cycle of topoisomerase II is shown in six discrete steps: 1) Topoisomerase II-DNA binding; 2) Pre-strand passage DNA cleavage/religation equilibrium; 3) ATP binding and DNA strand passage; 4) Post-strand passage DNA cleavage/religation equilibrium; 5) ATP hydrolysis and gate opening; 6) DNA release and enzyme turnover.

position the 5′ termini of the cleaved strands for religation (81) and noncovalent interactions to position the 3′ termini (82).

Step 3: Each protein monomer binds a molecule of ATP, resulting in a conformational change and passage of the intact T-segment DNA through the cleaved G-segment (67,68,83,84). Hydrolysis of one ATP molecule appears to stimulate the passage event (85). Translocation of the T-segment DNA through the N-terminal gate of topoisomerase II and closure of this gate forms what is termed a "protein clamp" (68,86). This conformation topologically links the enzyme to the DNA, allowing it to diffuse along the double helix without dissociation.

Step 4: Following DNA strand passage, the enzyme religates the cleaved double helix and establishes a post-strand passage cleavage/religation equilibrium (68).

Step 5: Upon hydrolysis of a second molecule of ATP, topoisomerase II undergoes another conformational change that results in the opening of the C-terminal gate (84,87). The T-segment DNA strand is released through this gate (67,68,86,88).

Step 6: Finally, the enzyme returns to its original conformation and can either remain associated with the DNA substrate for a new round of catalysis, or it can dissociate and begin catalysis on a new substrate (67,68).

Topoisomerase II-DNA Cleavage Complexes

Topoisomerase II-DNA cleavage complexes are transient in nature and their cellular concentration is tightly regulated (Figure 7). Cleavage complex formation is essential for topoisomerase II to perform is cellular functions

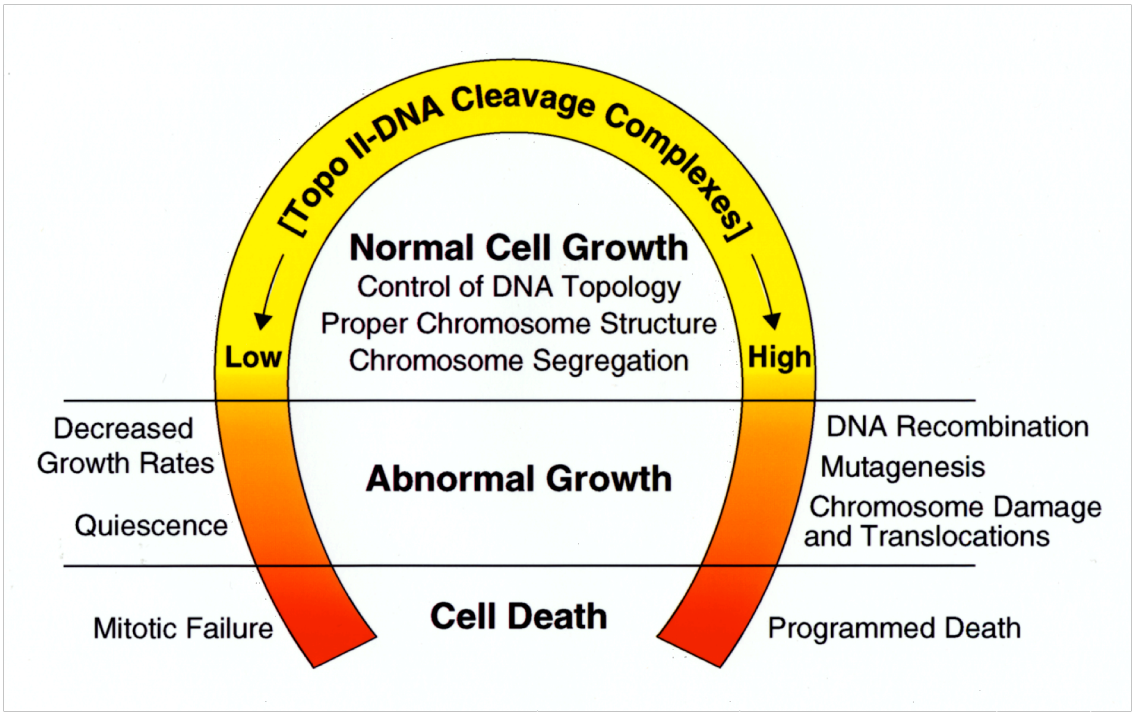

Figure 7. Effects of topoisomerase II-cleavage complexes in the cell. Topoisomerase II-DNA cleavage complexes are transient in nature, and their cellular concentration is tightly regulated. Cleavage complex formation is essential for topoisomerase II to perform is cellular functions. If the level of topoisomerase II-DNA cleavage complexes falls too low, cells are unable to undergo chromosome segregation, resulting in slow growth rates and ultimately cell death due to mitotic failure. Alternatively, if levels of cleavage complexes are too high, they can be converted to permanent strand breaks. The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple recombination/repair pathways. Accumulation of DNA breaks can lead to chromosome breakage and translocations. If the accumulation of breaks becomes overwhelming, they trigger cell death pathways.

(5,15,16). If the level of topoisomerase II-DNA cleavage complexes falls too low (*i.e.* enzyme activity is lowered), cells are unable to undergo chromosome segregation (7,12-16). This loss of enzyme activity results in slow growth rates and ultimately cell death due to mitotic failure.

While formation of the cleavage complex is essential to enzyme activity, it also is potentially deleterious to the cell. Transient, covalently linked topoisomerase II-DNA strand breaks are converted to permanent DNA strand breaks when nucleic acid tracking systems, such as replication or transcription complexes, attempt to traverse these topoisomerase-DNA roadblocks in the genetic material (Figure 8) (15,16,19,89,90). The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple recombination/repair pathways (15,16,76-78). Accumulation of DNA breaks can lead to chromosome breakage, DNA translocations, and cancer (Figure 8) (66,76,78). If the accumulation of breaks becomes overwhelming, they trigger cell death pathways (77). There are several different classes of topoisomerase II-targeting compounds that either stimulate or inhibit cleavage complex formation in cells.

Topoisomerase II-Targeting Agents

Beyond the critical physiological functions of type II topoisomerases, these enzymes are targets for some of the most important anticancer drugs currently used for treating human malignancies (15-19,91-94). One of these drugs, etoposide, has been used in the clinic since the 1960's (91,92,94). It is prescribed as treatment for a wide spectrum of leukemias, lymphomas, and solid tumors. The relative contributions of topoisomerase IIα and β to therapeutic outcomes are not clear at the present time. Because topoisomerase IIα is upregulated in many

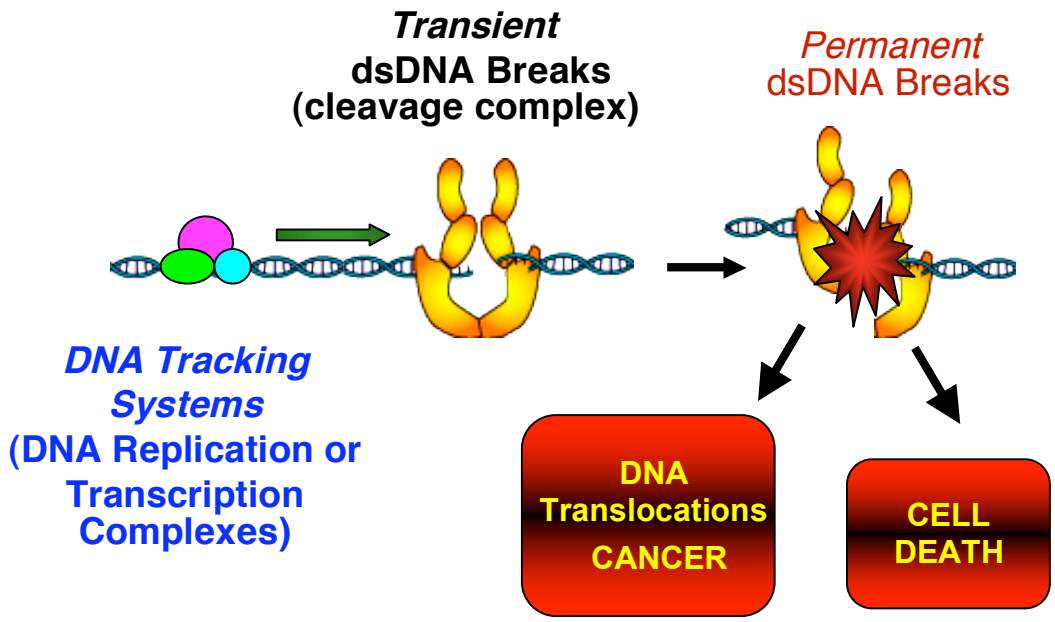

Figure 8. Conversion of transient topoisomerase II cleavage complexes to permanent double stranded DNA breaks. Transient, covalently linked topoisomerase II-DNA strand breaks are converted to permanent DNA strand breaks when DNA tracking systems, such as replication or transcription complexes, attempt to traverse these topoisomerase-DNA roadblocks in the genetic material. The resulting strand breaks initiate multiple recombination/repair pathways Accumulation of DNA breaks can lead to chromosome breakage, DNA translocations and cancer, and ultimately trigger cell death pathways.

cancer cells and is the isoform believed to be involved in DNA replication, it has been assumed to be an important drug target. In addition, drug interactions with topoisomerase IIβ in differentiated tissues, such as heart, are believed to contribute to the dose-limiting toxicity of some agents (95-98).

Topoisomerase II Poisons

Topoisomerase II-targeting agents represent a structurally diverse group of natural and synthetic compounds. Some of these compounds are depicted in Figure 9. Etoposide is a non-intercalative drug that was among the earliest anticancer agents identified as targeting topoisomerase II (15,16,91,92,94,99). TOP-53 is a non-intercalative drug that is a more potent derivative of etoposide (100,101). Genistein and CP-115,953 are non-intercalative compounds that share a similar core ring structure. Genistein is a naturally occurring bioflavanoid that is believed to possess chemopreventative properties (102-104). CP-115,953 belongs to the quinolone family (15,105-108), a drug class that includes a number of widely used antibacterials that target prokaryotic type II topoisomerases. TAS-103 displays strong interactions with DNA and is both an outside binder and an intercalating agent (109-111). Finally, amsacrine is an intercalative compound that is in clinical use (15,112,113). Each of these topoisomerase II-targeting drugs was utilized in the work described in chapter IV of this dissertation.

Although topoisomerase-targeted anticancer drugs come from several different structural classes, they all act by increasing levels of covalent topoisomerase-cleaved DNA complexes (*i.e.*, cleavage complexes) that are requisite intermediates in the catalytic cycles of these enzymes (15,16,19,89,90). When DNA tracking systems, such as replication forks, collide with these

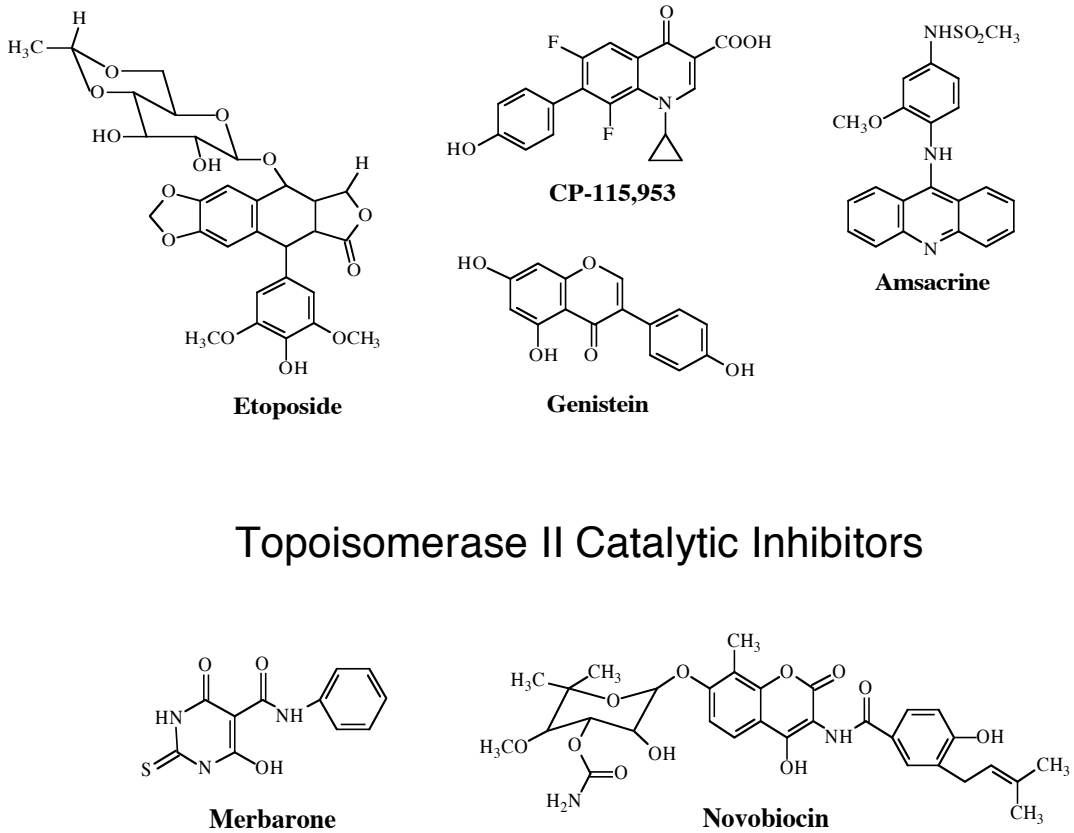

Figure 9. Topoisomerase II-targeting agents. The structures of four topoisomerase II poisons, etoposide, CP-115,953, genistein and amsacrine, are shown (top). These drugs function by increasing levels of topoisomerase II-mediated DNA cleavage complexes. The structures of two topoisomerase II catalytic inhibitors also are shown (bottom). These drugs function by blocking specific steps in the catalytic cycle of topoisomerase II.

complexes, transient enzyme-associated DNA breaks are converted to permanent breaks in the genome (15,16,19,34,90,114). As a result, anticancer agents convert topoisomerases from essential enzymes to potent cellular toxins, and are thus called topoisomerase II "poisons."

Topoisomerase II poisons increase levels of enzyme-DNA cleavage complexes by two non-mutually exclusive mechanisms. Some drugs, such as etoposide, genistein, TOP-53, amsacrine and TAS-103, act by inhibiting the ability of topoisomerase II to ligate the cleaved substrate (15,16,94,115,116). These drugs not only increase the level of cleavage complexes, but also increase the lifetime of these complexes. Other drugs, such as CP-115,953, have little effect on the rate of enzyme-mediated ligation and are believed to act primarily by enhancing the forward rate of cleavage complex formation (116-118). The exact mechanism by which this second group of drugs increases levels of DNA cleavage is unknown. They may specifically act to enhance the forward rate of DNA scission. Alternatively, they may have some effect on the DNA binding/dissociation equilibrium, as the level of topoisomerase II-mediated DNA cleavage is proportional to the amount of enzyme bound (118,119).

Drugs that target topoisomerases are believed to work at the enzyme-nucleic acid interface (15,16,119-124). However, intercalative agents, such as amsacrine and TAS-103, have two additional effects on DNA that could impact levels of topoisomerase-mediated scission in a geometry-specific manner. First, since these compounds locally underwind DNA, they induce compensatory unconstrained positive superhelical twists in distal regions of covalently closed circular molecules (125,126). Thus, as the concentration of an intercalating agent increases, a plasmid that is topologically negatively supercoiled would appear to

contain positive superhelical twists. Second, the accumulation of drugs in the double helix has the potential to inhibit enzyme binding or activity. Because the generation of positive superhelical twists by DNA intercalation induces torsional stress in the double helix, the ability of covalently closed molecules to absorb these compounds is limited. Since overwound plasmids are under positive torsional stress even in the absence of drugs, they cannot bind as many intercalative molecules as underwound DNA. These two characteristics of intercalative compounds have a profound effect on the ability of human type II topoisomerases to cleave negatively and positively supercoiled DNA substrates and will be discussed further in chapter IV of this dissertation.

Topoisomerase II Catalytic Inhibitors

While the only drugs currently being used in the clinic are topoisomerase II poisons, there is another class of topoisomerase II-targeting agents termed catalytic inhibitors. These drugs do not increase the level of enzyme-mediated DNA cleavage complexes. Instead, they block specific steps in the catalytic cycle of topoisomerase II (127-129). For example, agents such as novobiocin inhibit ATP binding (130,131). Merbarone, on the other hand, has been shown to block DNA cleavage mediated by topoisomerase II (128,132,133). These two catalytic inhibitors are depicted in Figure 9.

Whereas topoisomerase II poisons kill cells by fragmenting the genome, catalytic inhibitors kill cells by depriving them of the essential activity of topoisomerase II. Cells treated with catalytic inhibitors result in elongated and entangled chromosomes and ultimately die from mitotic failure (134-136). While topoisomerase II poisons are used as chemotherapeutic agents, catalytic

inhibitors have been used as modulators to increase the activity of other drugs (15,129,134). Additionally, some catalytic inhibitors have displayed potential anticancer activity in model organisms and currently are being tested in clinical trials (128,129,134,137).

Topoisomerase II and Cancer

Topoisomerase II as a Cellular Target for Anticancer Agents

Several pieces of evidence have been presented throughout the years indicating that certain anticancer drugs specifically target DNA topoisomerases. The initial suggestion that topoisomerases could be a target for such drugs came from the laboratory of Dr. Kurt Kohn in the late 1970s. This laboratory found that protein-associated DNA breaks were formed in cells treated with intercalative compounds (138,139). A few years later, Dr. Leroy Liu and co-workers published data demonstrating that certain anticancer drugs specifically stimulate topoisomerase II-mediated DNA cleavage both *in vitro* and *in vivo* (112,140-143).

In addition to this early data, Dr. John Nitiss and co-workers have developed yeast models for studying cellular resistance or hypersensitivity to topoisomerase II-targeted drugs (144-149). Cellular sensitivity to topoisomerase II-targeted drugs reflects the physiological level or activity of the enzyme (144,145,147,150,151). Cells that contain increased amounts of topoisomerase II display a hypersensitivity to drugs that target this enzyme. This is due to a higher level of topoisomerase II-mediated DNA strand breaks that cause cell death. In contrast, cells that contain decreased amounts of the enzyme display resistance to topoisomerase II-targeted drugs. This is due to a lower level of

topoisomerase II-mediated DNA cleavage, which is less toxic to cells. Finally, mutant type II topoisomerases that are hypersensitive or resistant to an anticancer drug *in vitro* have displayed the same hypersensitivity or resistance to the drug in cells (145,149,152-157).

Initiation of Cancer by Topoisomerase II

While topoisomerases are important targets for treating human cancers, there is some evidence suggesting that these enzymes also may be responsible for initiating cancers. More specifically, topoisomerase II-mediated DNA cleavage is believed to induce chromosomal translocations that lead to the development of specific types of leukemia (78,158-164). A small percentage of patients treated with etoposide-based chemotherapeutic regimens go on to develop specific treatment-related leukemias (163), the majority of which contain translocations at a breakpoint region in the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 (78,158,159,165-170). In correlation with these clinical data, etoposide was found to induce topoisomerase II-mediated DNA cleavage proximal to the chromosomal breakpoint region (171).

In addition to treatment-related leukemias, topoisomerase II is thought to be involved in the initiation of acute myeloid leukemias (AMLs) in infants (170,172-174). These AMLs also are the result of chromosomal translocations involving the *MLL* gene, although these translocations are observed *in utero* (174-176). Epidemiological studies have indicated that maternal consumption of foods during pregnancy that are high in naturally occurring topoisomerase II poisons increases the risk of developing infant AMLs ~10-fold (177,178). These findings

suggest that topoisomerase II-mediated DNA breaks may be initiating these translocations.

AMLs, along with non-lymphocytic leukemias, also have been linked to exposure to chemicals, such as benzene (179-186). Benzene is one of the top twenty production chemicals in the United States (187) and is both clastogenic and carcinogenic in humans (179-183,188). The mechanism by which benzene induces leukemias has not been fully elucidated, but it is thought to be through one of benzene's metabolites, 1,4-benzoquinione (189,190). Exposure of mammalian cells to 1,4-benzoquinone generates DNA mutations, insertions, deletions, and strand breaks (182,189,191-193). In addition, 1,4-benzoquinone was recently demonstrated to be a topoisomerase II poison (194).

The ability of topoisomerase II to cause rather than cure cancer is most likely related to the level of enzyme activity in a particular cell. If the concentration of topoisomerase II-mediated DNA cleavage in a cell is high, then recombination and repair pathways will be overwhelmed and cells will initiate apoptosis (77). However, if the concentration of topoisomerase II-mediated DNA cleavage is too low to initiate cell death, chromosomal breaks and translocations can result from normal cell survival pathways (66,76,78). These translocations can then ultimately lead to cancerous cell growth. Therefore, while topoisomerase II is an important target for cancer treatment, there also are potentially deleterious effects of targeting these enzymes.

Scope of the Dissertation

Topoisomerases play critical roles during DNA replication in eukaryotic cells (5,7,12-16). Since human topoisomerase I can relax positive or negative superhelical twists (14,19,195), it has been assumed that this enzyme acts ahead of replication forks to alleviate torsional stress. Conversely, because type II topoisomerases can untangle duplex DNA molecules (12,14-16,30), these enzymes are believed to act exclusively behind forks to resolve precatenanes or later in replication to unlink catenated daughter chromosomes. In marked contrast to this proposed segregation of function, models for anticancer drug action always place topoisomerase II ahead of approaching replication forks (15,16,90,196-198). This discrepancy raises the question of whether eukaryotic type II topoisomerases have normal physiological functions ahead of the DNA replication machinery.

If type II topoisomerases play a role ahead of replication forks in vertebrate species, then positively supercoiled DNA should be the preferred substrate for topoisomerase IIα, the isoform that is involved in replicative processes. Therefore, the goals of this dissertation are to 1) characterize the activity of human topoisomerase IIα and β on positively supercoiled DNA; 2) examine the effects of DNA supercoil geometry on topoisomerase-mediated DNA cleavage and enzyme response to anticancer drugs; and 3) explore the ability of topoisomerase IIα to recognize DNA supercoil geometry and determine what elements in the enzyme modulate substrate recognition.

Chapter I of this dissertation reviews both type I and type II topoisomerases, as well as the role of each enzyme in treating and/or initiating human cancers.

Chapter II describes the materials and methods utilized in the studies presented in Chapters III-V.

Chapter III of this dissertation characterizes the activity of human topoisomerase IIα and β on positively supercoiled DNA as compared to negatively supercoiled DNA. This work demonstrated that topoisomerase IIα, but not topoisomerase IIβ, preferentially relaxes positively supercoiled DNA over negatively supercoiled substrates. This preferential relaxation was not due to a greater binding affinity for positive supercoils or faster rates of religation with this substrate, and is most likely the result of faster DNA strand passage. In addition, topoisomerase IIα maintained lower levels of DNA cleavage intermediates with positively supercoiled plasmids, suggesting that the enzyme has the potential to relieve torsional stress ahead of approaching DNA tracking systems in an efficient and safe manner. The results of this study have been published (199).

Chapter IV of this dissertation examines the effects of positively supercoiled DNA on topoisomerase-mediated DNA cleavage, as well as the response to specific anticancer agents. This study includes human topoisomerase IIα and β, as well as human topoisomerase I. The human type II enzymes displayed lower levels of DNA cleavage in both the absence and presence of non-intercalative drugs. This was due primarily to lower baseline levels of cleavage, rather than decreased stimulation of scission by the drugs. Both topoisomerase IIα and β displayed higher relative cleavage enhancement with positively supercoiled substrates in the presence of intercalative drugs. This appeared to be the result of negative effects of high concentrations of intercalative compounds on negatively supercoiled DNA. Finally, topoisomerase I maintained higher levels of DNA

cleavage with a positively supercoiled substrate in the absence and presence of drug, indicating that this human enzyme may be an intrinsically more lethal target for anticancer drugs than either topoisomerase IIα or β. The results of this study have been published (200).

Chapter V of this dissertation explores the ability of topoisomerase II to recognize DNA supercoil geometry. While human topoisomerase IIα, but not topoisomerase IIβ, discerns supercoil geometry during DNA relaxation, both human isoforms discern the handedness of supercoils during DNA cleavage. These data suggest that type II topoisomerases must utilize distinct mechanisms to distinguish DNA geometry during these two processes. Several different enzymes were utilized to explore this hypothesis.

First, two chlorella virus type II topoisomerases (each naturally truncated at their C-terminal end) (201-204) were examined. Neither of these viral enzymes distinguished between negatively and positively supercoiled DNA during relaxation, but they each discerned DNA geometry during cleavage. Second, a truncation mutant of human topoisomerase IIα was created (205) and examined. This mutant enzyme also did not distinguish between negatively and positively supercoiled DNA during relaxation, but it discerned DNA geometry during cleavage. Finally, two mutants of topoisomerase IIα and β, in which the C-terminal domains were switched, were created and examined. The ability of topoisomerase IIα to preferentially relax positively supercoiled DNA was shown to be modulated by its C-terminal domain.

Taken together, these results suggest a bimodal recognition of DNA supercoil geometry by topoisomerase II, in which the enzyme uses elements in the C-terminal domain to sense the handedness of supercoils during DNA

relaxation and elements in the conserved N-terminal or central domains to recognize supercoil geometry during DNA cleavage. Finally, concluding remarks for this dissertation are found in Chapter VI.

CHAPTER II

METHODS

Materials

Archaeoglobus fulgidus reverse gyrase was expressed in *E. coli* C41(DE3) and purified according to Rodriguez (206). Human topoisomerase IIα, topoisomerase IIβ, hTop2αΔ1175, hTop2αCTDβ, hTop2βCTDα, and yeast topoisomerase II were expressed in *Saccharomyces cerevisiae* (50) and purified as described previously (157,207). *Drosophila melanogaster* topoisomerase II was purified from embryonic Kc cells as described by Shelton *et al*. (208). Human topoisomerase I was a gift from Dr. Mary Ann Bjornsti (St. Jude Children's Research Hospital). PBCV-1 topoisomerase II and CVM-1 topoisomerase II were expressed in *S. cerevisiae* and purified by a modification (204) of the procedure of Lavrukhin *et al*. (201). Negatively supercoiled pBR322 DNA was prepared from *E. coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Restriction enzymes, phosphatase, and T4 polynucleotide kinase were from New England Biolabs. [γ-^{32}P]ATP and genistein were obtained from ICN, etoposide and camptothecin were from Sigma, TOP-53 and TAS-103 were gifts from Taiho Pharmaceuticals, amsacrine was a gift from Bristol-Myers Squibb, and CP-115,953 was a gift from Pfizer Global Research. Etoposide, camptothecin, genistein, TOP-53, amsacrine, and CP-115,953 were stored at 4 °C as 10 or 20 mM stock solutions in 100% DMSO. TAS-103 was stored at 4 °C as a 10 mM stock solution in water. All other chemicals were analytical reagent grade.

Procedures

Preparation of Positively Supercoiled DNA

Positively supercoiled plasmid DNA was prepared by treating negatively supercoiled molecules with reverse gyrase (206). Reaction mixtures contained 35 nM negatively supercoiled pBR322 DNA and 420 nM reverse gyrase in a total of 500 μL of 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, and 1 mM ATP. Reactions were incubated at 95 °C for 5 min, halted by the addition of 13 μL of 375 mM EDTA, and cooled on ice. Proteinase K was added (10 μL of 4 mg/mL) and reactions were incubated at 45 °C for 30 min to digest the enzyme. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and positively supercoiled DNA was precipitated with cold ethanol. Plasmids were resuspended in 100 μL 5 mM Tris-HCl (pH 7.4) containing 500 μM EDTA. To make certain that differences between negatively and positively supercoiled substrates were not influenced by the temperature or other conditions used in the preparation protocol, negatively supercoiled plasmids were treated in a parallel fashion, except that reverse gyrase was omitted from reaction mixtures.

The average number of superhelical twists present in DNA substrates and the resulting σ values were determined by electrophoretic band counting relative to fully relaxed molecules. For negatively supercoiled substrates, time courses for the relaxation of pBR322 by topoisomerase I were resolved by electrophoresis in 1% agarose gels in TBE [100 mM Tris-borate (pH 8.3), 2 mM EDTA] containing 1–2 μg/mL chloroquine (Sigma) as running buffer. Optimal resolution was observed in 1 μg/mL chloroquine. For positively supercoiled substrates, time courses for the generation of positive superhelical twists by reverse gyrase were

resolved by electrophoresis as above in TBE containing 5–15 μg/mL netropsin B (Boehringer Mannheim). Optimal resolution was observed in 7.5 μg/mL netropsin B. Positively supercoiled bands also were counted in time courses of relaxation of positively supercoiled plasmid by topoisomerase I. Calculated σ values were consistent with those obtained from the reverse gyrase time courses.

DNA Relaxation

DNA relaxation assays were based on the procedure of Fortune and Osheroff (133) or Dickey *et al*. (204). Unless stated otherwise, reaction mixtures contained 1 nM human topoisomerase IIα, topoisomerase IIβ, PBCV-1 topoisomerase II or CVM-1 topoisomerase II, 0.2 nM yeast topoisomerase II, 2 nM *D. melanogaster* topoisomerase II, 4 nM hTop2αΔ1175, 2 nM hTop2αCTDβ or hTop2βCTDα, 5 nM negatively or positively supercoiled pBR322 DNA, and 1 mM ATP in a total of 20 μL of relaxation buffer [topoisomerase II α, topoisomerase IIβ, hTop2αΔ1175, hTop2αCTDβ, and hTop2βCTDα: 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 175 mM KCl, 5 mM $MgCl_2$, and 2.5% glycerol (v/v); yeast topoisomerase II: 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, and 2.5% glycerol (v/v); *D. melanogaster* topoisomerase II: 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, and 2.5% glycerol (v/v); PBCV-1 or CVM-1 topoisomerase II: 10 mM Tris-HCl (pH 8.5), 62.5 mM NaCl, 62.5 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl^2$, and 2.5% glycerol (v/v)]. Samples were incubated at optimal temperatures, 37 °C (human), 28 °C (yeast), 30 °C (*D. melanogaster* and CVM-1) or 25 °C (PBCV-1), and DNA relaxation was stopped by the addition of 3 μL of 0.77% SDS, 77 mM EDTA (pH 8.0). Samples were mixed with 2 μL of agarose gel loading buffer [60% sucrose in

10 mM Tris-HCl (pH 7.9)], heated at 45 °C for 2 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3), 2 mM EDTA.

Alternatively, DNA relaxation samples from assays using human topoisomerase IIα also were analyzed by two-dimensional gel electrophoresis. The first dimension was run for 2 h as described in the preceding paragraph. The gel was soaked for 2 h with gentle shaking in 200 ml of TBE containing 4.5 μg/mL chloroquine and run in the orthogonal dimension (90° clockwise) for 2 h in fresh TBE containing 4.5 μg/mL chloroquine.

All gels were stained with 1 μg/mL ethidium bromide, and DNA bands were visualized with ultraviolet light and quantified using an Alpha Innotech digital imaging system.

DNA Binding

The ability of human topoisomerase IIα, PBCV-1 topoisomerase II, CVM-1 topoisomerase II, and hTop2αΔ1175 to bind negatively and positively supercoiled DNA was assessed using a competitive nitrocellulose filter-binding assay. Binding mixtures contained 400 nM enzyme, 5 nM linear pBR322 DNA that was cleaved with Hind III and terminally labeled with [^{32}P]phosphate, and 0–20 nM negatively or positively supercoiled DNA in a total of 20 μL of binding buffer [topoisomerase IIα and hTop2αΔ1175: 10 mM Tris-HCl (pH 7.9), 100 or 175 mM KCl, 0.1 mM EDTA, and 2.5% glycerol (v/v); or PBCV-1 and CVM-1 topoisomerase II: 10 mM Tris-HCl (pH 8.5), 62.5 mM NaCl, 62.5 mM KCl, 0.1 mM EDTA, and 2.5% glycerol (v/v)]. Samples were incubated at optimal temperatures, 37 °C (human), 25 °C (PBCV-1) or 30 °C (CVM-1) for 6 min. Under the conditions of the assay, a DNA binding equilibrium was established in less

than 1 min. Nitrocellulose membranes (0.45 μm HA, Millipore) were prepared by incubation in binding buffer for 10 min. Samples were applied to the membranes and filtered in vacuo. Membranes were washed 3 times with 1 mL of binding buffer, dried, and submerged in 8 mL of scintillation fluid (Econo-Safe, Research Products International). Radioactivity remaining on the membranes was quantified using a Beckman LS 5000 TD Scintillation Counter. The percent linear DNA bound to each enzyme was determined based on the ratio of radioactivity on the membranes vs. that of the input DNA.

It should be noted that DNA binding experiments were performed in the absence of ATP and Mg^{2+}. This was done to prevent the formation of concatenated DNA multimers, which are too large to pass through the filter, or the generation of covalent enzyme-DNA cleavage complexes during the course of the assay.

ATP Hydrolysis

ATPase assays were performed as described by Osheroff *et al.* (67). Reaction mixtures contained 1 nM human topoisomerase IIα, 55 nM negatively or positively supercoiled pBR322 DNA, and 1 mM [γ-^{32}P]ATP in a total of 40 μL of relaxation buffer. Mixtures were incubated at 37 °C, and 2 μL samples were removed at time intervals up to 12 min and spotted on polyethyleneimine-impregnated thin layer cellulose chromatography plates (EMD Chemicals). Plates were developed by chromatography in freshly made 400 mM NH_4HCO_3 and analyzed using a Bio-Rad Molecular Imager FX. ATP hydrolysis was monitored by the release of free phosphate.

Plasmid DNA Cleavage

DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (133) or Dickey *et al*. (204). Reaction mixtures contained 0-800 nM topoisomerase IIα or hTop2αΔ1175, 0-1000 nM topoisomerase IIβ, 0-40 nM PBCV1 or CVM1, 10 nM negatively or positively supercoiled pBR322 DNA, and 5 mM $MgCl_2$ or $CaCl_2$ in a total of 20 μL of cleavage buffer [topoisomerase IIα, topoisomerase IIβ, or hTop2αΔ1175: 10 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.1 mM EDTA, and 2.5% glycerol (v/v); PBCV-1 topoisomerase II: 10 mM Tris-HCl (pH 8.5), 62.5 mM NaCl, 62.5 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl^2$, and 2.5% glycerol (v/v); or CVM-1 topoisomerase II: 10 mM Tris-HCl (pH 8.5), 120 mM KCl, 0.1 mM EDTA, 15 mM $MgCl^2$, and 2.5% glycerol (v/v)]. Assays were carried out in the absence of compound or in the presence of 0-100 μM etoposide, 50 μM genistein or TAS-103, 25 μM TOP-53, 5 μM CP-115,953, 0–500 μM amsacrine, or 0–25 μM ethidium bromide. Topoisomerase I DNA cleavage assays contained 11 nM human topoisomerase I and 10 nM negatively or positively supercoiled pBR322 DNA in a total of 20 μL of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 30 μg/ml BSA. Mixtures were incubated at optimal temperatures, 37 °C (human), 25 °C (PBCV-1) or 30 °C (CVM-1) for 6 min and enzyme-DNA cleavage complexes were trapped by the addition of 2 μL of 5% SDS (human) or 1% SDS (PBCV-1 and CVM-1), followed by 1 μL of 375 mM EDTA, pH 8.0 (human) or 2 μL 115 mM EDTA, pH 8.0 (PBCV-1 and CVM-1). Proteinase K (2 μl of 0.8 mg/mL) was added and samples were incubated at 45 °C for 30 min to digest the type II enzymes. Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 2 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3), 2 mM EDTA

containing 0.5 μg/mL ethidium bromide. DNA bands were visualized and quantified as described above and cleavage was monitored by the conversion of supercoiled plasmid DNA to linear molecules.

DNA cleavage reactions were performed in the absence of ATP so that the topological state of the DNA did not change during the course of the reaction. It should be noted that the nucleotide cofactor does not influence the mechanism of topoisomerase II-mediated DNA scission (15).

Site-specific DNA Cleavage

DNA sites cleaved by human topoisomerase IIα, PBCV-1 topoisomerase II and CVM-1 topoisomerase II in negatively and positively supercoiled DNA were mapped using a modification of the procedure of O'Reilly and Kreuzer (74,209). DNA cleavage mixtures contained 2.2 μM topoisomerase IIα, 120 nM PBCV-1 or CVM-1 topoisomerase II, 10 nM negatively or positively supercoiled pBR322 DNA, and $MgCl_2$ or $CaCl_2$ (human: 1 mM, PBCV-1: 2.5 mM, CVM-1: 15 mM) in a total of 160 μL of cleavage buffer. In some cases, reaction mixtures included 100 μM etoposide. Samples were incubated at optimal temperatures, 37 °C (human), 25 °C (PBCV-1) or 30 °C (CVM-1) for 6 min and enzyme-DNA cleavage complexes were trapped by the addition of 16 μL of 1% SDS followed by 8 μL of 375 mM EDTA, pH 8.0 (human) or 16 μL of 115 mM EDTA (PBCV-1 and CVM-1). Proteinase K (16 μL of 0.8 mg/mL) was added and mixtures were incubated at 45 °C for 30 min to digest the type II enzyme. DNA products were purified by passage through Qiaquick Spin Columns (Qiagen) as described by the manufacturer. DNA cleavage products were linearized by treatment with *Hin*dIII. Terminal 5′-phosphates were removed by treatment with calf intestinal

alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. Samples were treated with *Eco*RI and the singly-end labeled DNA products were purified by passage through a CHROMA SPIN+TE-10 column (Clontech). Loading buffer (40% formamide, 8.4 mM EDTA, 0.02% bromophenol blue, and 0.02% xylene cyanole FF) was added and samples were subjected to electrophoresis in 6% sequencing gels. Gels were fixed in 10% methanol/10% acetic acid and dried *in vacuo*. DNA cleavage products were visualized with a BioRad Molecular Imager FX.

The effects of ethidium bromide (0–10 μM) on the cleavage of linear DNA by topoisomerase IIα was monitored using the above protocol with the exception that a singly-end labeled linear pBR322 fragment (74,209) was used as the initial substrate. DNA cleavage was monitored by the loss of full-length linear molecules.

DNA Religation

DNA religation assays for different type II enzymes were carried out by one of two procedures. The first is a modification of the procedure of Kingma *et al*. (210) and was used to measure the religation abilities of topoisomerase IIα, PBCV-1 topoisomerase II and hTop2$\alpha\Delta$1175. DNA cleavage/ligation equilibria were established in topoisomerase II cleavage buffer as described above except that $MgCl_2$ in the reaction buffer was replaced by an equivalent concentration of $CaCl_2$. Topoisomerase II-DNA cleavage complexes were trapped by the addition of EDTA (pH 8.0) to a 6 mM (human) or 10 mM (PBCV-1) final concentration. NaCl was added to a 500 mM (human) or 250 mM (PBCV-1) final concentration in order to prevent re-cleavage of the DNA substrate. Religation was initiated by

the addition of $MgCl_2$ at a 0.25 mM (human) or 1 mM (PBCV-1) final concentration and terminated at times up to 60 s by the addition of 2 μL of 5% SDS (human) or 1 % SDS (PBCV-1).

The second method for DNA religation was carried out according to Dickey *et al*. (204) and was used to measure the religation activity of human topoisomerase IIα and CVM-1 topoisomerase II. DNA cleavage/ligation equilibria were established in topoisomerase II cleavage buffer as described above. Religation was initiated by shifting samples from 37 ºC (human) or 30 ºC (CVM-1) to 0 ºC (human) or -5 ºC (CVM-1) and terminated at times up to 60 s by the addition of 2 μL of 5% SDS (human) or 1% SDS (CVM-1). Assays were carried out in the absence of compound or in the presence of 100 μM etoposide.

All samples were processed and analyzed as described for plasmid DNA cleavage. The percent DNA cleavage at time 0 was set to 100% and the rate of religation was determined by quantifying the loss of cleaved DNA over time.

It should be noted that the religation activity of human topoisomerase IIα was examined by both methods described above with similar results. PBCV-1 topoisomerase II religation activity was examined only by the first method described, because a shift to lower temperature does not promote religation. CVM-1 topoisomerase II religation activity was examined only by the second method described, because $CaCl_2$ does not promote DNA cleavage by this type II enzyme.

DNA Intercalation

Intercalation reaction mixtures contained 220 nM topoisomerase IIα and 5 nM pBR322 DNA in a total of 20 μL of relaxation buffer that contained 1 mM

ATP, 0–25 μM ethidium bromide, 0–500 μM amsacrine, or 0–200 μM TAS-103. Mixtures were incubated at 37 °C for 6 min, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and added to 3 μL of 0.77% SDS, 77 mM EDTA (pH 8.0). Samples were mixed with 2 μL of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3), 2 mM EDTA. Gels were stained with 1 μg/mL ethidium bromide, and DNA bands were visualized as described for plasmid DNA cleavage.

The DNA intercalation assay is based on the fact that intercalative agents induce constrained negative supercoils and compensatory unconstrained positive superhelical twists in covalently closed circular DNA. Therefore, as the concentration of an intercalative compound increases, a plasmid that is negatively supercoiled or relaxed (*i.e.*, contains no superhelical twists) appears to become positively supercoiled. Treatment of an intercalated plasmid with topoisomerase IIα removes the unconstrained positive DNA superhelical twists. Subsequent extraction of the compound allows the local drug-induced unwinding to redistribute in a global manner and manifest itself as a net negative supercoiling of the plasmid. Thus, in the presence of an intercalative agent, topoisomerase treatment converts relaxed plasmids to negatively supercoiled molecules (see insets, Figures 23 and 24).

Construction of the hTop2αCTDβ and hTop2βCTDα Expression Vectors

The human topoisomerase IIβ gene in the YepWob6 plasmid was digested with *Kpn*I and *Sal*I restriction endonucleases. The resulting fragment of the topoisomerase IIβ gene was ligated using the same restriction sites into the

pUC18 expression plasmid. The Stratagene QuickChange II Site-directed Mutagenesis Kit was utilized to introduce an *Avr*II cut site at base 3159 (amino acid 1053) in the topoisomerase IIβ gene by PCR. The following sequences were used for the forward and reverse primers, respectively: 5′-GTTATTACGGTTTACGTAA-GGAGTGGCTCCTAGGAATGTTGGG - 3′ and 5′-CCCAACATTCCTAGGAGC-CACTCCTTACGTAAACCGTAATAAC- 3′. DNA was denatured at 95 °C for 5 min and subjected to 16 rounds of PCR using the following program: denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and primer extension at 68 °C for 8 min. Following the last round of PCR, primers were allowed to extend for an additional 5 min at 68 °C. The final product was purified by agarose gel electrophoresis and transformed into XL1-Blue *E. coli* cells. The purified plasmid was digested with *Kpn*I and *Sal*I and the topoisomerase IIβ fragment was ligated back into the original YepWob6 vector.

The human topoisomerase IIα gene in YepWob6 and the new topoisomerase IIβ gene both were digested with *Avr*II and *Xma*I, resulting in the isolation of each C-terminal domain fragment. Each C-terminal domain fragment and remaining vector fragment was gel purified. The isolated C-terminal domain fragments were then ligated to the opposite parent vector fragments and transformed into XL1-Blue *E. coli* cells. The C-terminal domain switched topoisomerase II constructs were sequenced to confirm the primary structures of the genes. The resulting plasmids carrying either hTop2αCTDβ or hTop2βCTDα were transformed in *S. cerevisiae* by heat shock.

CHAPTER III

HUMAN TOPOISOMERASE IIα RAPIDLY RELAXES POSITIVELY SUPERCOILED DNA: IMPLICATIONS FOR ENZYME ACTION AHEAD OF REPLICATION FORKS

Introduction

The movement of the replication machinery on DNA results in the accumulation of positive superhelical twists ahead of the fork and precatenanes behind (3,5-7,11). Although the torsional stress of DNA overwinding can be alleviated by the actions of an enzyme that generates single-stranded breaks in the double helix, the untangling of daughter chromosomes can only be accomplished by an enzyme that creates double-stranded breaks (5,7,12,14-16,30). Thus, it has been assumed that topoisomerase I functions ahead of the replication fork, while topoisomerase II acts behind.

There are two lines of evidence suggesting that type II topoisomerases have normal physiological functions ahead of DNA tracking systems, such as the replication fork. First, yeast topoisomerase II can compensate for the loss of topoisomerase I in *S. cerevisiae*, but loss of both enzymes abruptly halts DNA synthesis (8,9). This finding indicates that the type II enzyme can assume the role of topoisomerase I ahead of the replication machinery. Second, *E. coli* topoisomerase IV (whose functions in bacteria appear to parallel those of topoisomerase II in eukaryotes) can partially compensate for the loss of DNA gyrase during replication elongation (211,212). Moreover, topoisomerase IV

relaxes positive DNA supercoils ~20–fold faster than it does negative supercoils (213,214).

If type II topoisomerases play a role ahead of replication forks in vertebrate species, then positively supercoiled DNA should be the preferred relaxation substrate for topoisomerase IIα, the isoform that is involved in replicative processes. Results indicate that human topoisomerase IIα relaxes positively supercoiled plasmids >10–fold faster than negatively supercoiled molecules. In contrast, human topoisomerase IIβ, which is not required for DNA replication, shows no such preferential relaxation. Finally, topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled molecules. These enzymological properties are consistent with a topoisomerase that functions ahead of the replication machinery.

Results

Generation of Positively Supercoiled DNA Substrates

Steady-state interactions between eukaryotic topoisomerases and positively supercoiled DNA molecules have been examined previously (67,111,215-217). However, in most cases, positive superhelical twists were introduced in the DNA by the addition of intercalating agents such as ethidium bromide or chloroquine (67,111,215,216). Since these chemicals locally underwind the double helix, they generate a compensatory global overwinding elsewhere in covalently closed circular DNA substrates. Although previous studies have provided useful information, they suffer from a common deficiency; it is not always possible to dissociate the effects of DNA topology on topoisomerase action from those of the

intercalating agent. Thus, these studies have not been able to assess interactions between eukaryotic topoisomerases and positively supercoiled DNA in a quantitative manner.

To overcome this critical deficiency, we prepared positively supercoiled molecules by incubating pBR322 plasmid DNA with *A. fulgidus* reverse gyrase. This type IA topoisomerase is the only enzyme unique to hyperthermophiles and the only known topoisomerase that can actively introduce positive superhelical twists into DNA (206,218-221).

A time course for the conversion of negatively to positively supercoiled pBR322 DNA is shown in Figure 10. The initial plasmid substrate contained ~15 to 17 negative superhelical twists per molecule ($\sigma \approx$ -0.035 to –0.039) as determined by electrophoretic band counting in agarose gels containing chloroquine. This superhelical density is typical of plasmids isolated from *E. coli*. Reverse gyrase rapidly relaxed pBR322 and positive superhelical twists began to appear within the first minute of the reaction. As determined by electrophoretic band counting in gels containing netropsin B, positively supercoiled plasmids generated following a 5 min incubation with reverse gyrase contained ~15 to 17 positive superhelical twists per molecule ($\sigma \approx$ +0.035 to +0.039). The handedness of positively supercoiled DNA was confirmed by two-dimensional gel electrophoresis (Figure 11, *left panel*). Thus, the substrates employed for the experiments in this study contained equivalent numbers of superhelical twists, but were of opposite handedness.

When resolved by one-dimensional gel electrophoresis, relaxation mixtures were run in the absence of intercalating dyes and subsequently stained with ethidium bromide. Despite the opposite handedness of the DNA substrates

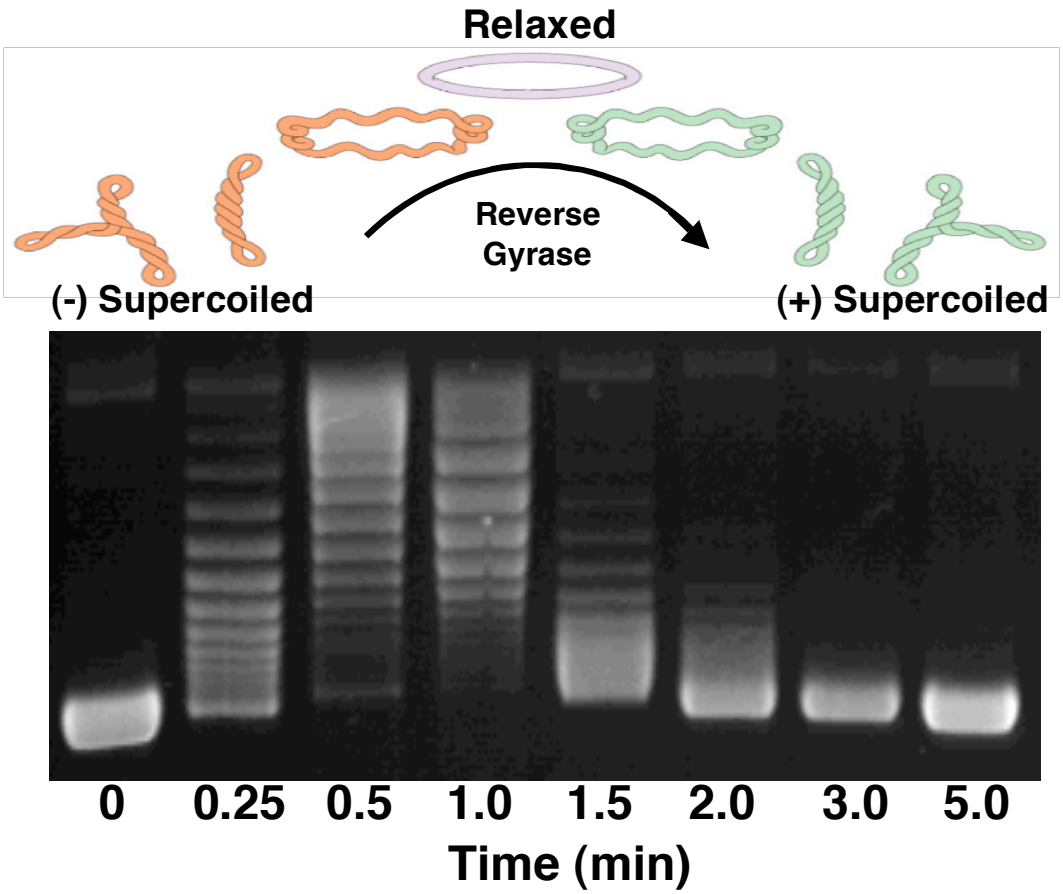

Figure 10. Generation of positively supercoiled DNA by *A. fulgidus* reverse gyrase. The positions of negatively (-) supercoiled, relaxed and positively (+) supercoiled DNA are indicated. Negatively supercoiled pBR322 plasmid DNA was incubated with reverse gyrase for the indicated times. The extent of positive supercoiling was monitored by agarose gel electrophoresis.

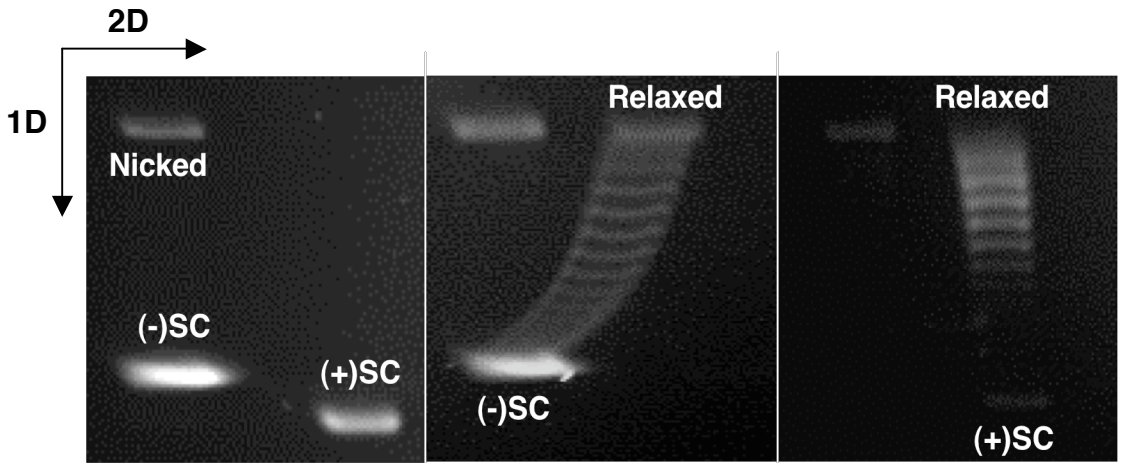

Figure 11. Two-dimensional gel electrophoresis of negatively and positively supercoiled DNA relaxation by human topoisomerase IIα. Ethidium bromide-stained agarose gels displaying representative relaxation reactions of negatively supercoiled [(-) SC] or positively supercoiled [(+) SC] pBR322 plasmid DNA by human topoisomerase IIα are shown. The positions of negatively supercoiled, positively supercoiled, and nicked circular DNA in the absence of enzyme are shown in the left panel. Relaxation of negatively supercoiled DNA after 30 min is shown in the center panel. Relaxation of positively supercoiled DNA after 5 min is shown in the right panel. The positions of fully relaxed DNA products are indicated (Relaxed).

employed, similar resolution of negatively and positively supercoiled plasmids was observed. However, it is notable that positively supercoiled substrates bind less ethidium bromide than negatively supercoiled plasmids. Therefore, to ensure that equal amounts of initial substrates were used in all experiments, DNA concentration was independently assessed by spectrophotometric analysis and by ethidium bromide staining of linearized plasmid substrates (see insets, Figures 12 and 17).

Human Topoisomerase IIα Preferentially Relaxes Positively Supercoiled DNA

If type II topoisomerases play a role ahead of replication forks in vertebrates, then positively supercoiled DNA might be the preferred relaxation substrate for topoisomerase IIα, the isoform that is involved in replicative processes. Therefore, to characterize interactions between human topoisomerase IIα and DNA substrates found ahead of replication forks, we assessed the ability of the enzyme to relax positively supercoiled molecules (Figures 11 and 12).

Compared to relaxation of negatively supercoiled substrates, the enzyme removed positive superhelical twists at a much higher rate. In addition, topoisomerase IIα relaxed positive supercoils in a more distributive fashion. Because of this difference, relaxation rates were quantified by one-dimensional gel analysis using two complementary methods: gain of fully relaxed product or loss of fully supercoiled substrate (Figure 12). As determined by the former method, the rate of relaxation of positively supercoiled DNA by topoisomerase IIα is >30–fold higher than the rate of relaxation of negatively supercoiled molecules. As determined by the latter method, it is nearly 10–fold higher.

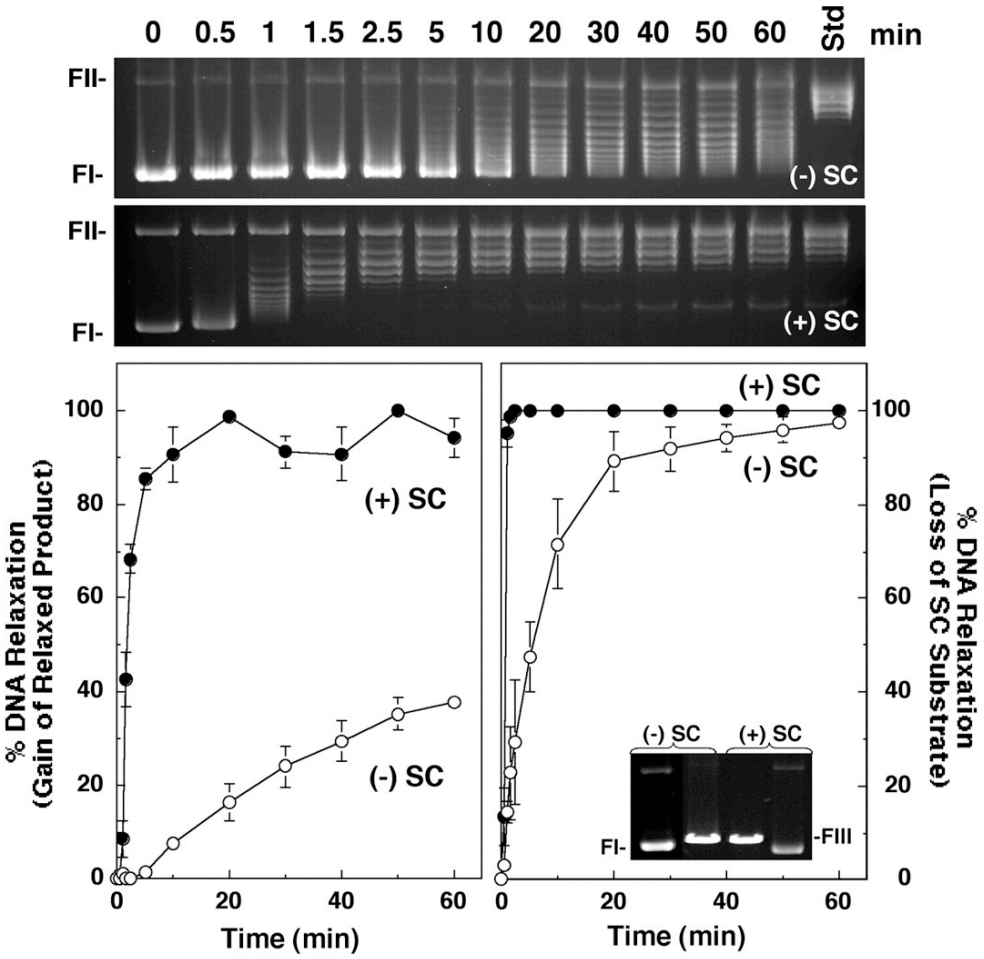

Figure 12. Human topoisomerase IIα relaxes positively supercoiled DNA faster than negatively supercoiled molecules. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *top gel*] or positively supercoiled [(+) SC; *bottom gel*] pBR322 plasmid DNA by human topoisomerase IIα are shown. Relaxed DNA standards (Std) were generated by incubation with 20 nM enzyme for 60 min. The positions of supercoiled plasmid DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Error bars represent the standard error of the mean of two independent assays. Positively supercoiled DNA binds less ethidium bromide than does negatively supercoiled DNA. To demonstrate that equal amounts of the two substrates were employed in assays, linear digests of the plasmids are shown in the *inset*. Supercoiled plasmid DNA (form 1, FI) and linear molecules (form III, FIII) are indicated.

Similar differences in relaxation rates were observed when reaction mixtures were resolved by two-dimensional gel electrophoresis (Figure 11). The *center panel* shows the results of a 30 min relaxation assay with negatively supercoiled plasmid, and the *right panel* shows a 5 min assay with positively supercoiled substrate. It should be noted that as the DNA molecules approached their fully relaxed state, underwound plasmids remained slightly negatively supercoiled and overwound plasmids remained slightly positively supercoiled. However, since the buffer conditions of the DNA relaxation assays and the gel electrophoresis are not identical, it is possible that the final distribution of reaction products differs somewhat.

The degree of DNA overwinding that occurs ahead of DNA replication forks in human cells is not known. However, single molecule experiments suggest that DNA ahead of tracking systems can reach a σ value as high as +0.110 (222). DNA buckling (*i.e.*, the transition to non-B-form structures) was not observed in these experiments below a σ value of +0.058 (222). Therefore, we believe that the positively supercoiled substrates used in our DNA relaxation assays ($\sigma \approx +0.035$ to +0.039) should be free of unusual non-B structures.

The salt concentration utilized for DNA relaxation experiments, 175 mM KCl, represents the optimal condition for the relaxation of negatively supercoiled substrates. To determine whether the switch from a processive to a distributive reaction contributed to the preferential relaxation of positively supercoiled plasmids, additional assays were carried out at lower ionic strengths. The relaxation of positively supercoiled DNA switched to a fully processive reaction at 100 mM KCl (Figure 13). Even under fully processive relaxation conditions, positively supercoiled DNA substrates were relaxed at a rate that was ~10-fold

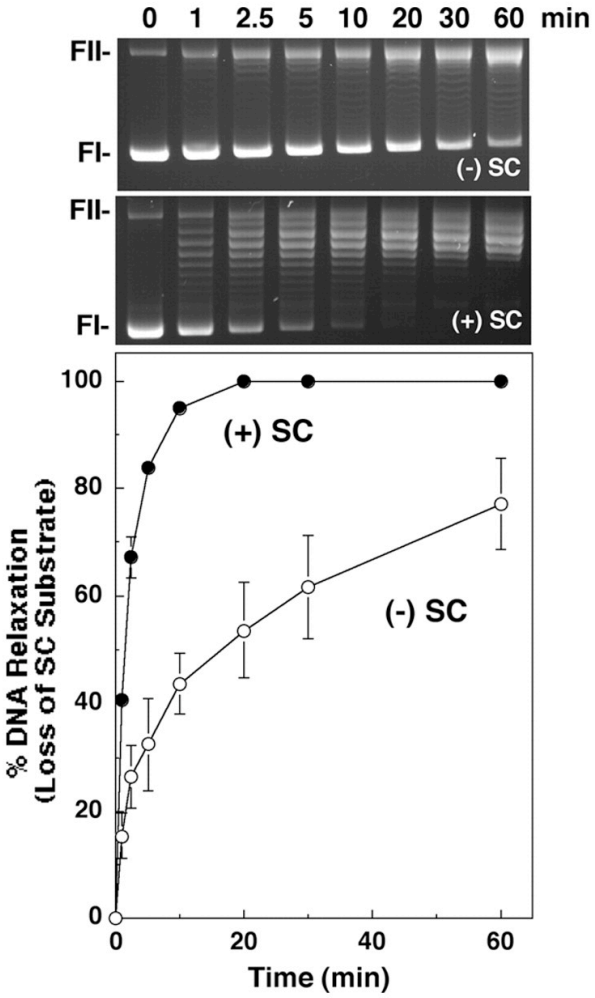

Figure 13. Human topoisomerase IIα relaxes positively supercoiled DNA faster than negatively supercoiled DNA under processive conditions. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *top gel*] or positively supercoiled [(+) SC; *bottom gel*] pBR322 plasmid DNA by human topoisomerase IIα in 100 mM KCl are shown. The positions of supercoiled plasmid DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. Since reactions with both substrates were processive, DNA relaxation was quantified by the loss of supercoiled substrate. Error bars represent the standard deviation of four independent assays.

faster than that observed with negatively supercoiled plasmids. Therefore, the processivity of DNA relaxation by human topoisomerase IIα does not appear to be the underlying basis for the preferential removal of positive superhelical twists.

Human Topoisomerase IIβ Does Not Preferentially Relax Positively Supercoiled DNA

Although topoisomerase IIα and β differ dramatically in their physiological regulation and functions, the two isoforms display only minimal differences in their enzymatic properties (15,16,33,35,223). Since topoisomerase IIβ is not believed to play a role in DNA replication, it was of interest to determine whether this isoform also displayed a preference for positively supercoiled molecules. As seen in Figure 14, topoisomerase IIβ relaxed positive and negative DNA supercoils at similar rates (less than 2–fold difference). Once again, relaxation of positively supercoiled molecules appeared to be more distributive. The recognition of positively supercoiled DNA by topoisomerase IIα represents the first major enzymological difference between the two isoforms. Furthermore, it is consistent with the proposal that topoisomerase IIα, and not topoisomerase IIβ, functions in DNA replication.

Effects of DNA Supercoil Geometry on Substrate Binding by Human Topoisomerase IIα

Previous studies indicate that the ability of human topoisomerase IIα to recognize DNA sequence or damage is controlled by chemical steps in the catalytic cycle of the enzyme rather than by alterations in substrate binding affinity (210,224,225). In contrast, the ability of eukaryotic type II topoisomerases to distinguish negatively supercoiled DNA from relaxed substrates appears to be

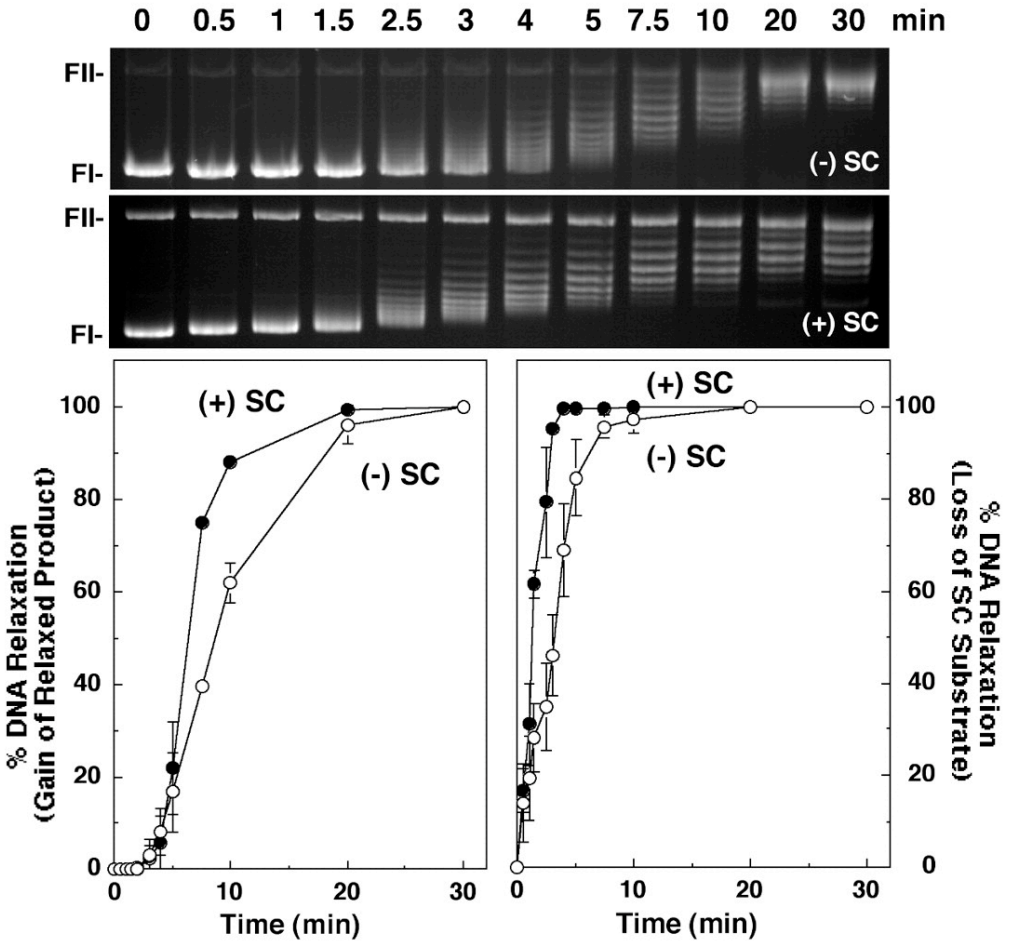

Figure 14. Human topoisomerase IIβ does not preferentially relax positively supercoiled DNA. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *top gel*] or positively supercoiled [(+) SC; *bottom gel*] pBR322 plasmid DNA by human topoisomerase IIβ are shown. The positions of supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Error bars represent the standard error of the mean or standard deviation of two or three independent assays, respectively.

governed primarily by binding interactions (67,69,215,226). These enzymes differentiate between supercoiled and relaxed DNA topoisomers by preferentially interacting with DNA crossovers, which are more prevalent in supercoiled molecules (69,215). However, since both negatively and positively supercoiled molecules contain DNA nodes, albeit of opposite handedness, it is not known how topoisomerase IIα discriminates between underwound and overwound DNA.

As a first step towards addressing this critical issue, the relative affinity of the human enzyme for negatively and positively supercoiled plasmids was determined using a competitive DNA binding assay (Figure 15). In this system, the ability of supercoiled plasmids to compete with radiolabeled linear pBR322 molecules for binding to topoisomerase IIα was monitored on nitrocellulose filters. The enzyme was exposed to both DNA substrates simultaneously, and the 6 min time point used for the assay ensured that a binding equilibrium was established. DNA binding was analyzed at the two salt conditions, 175 and 100 mM KCl, used for DNA relaxation assays.

At either ionic strength, topoisomerase IIα displayed a slightly higher affinity (~2–fold) for negatively supercoiled plasmids as compared to positively supercoiled substrates (Figure 15). This finding indicates that the rapid relaxation of positively supercoiled molecules by human topoisomerase IIα is not due to a higher binding affinity of the enzyme for its initial DNA substrate.

Effects of DNA Supercoil Geometry on ATP Hydrolysis by Human Topoisomerase IIα

DNA-bound topoisomerase II hydrolyzes more ATP than does free enzyme (67,84,227-230). It has been proposed that the stimulation of ATP hydrolysis by

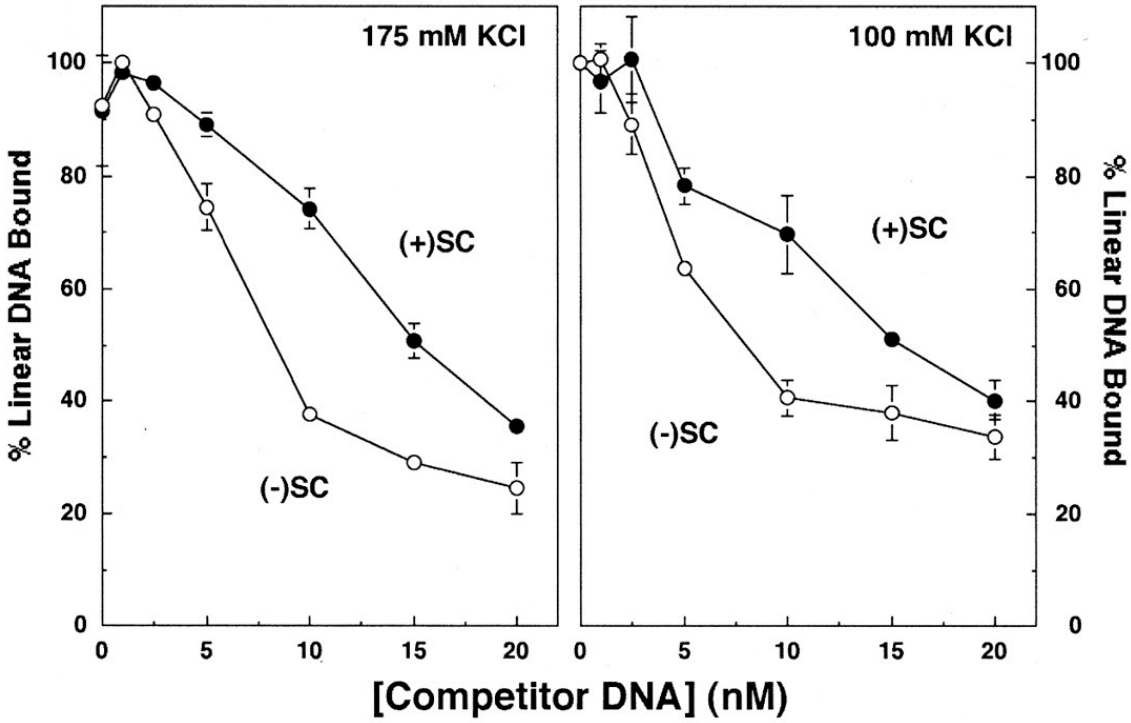

Figure 15. Binding of human topoisomerase IIα to negatively and positively supercoiled DNA. The ability of 0–20 nM negatively supercoiled [(-) SC] or positively supercoiled [(+) SC] pBR322 plasmid DNA to compete with the binding of 5 nM [^{32}P]-labeled linear pBR322 DNA by human topoisomerase IIα is shown. Percent linear DNA bound was determined by the ratio of cpm retained on a nitrocellulose filter *vs.* the input amount of radioactivity. Error bars represent the standard deviation of three independent assays.

DNA represents a non-productive cycling of the enzyme in the "closed-clamp" form and actually decreases the efficiency of ATP utilization (230). Therefore, if topoisomerase IIα relaxes positively supercoiled substrates more efficiently than it does negatively supercoiled DNA, it might hydrolyze lower levels of ATP during the course of the reaction. As seen in Figure 16, the rate of ATP hydrolysis in the presence of positively supercoiled plasmid was ~2–fold lower than the rate observed in reactions that contained negatively supercoiled DNA.

Human Topoisomerase IIα and β Maintain Lower Levels of DNA Cleavage Complexes with Positively Supercoiled Substrates

As a prerequisite to the strand passage event, topoisomerase II creates transient double-stranded breaks in its DNA substrate. To maintain genomic integrity during this process, the enzyme forms covalent bonds between active site tyrosyl residues and the 5'-terminal phosphates of the cleaved DNA (5,15,16,30,51). However, two negative outcomes are possible if a DNA tracking system, such as the replication machinery, collides with one of these cleavage complexes. First, the collision can disrupt the cleavage complex, making it impossible for the enzyme to ligate the cleaved strands (198,231). Second, the collision can arrest the progress of the replication fork, which triggers fork restart and recombination pathways (88). Both of these outcomes eventually lead to the formation of double-stranded breaks in the genome (15,16,76,196-198,232).

Since the DNA that precedes the replication fork is overwound, we characterized the ability of human topoisomerase IIα and β to cleave positively supercoiled plasmid molecules (Figure 17 and 18, respectively). Over a range of enzyme:plasmid ratios, levels of DNA scission by topoisomerase IIα were 3– to

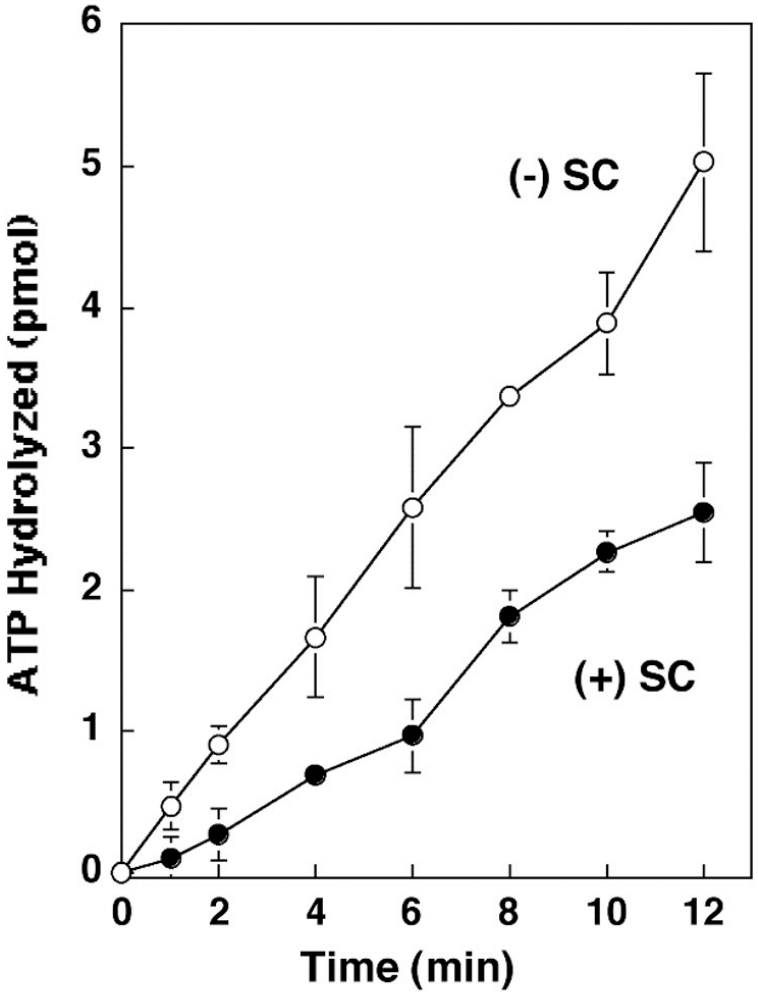

Figure 16. Hydrolysis of ATP by human topoisomerase IIα in the presence of negatively and positively supercoiled DNA. A time course for the hydrolysis of ATP in the presence of negatively supercoiled [(-) SC] or positively supercoiled [(+) SC] pBR322 plasmid DNA is shown. ATPase activity was monitored by quantifying the release of free phosphate from [γ-^{32}P]ATP. Error bars represent the standard deviation of three independent assays.

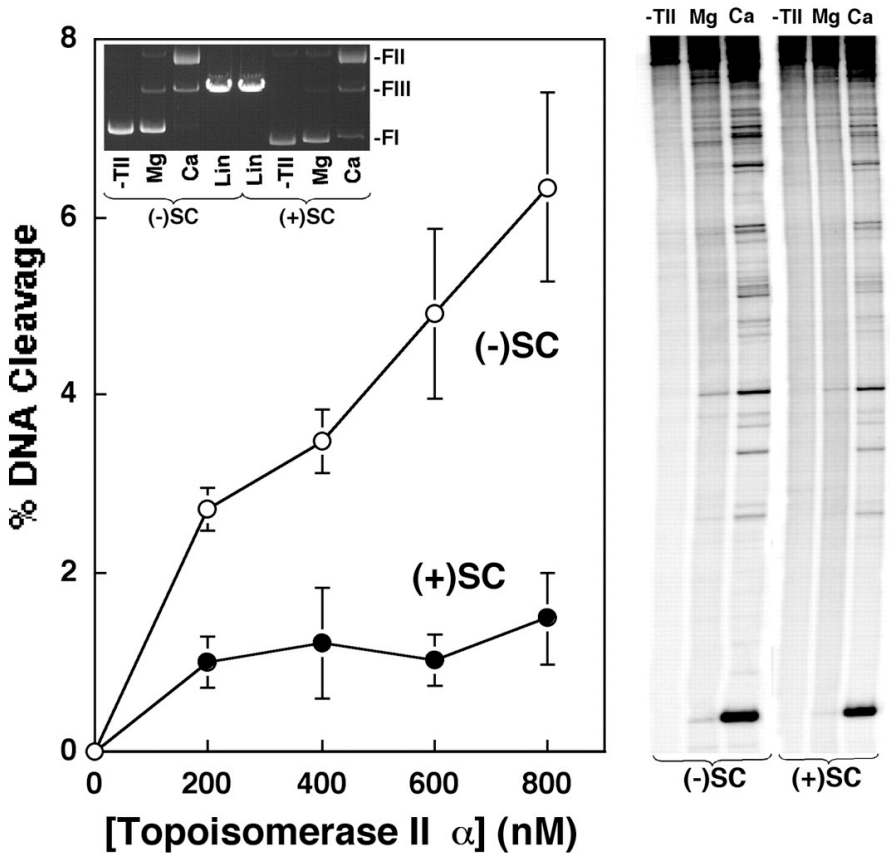

Figure 17. Human topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules. *Left*, the ability of 0–800 nM human topoisomerase IIα to cleave negatively supercoiled [(-) SC] pBR322 plasmid DNA or positively supercoiled [(+) SC] molecules is shown. Assays employed Mg^{2+} as the divalent cation. Error bars represent the standard deviation of three to four independent experiments. The *inset* shows a representative ethidium bromide-stained agarose gel of DNA cleavage assays that utilized either Mg^{2+} or Ca^{2+} as the divalent cation. Supercoiled DNA from reactions that lacked topoisomerase IIα (-TII) and a linear (Lin) DNA standard are shown. The positions of supercoiled (form I, FI), nicked circular (form II, FII), and linear molecules (form III, FIII) are indicated. *Right*, DNA sites cleaved by human topoisomerase IIα were mapped in negatively supercoiled or positively supercoiled plasmid substrates. Reaction products are representative of three independent experiments. Reactions utilized either Mg^{2+} or Ca^{2+} as the divalent cation. DNA from reactions that lacked topoisomerase IIα (-TII) are shown.

4–fold lower with positively supercoiled substrates than observed with negatively supercoiled plasmids. Similar results were seen when the physiological divalent cation, Mg^{2+}, was replaced with Ca^{2+} (Figure 17, *inset*). This latter divalent cation supports higher levels of DNA cleavage (73,74). Although DNA scission was reduced with overwound substrates, the site specificity of cleavage, as well as the relative site utilization, was identical with positively and negatively supercoiled DNA (Figure 17).

In contrast to relaxation assays, DNA cleavage results were similar for topoisomerase IIα and β. Topoisomerase IIβ maintained lower (~3– to 4–fold) levels of cleavage complexes with positively supercoiled DNA as compared to negatively supercoiled substrates (Figure 18). These results suggest that the human type II enzyme utilizes two distinct mechanisms to distinguish supercoil geometry during DNA relaxation and cleavage. This hypothesis will be discussed further in chapter V of this dissertation.

Discussion

Previous *in vivo* studies on *S. cerevisiae* topoisomerase II (8,9) and *E. coli* topoisomerase IV (211,212) suggest a role for type II enzymes ahead of the replication fork. This suggestion is supported by *in vitro* studies, which indicate that topoisomerase IV preferentially relaxes positively supercoiled DNA (213,214). To determine whether mammalian type II topoisomerases also display catalytic properties consistent with functions ahead of a replication fork, we characterized the ability of human topoisomerase IIα and β to relax positively supercoiled DNA.

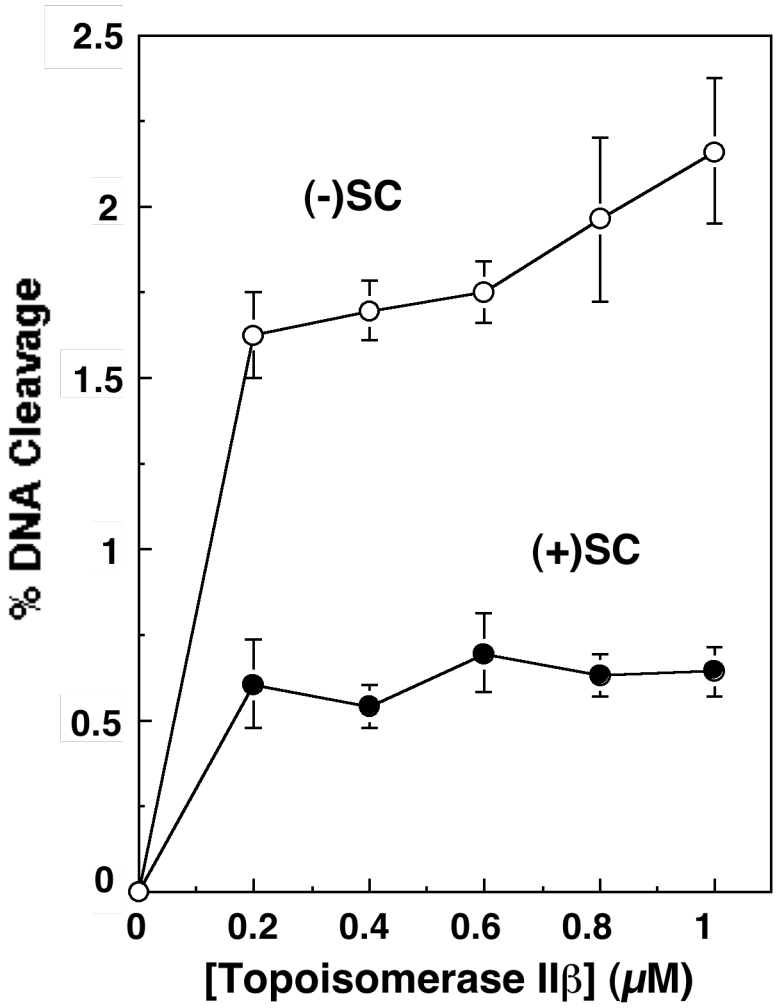

Figure 18. Human topoisomerase IIβ maintains lower levels of DNA cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules. The ability of 0- 1 μM topoisomerase IIβ to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA is shown. Error bars represent the standard deviation of four independent assays.

Topoisomerase IIα displayed two important enzymological characteristics that would be beneficial to an enzyme that operates ahead of the replication machinery. First, the enzyme removed positive superhelical twists at a rate that was >10–fold faster than it did negative superhelical twists. Thus, topoisomerase IIα displays preferential activity with the DNA substrates that accumulate ahead of the fork. Furthermore, the rapid rate of catalysis with overwound substrates makes it less likely that the replication machinery would collide with a molecule of topoisomerase IIα acting on the pre-replicated DNA. Second, the enzyme maintained lower levels of DNA cleavage complexes with positively supercoiled substrates. This decreases the probability that a collision with a replication fork would result in the formation of a topoisomerase II-associated double-stranded break in the genetic material. Taken together, these properties suggest that human topoisomerase IIα has the potential to alleviate torsional stress ahead of replication forks in an efficient and safe manner.

In contrast to the α isoform, topoisomerase IIβ, which is not believed to function in DNA replication, relaxed positively and negatively supercoiled substrates at similar rates. Relatively few studies on the enzymatic properties of topoisomerase IIβ have been reported. However, in every fundamental mechanistic aspect that has been compared, topoisomerase IIα and β are virtually identical (33,35,223). Results of the present study indicate a major difference between these two isoforms that is consistent with their proposed physiological roles. It is not clear how topoisomerase IIα, but not β, is able to distinguish supercoil geometry during DNA relaxation. However, based on structural studies on DNA gyrase and topoisomerase IV, coupled with nucleic acid

modeling and binding experiments, it has been proposed that this ability resides in the C-terminal domain of bacterial type II enzymes (62-64,217).

The amino acid sequence of the C-terminal domain varies considerably from species to species. Although topoisomerase IIα and β possess a high degree of amino acid sequence identity in their catalytic cores (~79% identity), the two enzymes diverge considerably in their C-terminal domains (~31% identity) (233). The present observations make it tempting to speculate that the C-terminal domain of human topoisomerase IIα plays a role in sensing the geometry of DNA substrates. Studies with deletion mutants of the human enzyme currently are underway to test this hypothesis.

In contrast to DNA relaxation assays, topoisomerase IIα and β both displayed lower levels of DNA cleavage with positively supercoiled substrates. The results suggest a bimodal recognition of DNA geometry by human type II topoisomerases. This idea will be discussed in depth in chapter V of this disseration.

Lower eukaryotic species, such as *S. cerevisiae* and *Drosophila* contain only one isoform of topoisomerase II (31,32). Single molecule experiments suggest that *Drosophila* topoisomerase II removes DNA supercoils of different handedness at similar rates (217). Steady-state experiments conducted as part of the present work support this conclusion (Figure 19). *Drosophila* topoisomerase II relaxed positively supercoiled plasmids at a rate that was marginally (<2–fold) higher than obtained with negatively supercoiled molecules (Figure 19, *left panel*). Similar results were observed with *S. cerevisiae* topoisomerase II, which relaxed positively supercoiled plasmids ~2 to 3 times faster (Figure 19, *right panel*). It is not known why the ability to discern the handedness of DNA supercoils is not

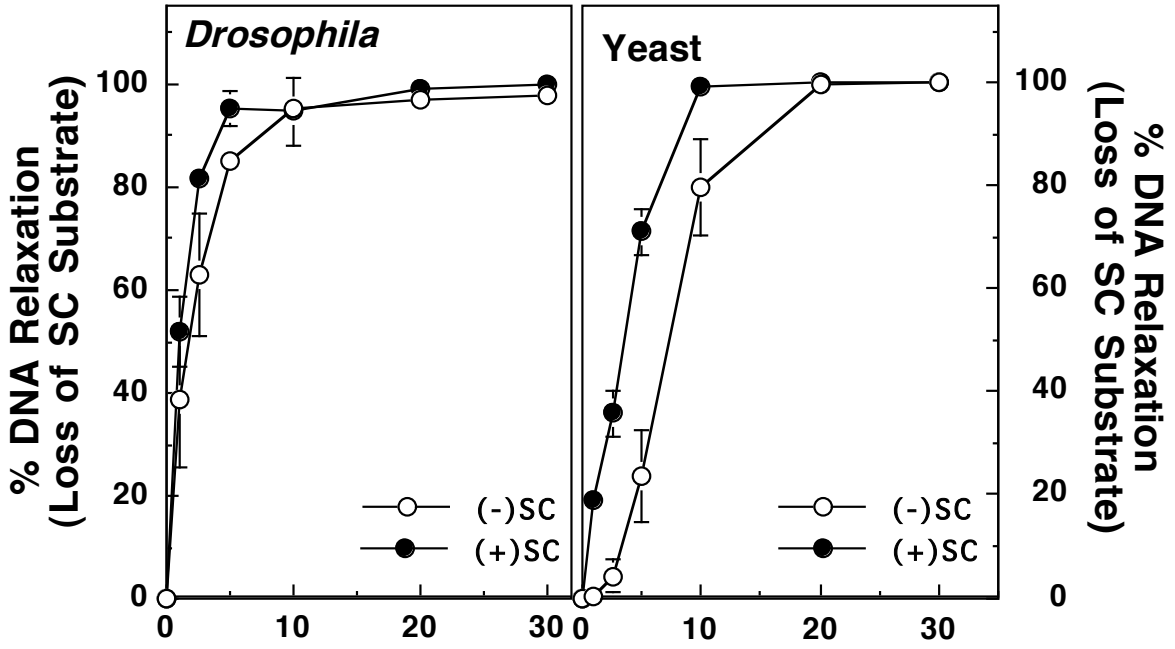

Figure 19. *Drosophila* and yeast topoisomerase II display similar relaxation rates with negatively and positively supercoiled DNA. Time courses for relaxation of negatively supercoiled [(-) SC] or positively supercoiled [(+) SC] pBR322 plasmid DNA by *Drosophila* topoisomerase II (*left panel*) or yeast topoisomerase II (*right panel*) are shownDNA relaxation was quantified from either the loss of supercoiled substrate. Error bars represent the standard deviation or standard error of the mean of three or two independent assays, respectively.

extended (or is weakly extended) to eukaryotic organisms that contain only a single isoform of topoisomerase II. However, it may be related to the distinct separation of physiological functions of topoisomerase IIα and β in vertebrate species.

Human topoisomerase IIα and *E. coli* topoisomerase IV (213,214) both preferentially relax positively supercoiled DNA. Although neither enzyme displays an increased affinity for positive DNA supercoils, they exhibit different attributes with overwound molecules with regard to DNA cleavage. Whereas topoisomerase IIα maintains lower levels of cleavage complexes with positively supercoiled substrates, topoisomerase IV displays higher amounts of DNA scission (213). Thus, there appears to be at least one fundamental difference between the modalities these two enzymes use to distinguish the handedness of superhelical twists.

In summary, human topoisomerase IIα preferentially removes positive superhelical twists from DNA. This finding suggests that positively supercoiled DNA is the preferred physiological substrate for this enzyme and implies that topoisomerase IIα plays a role in relieving torsional stress that accumulates in front of the replication machinery or other DNA tracking systems. The high rates of DNA relaxation observed with positively supercoiled substrates, coupled with the low levels of DNA cleavage, makes topoisomerase IIα ideally suited to function ahead of replication forks.

CHAPTER IV

GEOMETRY OF DNA SUPERCOILS MODULATES TOPOISOMERASE-MEDIATED DNA CLEAVAGE AND ENZYME RESPONSE TO ANTICANCER DRUGS

Introduction

Humans encode three different type I topoisomerases, topoisomerase I, IIIα, and IIIβ (17,234-237). Of these enzymes, only topoisomerase I currently is exploited for cancer chemotherapy (17,19,26). This enzyme is the target for an emerging class of drugs based on the parent compound, camptothecin (17,19,26,27). One important aspect of topoisomerase I-targeted agents is that they are active against a spectrum of cancers that currently have few, if any, other treatment options.

Type II topoisomerases are targets for a number of well-established chemotherapeutic agents (15-19,90-92). One of these drugs, etoposide, has been used in the clinic since the 1960's (91,92,94). It is prescribed as treatment for a wide spectrum of leukemias, lymphomas, and solid tumors. The relative contributions of topoisomerase IIα and β to therapeutic outcomes are not clear at the present time. However, drug interactions with topoisomerase IIβ in differentiated tissues, such as heart, are believed to contribute to the dose-limiting toxicity of some agents (95-98).

Although topoisomerase-targeted anticancer drugs come from several different structural classes, they all act by increasing levels of covalent topoisomerase-cleaved DNA complexes (*i.e.*, cleavage complexes) that are

requisite intermediates in the catalytic cycles of these enzymes (15,16,19,89,90). Cleavage complexes are transient in nature. However, they are converted to permanent DNA strand breaks when nucleic acid tracking systems, such as replication or transcription complexes, attempt to traverse these covalent topoisomerase-DNA roadblocks in the genetic material (15,16,19,89,90). The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple recombination/repair pathways (15,16,76-78). If the accumulation of strand breaks becomes overwhelming, they trigger cell death pathways (77).

Despite the importance of DNA tracking enzymes to drug efficacy, relationships between the geometry of DNA supercoils and the activity of topoisomerase-targeted anticancer agents have not been characterized. Thus, the ability of drugs to induce DNA cleavage mediated by human topoisomerase IIα, topoisomerase IIβ, and topoisomerase I was assessed with positively supercoiled substrates. Results indicate that the geometry of DNA supercoils can significantly diminish or enhance the actions of anticancer drugs in an enzyme-dependent manner. Additionally, results provide an explanation for the differential effects of intercalative and non-intercalative drugs on DNA scission mediated by type II topoisomerases.

Results

Effects of DNA Supercoil Geometry on Drug-induced DNA Cleavage Mediated by Human Topoisomerase IIα.

Collisions with DNA tracking systems are critical for the conversion of transient topoisomerase-DNA cleavage complexes to permanent strand breaks (15,16,19,89,90). Since the double helix is acutely overwound immediately ahead of tracking systems (3,5-7,11,238-240), cleavage complexes most likely to produce permanent strand breaks should be formed between topoisomerases and positively supercoiled DNA. Unfortunately, relatively little is known about the interaction between these enzymes and positively supercoiled substrates.

Data in chapter III demonstrated that human topoisomerase IIα maintains a lower concentration of DNA cleavage complexes with overwound than with underwound substrates. Levels of DNA cleavage mediated by topoisomerase IIα in the presence of either Mg^{2+} or Ca^{2+} are ~3– to 4–fold lower with positively supercoiled DNA (Figures 17 and 20). The decreased baseline levels of DNA cleavage observed with overwound DNA may reflect, at least in part, somewhat lower binding affinity (~2–fold) that topoisomerase IIα displays for positively over negatively supercoiled molecules (Figure 15). The lower level of cleavage notwithstanding, the site specificity of DNA scission is not affected by the handedness of the substrate (Figures 17 and 20, *right panels*).

Many anticancer drugs kill cells by increasing levels of double-stranded DNA breaks generated by topoisomerase II (15,16,19,89,90). As a first step towards characterizing the effects of these drugs on enzyme-DNA cleavage complexes formed with positively supercoiled substrates, the ability of etoposide

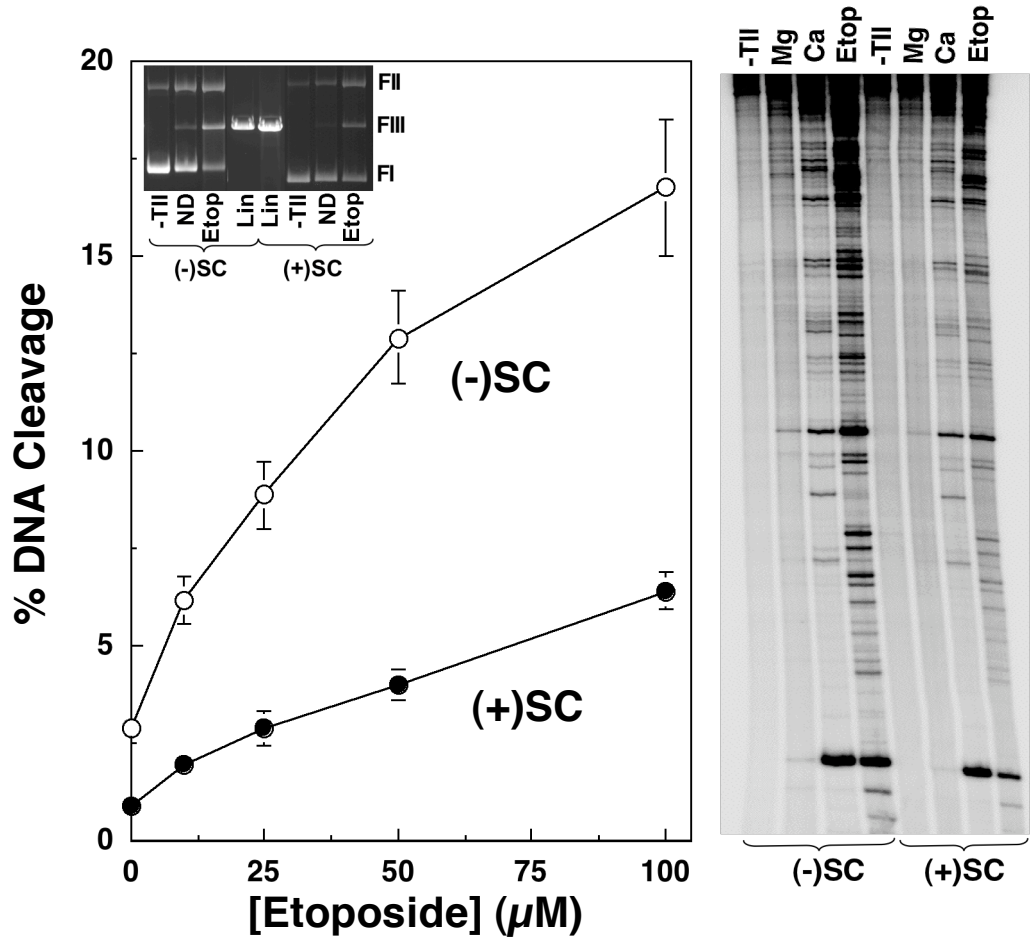

Figure 20. Human topoisomerase IIα maintains lower levels of DNA cleavage complexes with positively supercoiled plasmids in the absence or presence of etoposide. The ability of topoisomerase IIα to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA in the presence of 0–100 μM etoposide is shown (left panel). Assays employed Mg^{2+} as the divalent cation. Error bars represent the standard deviation of four independent assays. The inset shows a representative ethidium bromide-stained agarose gel of DNA cleavage assays with negatively and positively supercoiled plasmids in the absence of topoisomerase IIα (-TII) and in the absence (no drug, ND) or presence (Etop) of etoposide. Linear DNA standards (Lin) also are shown. The positions of supercoiled (form I, FI), nicked circular (form II, FII), and linear molecules (form III, FIII) are indicated. DNA sites cleaved by topoisomerase IIα were mapped in negatively or positively supercoiled plasmids (right panel). Products of DNA cleavage assays were linearized and singly-end labeled with [^{32}P]phosphate. The autoradiogram is representative of three independent assays. Reactions were carried out in the presence of 0 or 100 μM etoposide, and utilized either Mg^{2+} or Ca^{2+} as the divalent cation. DNA from reactions that lacked topoisomerase IIα (-TII) are shown.

to induce DNA cleavage by human topoisomerase IIα was assessed. Etoposide was chosen for initial experiments because its mechanism of action is well defined (15,16,94,115,116,119,241). In addition, the drug is not intercalative in nature and displays little, if any, affinity for DNA (15,16,94,241,242). Over a range of drug concentrations, DNA cleavage induced by etoposide was ~3– to 4–fold lower with positively as compared to negatively supercoiled molecules (Figure 20). Moreover, as observed in the absence of drug, the geometry of DNA supercoils did not affect the site specificity of etoposide-induced scission (Figure 20, *right panel*).

Etoposide increases levels of topoisomerase II-DNA cleavage complexes primarily by inhibiting the ligation of cleaved strands (15,16,93,114,115). Therefore, the effects of etoposide on the ability of topoisomerase IIα to ligate positively or negatively supercoiled molecules were determined. As seen in Figure 21, the handedness of superhelical twists had little impact on rates of DNA ligation in either the presence or absence of etoposide. Thus, the decreased levels of DNA strand breaks induced by etoposide in positively supercoiled substrates do not result from a decreased inhibition of ligation.

Despite the lower absolute percent DNA cleaved with positively supercoiled substrates, the relative enhancement of scission by etoposide with overwound and underwound substrates was similar. For example, at 50 μM drug, this enhancement was ~4.5–fold and ~3.6-fold for positively and negatively supercoiled DNA, respectively (Figures 20 and 22, Table 1). These data yield a "superhelical specificity" for etoposide (*i.e.*, relative cleavage enhancement observed with positively supercoiled DNA divided by the relative cleavage enhancement with negatively supercoiled DNA) of ~1.3 (Table 1). It should be

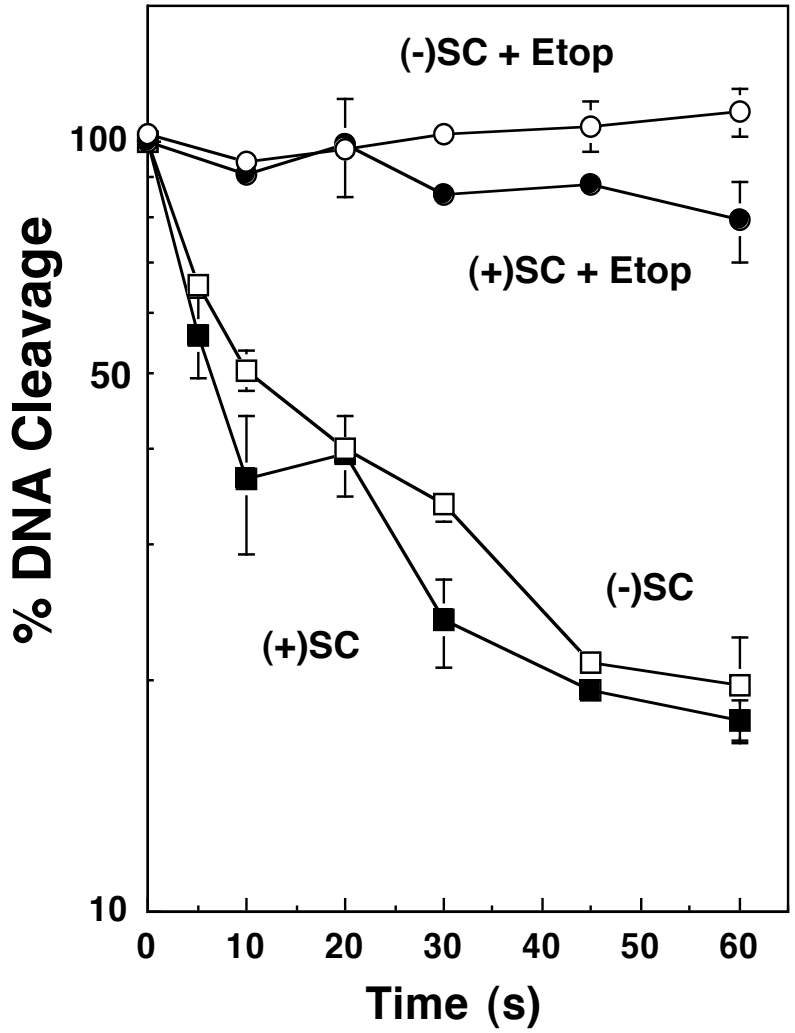

Figure 21. Etoposide inhibits the ligation of negatively or positively supercoiled DNA by human topoisomerase IIα. A time course of ligation in the presence of negatively [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid is shown. DNA ligation was monitored in the absence (boxes) or presence (circles) of 100 μM etoposide (+Etop). The initial level of DNA cleavage was set to 100% and the rate of ligation was determined by quantifying the loss of the cleaved DNA over time. Error bars represent the standard deviation of three independent assays.

noted that the superhelical specificity for etoposide varied little (1.0–1.3) over the entire range of drug concentration examined. These correlations, together with the ligation and site-specificity data, imply that interactions between etoposide and topoisomerase IIα are not altered significantly by the handedness of DNA. Rather, the decrease in drug-induced cleavage of positively supercoiled DNA reflects the reduced baseline (*i.e.*, non-drug) level of enzyme-mediated scission with this substrate.

In light of this finding, the influence of DNA geometry on the actions of other topoisomerase II-targeted drugs, including, TOP-53, genistein, CP-115,953, TAS-103 and amsacrine, was characterized. These drugs are described in detail in chapter I of this dissertation.

All of the above compounds increased DNA cleavage mediated by human topoisomerase IIα. Data for 50 μM drugs are shown in Figure 22 and Table 1. The only exception is CP-115,953, for which data are shown for 5 μM drug (higher drug concentrations were not used because they induced multiple DNA cleavage events per plasmid). As found for etoposide, most induced higher absolute levels of scission with negatively supercoiled substrates, but displayed greater relative cleavage enhancement with positively supercoiled DNA. The non-intercalative drugs displayed superhelical specificities between 1.3 and 1.8. These values remained constant from the lowest drug concentrations examined (5 or 10 μM) up to concentrations that induced multiple DNA cleavage events per plasmid (50 or 100 μM). It was not possible to determine superhelical specificities for CP-115,953 above 5 μM due to the reason discussed above. Taken together, these results suggest that the superhelical specificity for DNA cleavage with non-intercalative topoisomerase II poisons is independent of drug concentration.

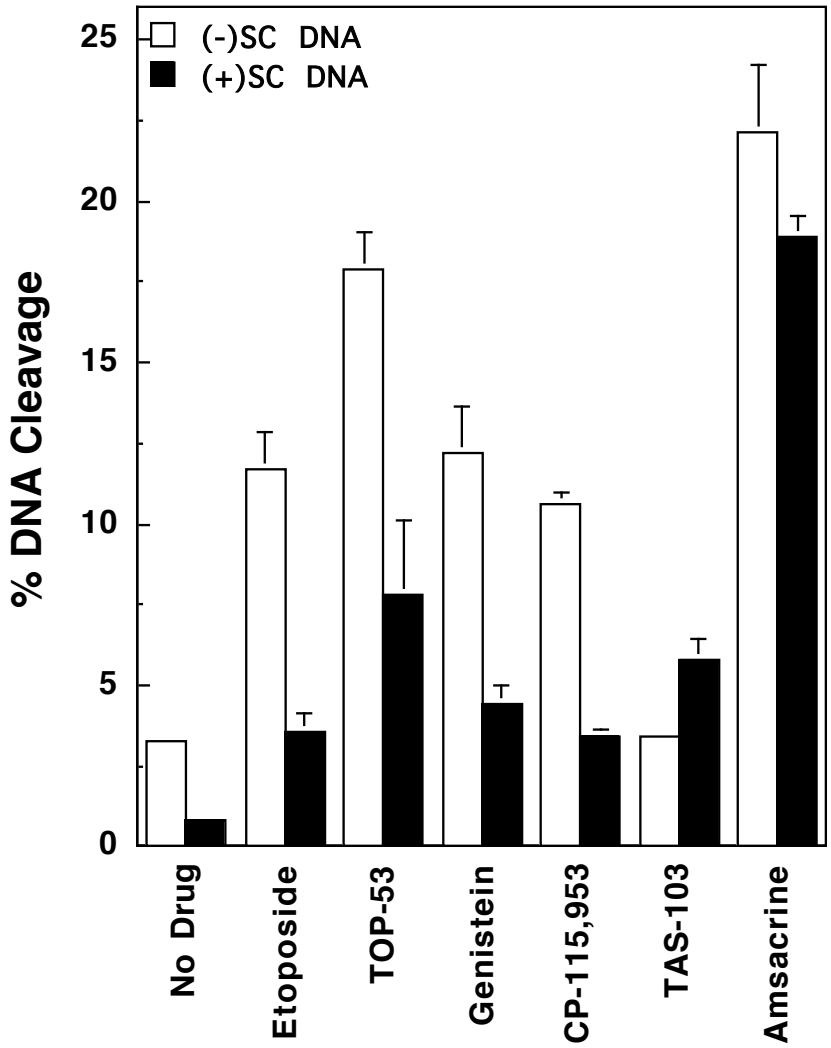

Figure 22. Effects of DNA superhelical geometry on drug-induced DNA cleavage mediated by human topoisomerase IIα. The ability of topoisomerase IIα to cleave negatively [(-)SC DNA, open bars] or positively [(+)SC DNA, closed bars] supercoiled pBR322 plasmid DNA in the presence of various topoisomerase II-targeted drugs is shown. All drugs were used at a concentration of 50 μM, except for CP-115,953, which was used at 5 μM (multiple DNA cleavage events per plasmid were observed at higher concentrations of CP-115,953). Error bars represent the standard deviation of 3 or 4 independent assays.

Table 1. Relative DNA cleavage enhancement of human topoisomerase IIα by anticancer agents.

Drug[a]	Relative Cleavage Enhancement (-)SC DNA	Relative Cleavage Enhancement (+)SC DNA	Superhelical Specificity [(+)SC/(-)SC]
Etoposide	3.6 ± 0.4	4.5 ± 0.4	1.3
TOP-53	5.4 ± 0.5	9.8 ± 1.1	1.8
Genistein	3.7 ± 0.4	5.7 ± 0.5	1.5
CP-115,953	3.2 ± 0.2	4.4 ± 0.6	1.4
TAS-103	1.1 ± 0.1	7.8 ± 0.9	7.1
Amsacrine	7.5 ± 0.3	22.6 ± 0.7	3.0

[a]All drugs were at 50 μM, with the exception of CP-115-953, which was at 5 μM (multiple DNA cleavage events per plasmid were observed at higher concentrations of CP-115,953).

The superhelical specificities of the two intercalative drugs (TAS-103 and amsacrine) at 50 μM, 7.1 and 3.0, respectively, were greater than those seen for the non-intercalative compounds. These higher values suggest that DNA geometry may influence the actions of intercalative compounds towards topoisomerase IIα beyond their effects on baseline DNA cleavage mediated by the enzyme.

Drugs that target topoisomerases are believed to work at the enzyme-nucleic acid interface (15,16,119-124). However, intercalative agents have two additional effects on DNA that could impact levels of topoisomerase-mediated scission in a geometry-specific manner. First, since these compounds locally underwind DNA, they induce compensatory unconstrained positive superhelical twists in distal regions of covalently closed circular molecules (125,126). Thus, as the concentration of an intercalating agent increases, a plasmid that is topologically negatively supercoiled would appear to contain positive superhelical twists. As discussed above, baseline levels of DNA cleavage mediated by topoisomerase IIα are lower with positively supercoiled substrates. Consequently, the apparent change in DNA topology induced by intercalation could diminish the ability of a compound to enhance cleavage with underwound substrates. In contrast, the apparent geometry of a positively supercoiled plasmid (which already is overwound) would not change substantially upon addition of an intercalative drug.

Second, the accumulation of drugs in the double helix has the potential to inhibit enzyme binding or activity. Because the generation of positive superhelical twists by DNA intercalation induces torsional stress in the double helix, the ability of covalently closed molecules to absorb these compounds is

limited. Since overwound plasmids are under positive torsional stress even in the absence of drugs, they cannot bind as many intercalative molecules as underwound DNA. Therefore, enzyme activity on positively supercoiled substrates is less likely to be inhibited by the accumulation of bound drug.

Two independent experiments were carried out to determine whether the above effects contribute to the higher superhelical specificity of intercalative agents. The first utilized ethidium bromide, a classical intercalating agent that does not enhance topoisomerase II-mediated DNA scission. When ethidium bromide was included in reaction mixtures, there was a precipitous drop in the ability of human topoisomerase IIα to cleave negatively supercoiled pBR322 (Figure 23). Levels of cleavage decreased ~6–fold at 10 μM ethidium bromide, which corresponds to the concentration at which "full" intercalation was observed (see *inset*). Beyond 10 μM, little additional inhibition was observed. Thus, the decrease in DNA cleavage induced by ethidium bromide correlates with the change in the apparent supercoiled state of the plasmid substrate. Consistent with this conclusion, ethidium bromide had a much smaller effect on DNA cleavage when the initial substrate was positively supercoiled (Figure 23). Furthermore, once the concentration of ethidium bromide exceeded 10 μM, DNA cleavage levels for positively and negatively substrates were virtually identical.

To determine whether ethidium bromide accumulation on the double helix affects the DNA cleavage activity of topoisomerase IIα independent of changes in DNA geometry, a linear substrate was employed (Figure 23, *right panel*). Since linear molecules are not topologically constrained, DNA intercalation does not induce torsional stress or positive supercoiling in these substrates. The addition of 10 μM ethidium bromide only had a minor effect on levels of DNA cleavage

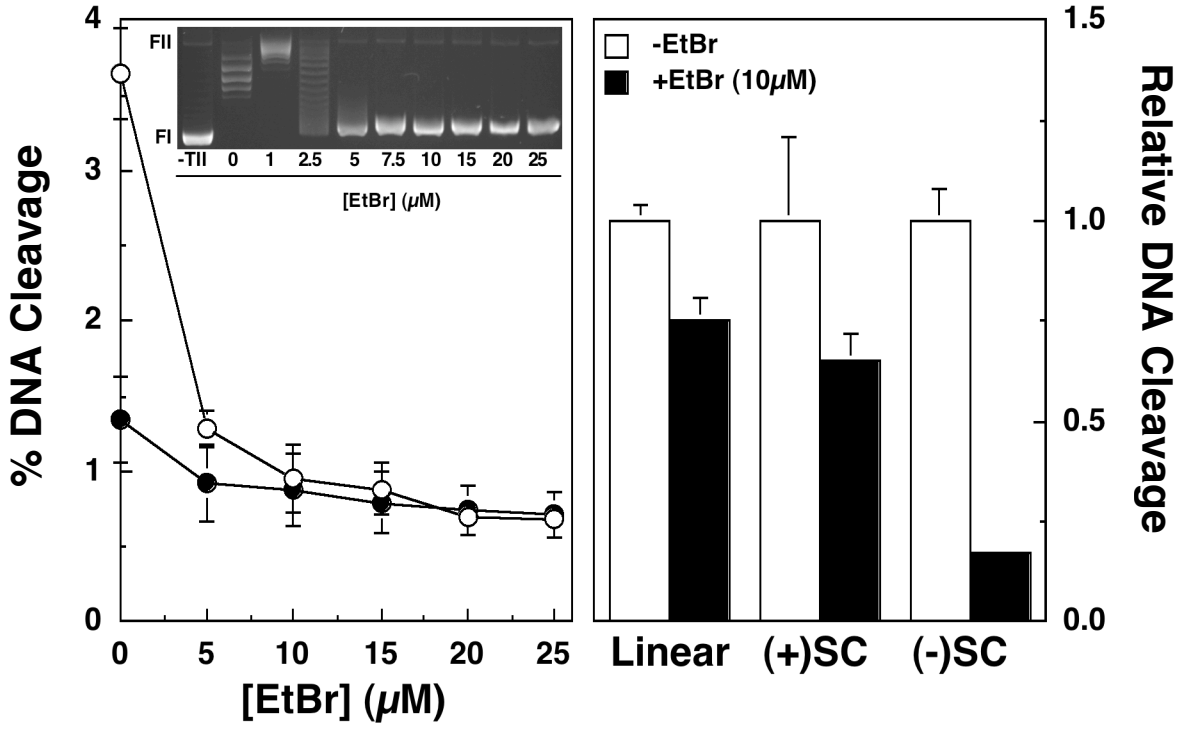

Figure 23. Effects of ethidium bromide intercalation on DNA cleavage mediated by human topoisomerase IIα. The ability of topoisomerase IIα to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA in the presence of 0–25 μM ethidium bromide is shown (left panel). Error bars represent the standard deviation of three independent assays. The inset shows a representative gel of DNA intercalation assays using negatively supercoiled plasmids in the absence of topoisomerase IIα (-TII), or in the absence (0 μM) or presence (1–25 μM) of ethidium bromide (see Experimental Procedures for the interpretation of intercalation assays). The positions of supercoiled (FI) and nicked circular (FII) molecules are indicated. Relative DNA cleavage of linear plasmid, or negatively or positively supercoiled molecules in the absence (-EtBr, open bars) or presence (+EtBr, closed bars) of 10 μM eithdium bromide also is depicted (right panel). Relative DNA cleavage was calculated by normalizing levels of scission in the absence of ethidium bromide to a value of 1.0. Error bars represent the standard deviation of three independent assays for supercoiled substrates, or the standard error of the mean for two independent assays for linear DNA.

with linear pBR322. These results suggest that ethidium bromide inhibits DNA cleavage mediated by human topoisomerase IIα primarily by altering the apparent topology of the DNA substrate and to a lesser extent by accumulation within the double helix.

The second experiment examined the effects of a broad concentration range of amsacrine or TAS-103 on DNA scission mediated by topoisomerase IIα. In contrast to ethidium bromide, these two intercalating drugs are potent topoisomerase II poisons that enhance DNA cleavage (15,112,113). The initial study examined amsacrine. When negatively supercoiled plasmids were employed as the substrate, peak levels of cleavage were observed between 25 and 50 μM drug (Figures 22 and 24, *left panel*). This is the amsacrine concentration range in which changes in DNA topology begin to appear (Figure 24, *left panel inset*). Cleavage levels dropped ~50% between 25 and 250 μM drug and plateaued thereafter (Figure 24, *left panel*). It is notable that "full" intercalation is observed at an amsacrine concentration of ~200 μM (see *inset*). As discussed above, these findings are consistent with the suggestion that the rise in drug-induced DNA cleavage by amsacrine is attenuated by the concomitant fall in baseline scission caused, at least in part, by the apparent change in the geometry of the plasmid substrate.

A different pattern was seen with positively supercoiled substrates. Levels of DNA scission peaked between 100 and 200 μM amsacrine and remained constant at higher drug concentrations (Figure 24, *left panel*). Because of the changes in cleavage enhancement seen with negatively and positively supercoiled substrates, the superhelical specificity of amsacrine-induced DNA cleavage was concentration-dependent. Values were in the range of 1.0–1.5 at drug

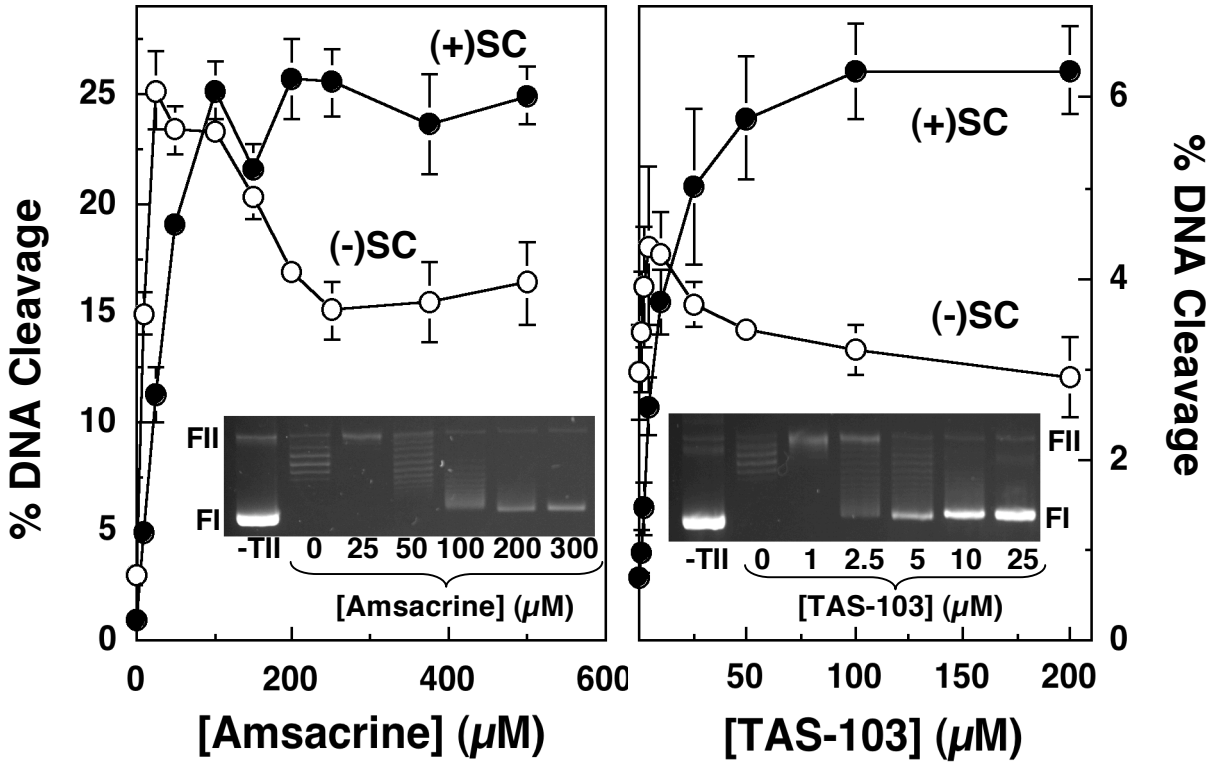

Figure 24. Effects of amsacrine and TAS-103 intercalation on DNA cleavage mediated by human topoisomerase IIα. The ability of topoisomerase IIα to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA in the presence of 0–500 μM amsacrine (left panel) or 0–200 μM TAS-103 (right panel) is shown. Error bars represent the standard deviation of three independent assays. The insets show representative DNA intercalation assays in the absence of topoisomerase IIα (-TII), or in the absence (0 μM) or presence of the respective drug (see Experimental Procedures for the interpretation of intercalation assays). The positions of supercoiled (FI) and nicked circular (FII) molecules are indicated.

concentrations that did not substantially alter the apparent topology of negatively supercoiled substrates (<25 μM), increased to 3.0–3.5 at concentrations that began to affect apparent topology (25–150 μM), and plateaued at ~5.1 at concentrations that induced full intercalation. It is notable that the plateau level of cleavage observed with positively supercoiled DNA was higher than that observed with negatively supercoiled molecules. It is unlikely that amsacrine intercalation could make negatively supercoiled plasmids appear to be more overwound than positively supercoiled molecules at a comparable drug concentration (see Figure 23). Therefore, it is concluded that the preferential accumulation of amsacrine in underwound substrates also contributes to the decrease in DNA cleavage observed with negatively supercoiled molecules at high drug concentrations.

To determine whether the concentration dependence of superhelical specificity is a general feature of intercalative drugs, the ability of TAS-103 to induce DNA cleavage by topoisomerase IIα was examined (Figure 24, *right panel*). Results were similar to those seen with amsacrine. Scission of negatively supercoiled plasmid rose initially, peaked at drug concentrations that began to alter the apparent topology of the substrate, and fell to approximately baseline thereafter. Conversely, scission of positively supercoiled plasmids increased and remained high over the entire range of TAS-103 examined (Figure 24, *right panel*). As a result, the superhelical specificity of TAS-103, like amsacrine, was concentration dependent. Values were 1.2–1.6 at drug concentrations that did not substantially alter the apparent topology of negatively supercoiled substrates (<5 μM), increased to 2.6–3.7 at concentrations that begin to affect apparent topology (5–10 μM), and plateaued at 7.1–9.3 at concentrations that induced full

intercalation. This concentration dependence for superhelical specificity is in sharp contrast to those calculated for non-intercalative compounds and may be a defining distinction between intercalative and non-intercalative topoisomerase II poisons.

Effects of DNA Supercoil Geometry on Drug-induced DNA Cleavage Mediated by Human Topoisomerase IIβ.

In contrast to human topoisomerase IIα, topoisomerase IIβ is not required for DNA replication and does not preferentially remove positive superhelical twists (13,34,38,42,44,65). However, the effects of nucleic acid geometry on DNA cleavage mediated by the β isoform have never been examined. Since topoisomerase IIβ is an important target for anticancer drugs (97) and also appears to mediate some of the toxic effects of these agents in differentiated tissues (95-97), the ability of the enzyme to cleave positively vs. negatively supercoiled substrates in the presence of topoisomerase II-targeted drugs was assessed.

Similar to topoisomerase IIα, topoisomerase IIβ maintains lower (~3– to 4–fold) levels of cleavage complexes with positively supercoiled DNA in the absence of drug (Figures 18 and 25). The enzyme also displayed lower (~2–fold) cleavage activity with positively supercoiled substrates over a range of etoposide concentrations (Figure 25). However, the effects of DNA geometry on drug-induced scission were not as pronounced with topoisomerase IIβ as they were with the α isoform. As seen in Figure 26, the percentage of positively supercoiled molecules cleaved in the presence of several drugs approached or actually exceeded that observed with negatively supercoiled substrates. Consequently,

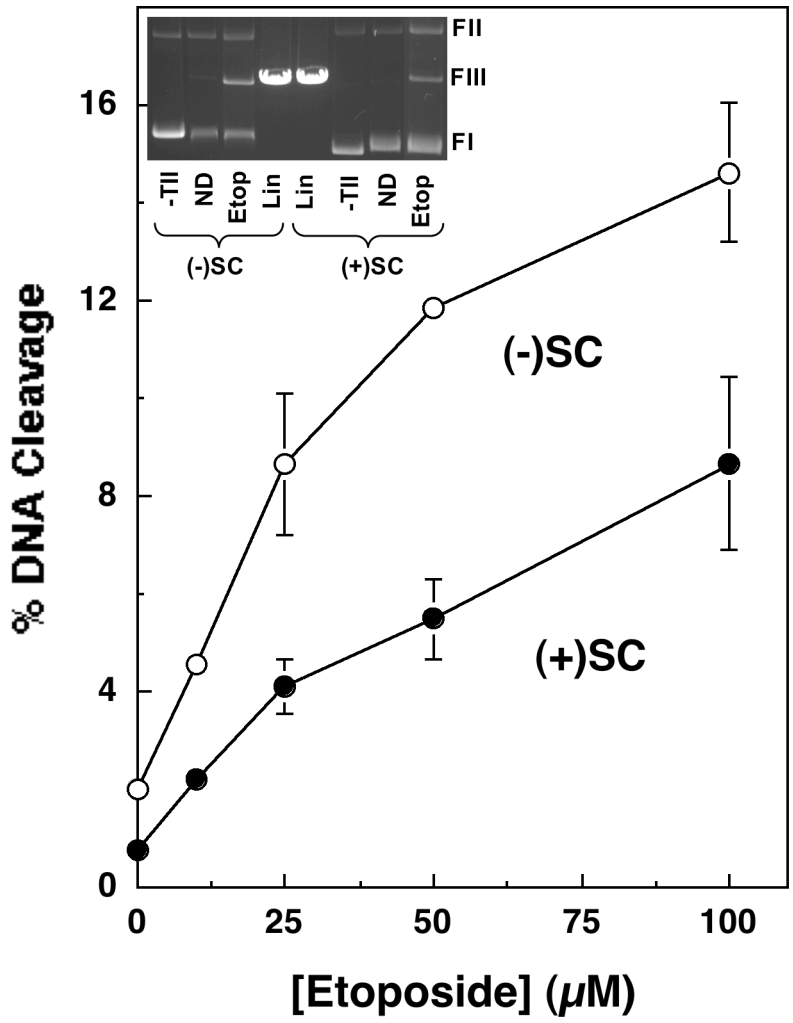

Figure 25. Human topoisomerase IIβ maintains lower levels of DNA cleavage complexes with positively supercoiled plasmids in the presence of etoposide. The ability of topoisomerase IIβ to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA in the presence of 0–100 μM etoposide is shown. Error bars represent the standard deviation of three independent assays. The inset shows a representative ethidium bromide-stained agarose gel of DNA cleavage assays with negatively and positively supercoiled plasmids in the absence of topoisomerase IIβ (-TII) and in the absence (no drug, ND) or presence (Etop) of etoposide. Linear DNA standards (Lin) also are shown. The positions of supercoiled (FI), nicked circular (FII), and linear molecules (FIII) are indicated.

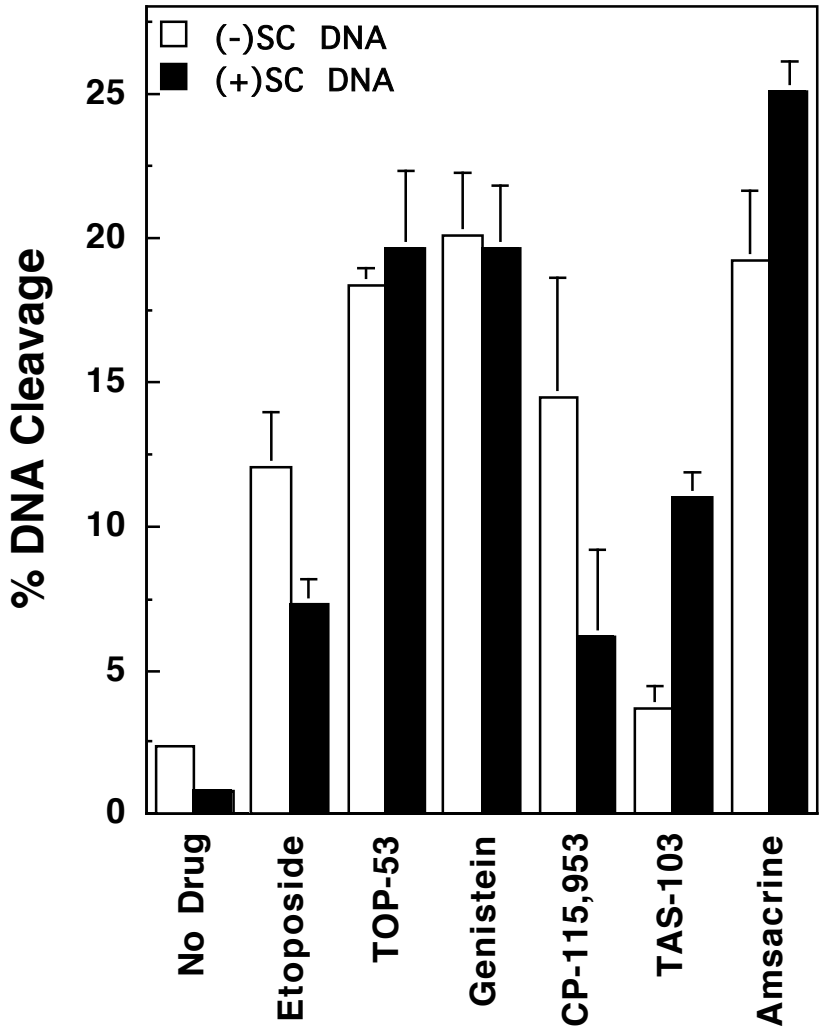

Figure 26. Effects of DNA superhelical geometry on drug-induced DNA cleavage mediated by human topoisomerase IIβ. The ability of topoisomerase IIβ to cleave negatively [(-)SC DNA, open bars] or positively [(+)SC DNA, closed bars] supercoiled pBR322 plasmid DNA in the presence of various topoisomerase II-targeted drugs is shown. All drugs were used at a concentration of 50 μM, except for CP-115,953, which was used at 5 μM (multiple DNA cleavage events per plasmid were observed at higher concentrations of CP-115,953). Error bars represent the standard deviation of 3 or 4 independent assays.

the calculated superhelical specificities for most drugs were somewhat higher for topoisomerase IIβ than they were for topoisomerase IIα (Table 2). Once again, the superhelical specificity of the non-intercalative compounds was concentration-independent and remained constant at all drug concentrations examined. Furthermore, the highest superhelical specificities were observed for the two intercalative drugs, TAS-103 and amsacrine.

To further analyze this finding, the effects of amsacrine and TAS-103 on DNA scission mediated by topoisomerase IIβ were examined over a broad concentration range (data not shown). As found with the α isoform, levels of negatively supercoiled DNA cleaved by topoisomerase IIβ peaked at drug concentrations at which changes in apparent topology were obvious, dropped, and plateaued at concentrations that induced full intercalation. In contrast, levels of cleavage with positively supercoiled substrates increased and remained high over the entire drug ranges examined. Thus, as discussed above for topoisomerase IIα, the superhelical specificities for amsacrine and TAS-103 were concentration-dependent with topoisomerase IIβ, and rose from initial values of 1.2 and 1.7, respectively, at low concentrations, to 8.3 and 9.1, respectively, at high concentrations. These results further suggest that drug-induced cleavage of underwound substrates by topoisomerase IIβ is attenuated by the ability of intercalative agents to change the apparent geometry of DNA and by increased drug accumulation on negatively supercoiled molecules.

Table 2. Relative DNA cleavage enhancement of human topoisomerase IIβ by anticancer agents.

Drug[a]	Relative Cleavage Enhancement (-)SC DNA	Relative Cleavage Enhancement (+)SC DNA	Superhelical Specificity [(+)SC/(-)SC]
Etoposide	4.9 ± 0.3	8.2 ± 0.5	1.6
TOP-53	7.9 ± 0.6	20.9 ± 0.9	2.7
Genistein	8.5 ± 0.2	22.0 ± 2.1	2.6
CP-115,953	5.6 ± 1.8	10.4 ± 4.8	1.9
TAS-103	1.7 ± 0.3	12.6 ± 1.3	7.3
Amsacrine	7.8 ± 1.1	31.7 ± 4.7	4.1

[a]All drugs were at 50 μM, with the exception of CP-115-953, which was at 5 μM (multiple DNA cleavage events per plasmid were observed at higher concentrations of CP-115,953).

Effects of DNA Supercoil Geometry on Drug-induced DNA Cleavage Mediated by Human Topoisomerase I.

Topoisomerase I is an important target for several new anticancer drugs that are based on camptothecin, a naturally occurring non-intercalative compound found in the bark of the Chinese tree *Camptotheca acuminata* (17,19,26-28). These drugs kill cells by increasing levels of single-stranded DNA breaks (*i.e.*, nicks) that are generated by the type I enzyme (17,19,89). Since one of the major functions of topoisomerase I is to alleviate torsional stress that builds up ahead of replication forks and transcription complexes (13,14,19), the effects of nucleic acid geometry on enzyme-mediated DNA cleavage was examined in the absence or presence of camptothecin (Figure 27).

In sharp contrast to the type II enzymes, human topoisomerase I maintained a higher level of cleavage intermediates with overwound DNA. Three times more nicked molecules were generated with positively supercoiled plasmids than were observed with negatively supercoiled substrates (see bar graph inset). A far more striking effect of DNA geometry on topoisomerase I was seen in the presence of camptothecin (Figure 27). Over a concentration range of 1–25 μM drug, the enzyme generated dramatically higher levels of nicked DNA with positively supercoiled substrates. For example, at 1 μM camptothecin, ~16 times more DNA cleavage was observed with positively as compared to negatively supercoiled pBR322, yielding a superhelical specificity of 4.7. Together with the findings for the type II enzymes, these results demonstrate that the handedness of DNA supercoils has a profound influence on DNA cleavage reactions mediated by human topoisomerases.

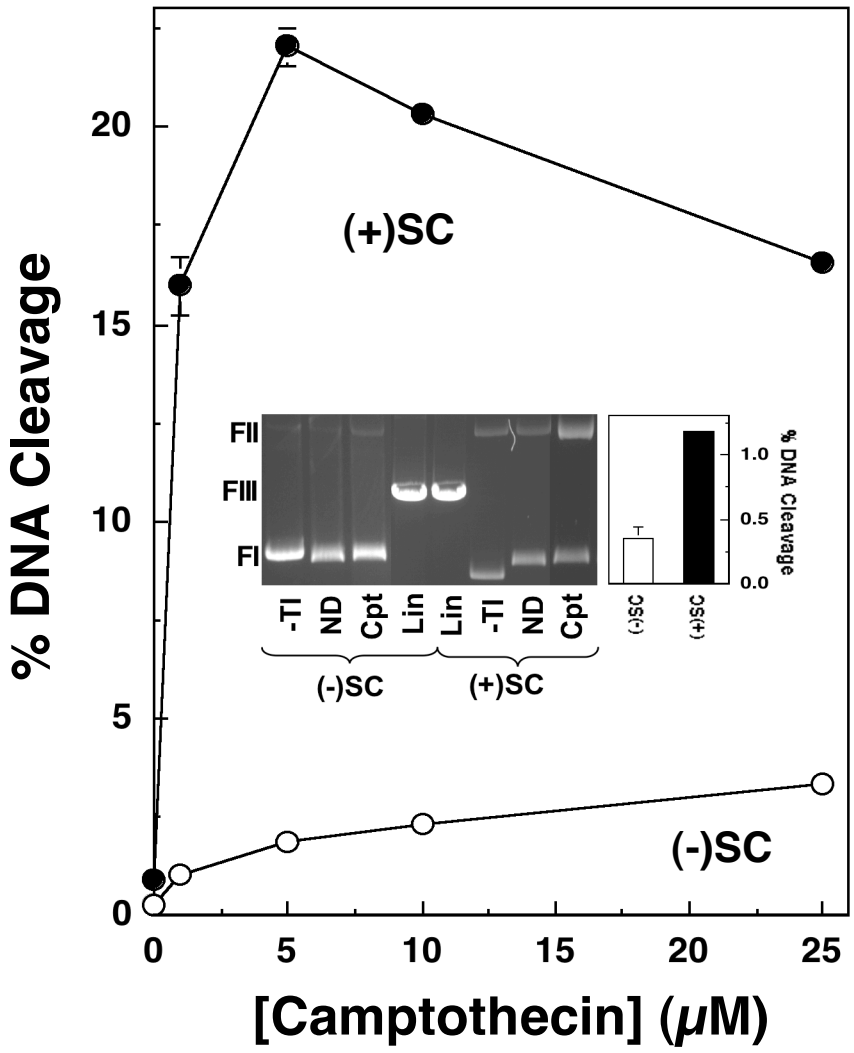

Figure 27. Human topoisomerase I maintains higher levels of DNA cleavage complexes with positively supercoiled plasmids in the absence or presence of camptothecin. The ability of topoisomerase I to cleave negatively [(-)SC, open circles] or positively [(+)SC, closed circles] supercoiled pBR322 plasmid DNA in the presence of 0–25 μM campthothecin is shown. Error bars represent the standard deviation of three independent assays. The inset shows a representative ethidium bromide-stained agarose gel of DNA cleavage assays with negatively and positively supercoiled plasmids in the absence of topoisomerase I (-TI) and in the absence (no drug, ND) or presence (Cpt) of 5 μM camptothecin. Linear DNA standards (Lin) also are shown. The positions of supercoiled (FI), nicked circular (FII), and linear molecules (FIII) are indicated. The inset also shows a bar graph highlighting topoisomerase I-mediated DNA cleavage of negatively (open bar) and positively (closed bar) supercoiled DNA in the absence of drug.

Discussion

Beyond their critical physiological functions, topoisomerases are targets for a number of important anticancer drugs (15-19,91-94). While these agents all increase the concentration of topoisomerase-generated breaks in the genetic material (15,16,19,89,90), their ability to kill cells requires the actions of DNA tracking systems, such as replication or transcription complexes (15,16,19,89,90). Collisions between tracking systems and cleavage complexes convert these transient enzyme intermediates to permanent DNA strand breaks either directly, or by the induction of recombination (15,16,76-78). It is the accumulation of these permanent strand breaks that ultimately triggers cell death pathways (77).

Previous studies on the interaction of anticancer drugs and topoisomerases have used negatively supercoiled or linear DNA as cleavage substrates. However, the movement of enzymes through the double helix leads to the formation of overwound DNA ahead of tracking systems (3,5-7,11,238-240). As a result, the cleavage complexes most likely to produce permanent DNA strand breaks should form between topoisomerases and positively supercoiled DNA. Therefore, the ability of human topoisomerase IIα and β, and topoisomerase I to cleave positively supercoiled molecules was assessed in the absence or presence of anticancer drugs.

As discussed in chapter III of this dissertation, topoisomerase IIα and β both maintain ~3– to 4–fold lower levels of cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules. This decrease in nucleic acid scission may benefit the cell, because it makes it less likely that the

actions of a DNA tracking system will generate permanent topoisomerase II-associated strand breaks during normal cellular processes.

Topoisomerase IIα also maintains lower concentrations of drug-induced cleavage intermediates with positively supercoiled substrates. With the non-intercalative agents examined, the relative enhancement of DNA scission seen with overwound DNA is similar to that observed with underwound molecules. Furthermore, the superhelical specificity for DNA cleavage appears to be independent of drug concentration. Consequently, it is proposed that decreased drug efficacy is due primarily to a drop in baseline levels of cleavage mediated by topoisomerase IIα, rather than an altered interaction with the enzyme-DNA complex.

Results were somewhat different for topoisomerase IIβ. Whereas some non-intercalative drugs such as etoposide and CP-115,953, follow trends similar to those seen for the α isoform, others, such as genistein and TOP-53, induced equivalent levels of cleavage with positively and negatively supercoiled plasmids. The underlying reason for the increased drug effect with positively supercoiled DNA is not known. However, this finding suggests that in some cases, drug-induced stimulation of DNA cleavage by topoisomerase IIβ is more likely to generate permanent strand breaks than would topoisomerase IIα under comparable circumstances. This difference notwithstanding, the superhelical specificity for DNA cleavage with non-intercalative drugs was once again concentration-independent.

Consistently, with both topoisomerase IIα and β, intercalative drugs displayed higher relative cleavage enhancement in the presence of positively rather than negatively supercoiled DNA. This superhelical specificity did not

correlate with a greater intrinsic drug activity with overwound substrates. Rather, it appears to result from the apparent positive supercoiling of underwound molecules and the preferential accumulation of intercalative drugs in negatively supercoiled substrates. At higher drug concentrations, these effects attenuate the stimulation of topoisomerase II-mediated DNA cleavage of negatively supercoiled molecules and result in an increased superhelical specificity. As a result, they probably are responsible for the characteristic "bell-shaped curve" observed for the enhancement of scission by intercalative anticancer drugs. In contrast, DNA cleavage by topoisomerase II rises and plateaus with positively supercoiled substrates. This finding implies that intercalative agents are able to maintain their effectiveness ahead of DNA tracking systems, even at high drug concentrations.

Results with topoisomerase I were unexpected. Despite the fact that this enzyme characteristically functions to alleviate overwinding ahead of DNA tracking systems (14,17,19), it maintains ~3 times higher levels of cleavage complexes with positively supercoiled substrates. Thus, under normal physiological circumstances, topoisomerase I is inherently more likely to trigger the formation of permanent DNA strand breaks than either type II isoform. The cellular ramifications of this enzyme feature are unclear at the present time.

The influence of DNA topology on topoisomerase I-mediated scission is even more dramatic in the presence of camptothecin. This finding suggests that topoisomerase I may be an intrinsically more lethal target for anticancer drugs than is topoisomerase IIα or β.

In summary, numerous factors influence the effectiveness of topoisomerase-targeted anticancer drugs, including the concentration, localization, and roles

played by each enzyme in specific nucleic acid processes. Results of the present study indicate that the topological state of the genetic material also has a profound influence on topoisomerase-mediated DNA cleavage and the response of topoisomerase IIα, topoisomerase IIβ, and topoisomerase I to anticancer drugs. Although topoisomerase IIα is the enzyme that is most frequently targeted by chemotherapeutic regimens, it may actually be the topoisomerase that is least likely to generate cleavage complexes that are converted to permanent DNA strand breaks in treated cells. Alternatively, all other things being equal, topoisomerase I appears to be the enzyme most likely to fragment the genome. This may be one of the reasons why camptothecin-based drugs display a spectrum of activity that exceeds those of other established anticancer agents.

CHAPTER V

BIOMODAL RECOGNITION OF DNA SUPERCOIL GEOMETRY BY TYPE II TOPOISOMERASES

Introduction

Recently, it was demonstrated that some type II enzymes are capable of discerning the geometry of DNA supercoils. Human topoisomerase IIα (chapter III, Figure 12) and *E. coli* topoisomerase IV (213,214) both relax positive superhelical twists more than an order of magnitude faster than they do negative superhelical twists. In contrast, yeast and *Drosophila* topoisomerase II (chapter III, Figure 19), and human topoisomerase IIβ (chapter III, Figure 14) relax negatively and positively supercoiled plasmids at similar rates (217). Although type II topoisomerases possess a high degree of amino acid sequence identity in their N-terminal and central homology domains (GyrB and GyrA domains, respectively), these enzymes diverge considerably in their C-terminal domains (14,15,18,243,244). Based on these amino acid sequence differences, it was suggested in chapter III of this dissertation that the ability of human topoisomerase IIα to preferentially relax positive DNA supercoils resides in the C-terminal domain of the protein. Structural and modeling studies of topoisomerase IV and DNA gyrase, coupled with nucleic acid binding experiments, have led to similar conclusions for the bacterial type II enzymes (62-64).

In addition to their ability to distinguish supercoil geometry during DNA relaxation, type II topoisomerases also discern the handedness of supercoils

during the DNA cleavage event (chapter III, Figures 17 and 18). Both topoisomerase IIα and β maintain lower levels (3– to 4–fold) of cleavage intermediates when positively supercoiled substrates are employed. Because the human isoforms display different results in DNA relaxation and cleavage assays, the type II enzyme must utilize distinct mechanisms to distinguish DNA geometry during these two processes.

To more fully assess the recognition of DNA geometry by type II topoisomerases, two groups of enzymes were studied (Figure 28 and 29). The first group consisted of type II topoisomerases that lack the C-terminal protein domain. Two of these enzymes are encoded by *Paramecium bursaria* chlorella virus-1 (PBCV-1) and chlorella virus Marburg-1 (CVM-1) (201-204). They are homologous to human topoisomerase IIα, but naturally lack the C-terminal domain (Figure 28). Chlorella virus type II topoisomerases display high levels of DNA cleavage, but in other respects are similar to eukaryotic topoisomerase II. The third enzyme in this group, hTop2αΔ1175, is a deletion mutant of human topoisomerase IIα that is truncated at the amino acid residue that corresponds to the C-terminus of PBCV-1 topoisomerase II (Figure 28) (205). These three truncated enzymes allow for the examination of the recognition of DNA supercoil geometry in the absence of the variable C-terminal domain, which is thought to be involved in modulating DNA relaxation by topoisomerase II.

The second group of enzymes studied consists of two additional mutant enzymes of human topoisomerase IIα in which the C-terminal domains have been switched (Figure 29). Results indicate that topoisomerase II uses elements in the conserved N-terminal or central domains to recognize supercoil geometry

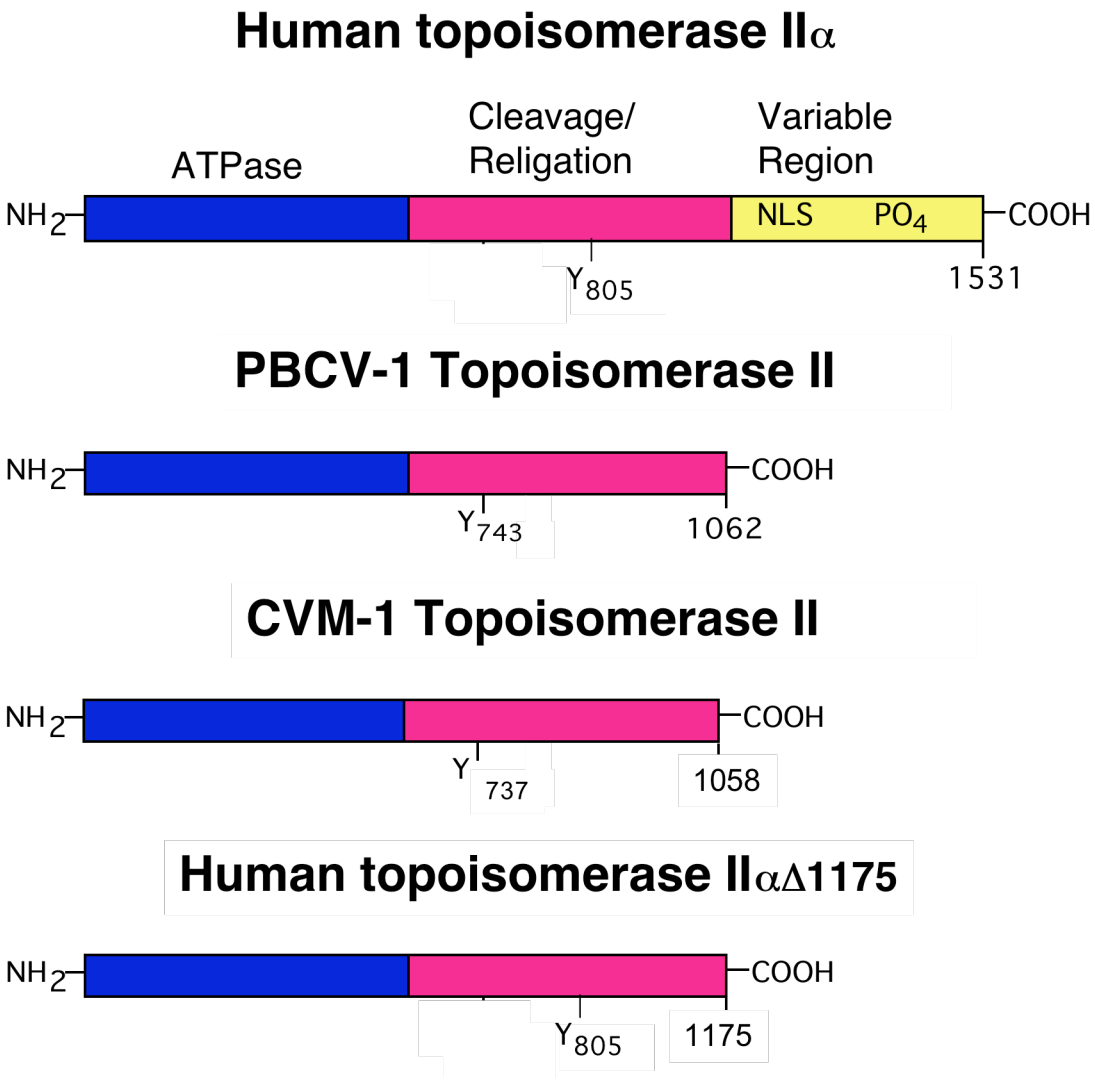

Figure 28. Domain structures of type II topoisomerases lacking the C-terminal variable region. The domain structures of human topoisomerase IIα, PBCV-1 topoisomerase II, CVM-1 topoisomerase II and a C-terminal truncation mutant of human topoisomerase IIα (hTop2αΔ1175) are shown. The human and viral type II enzymes contain similar ATPase (blue) and cleavage/religation domains (pink). The active site tyrosine residues (human: Y805, PBCV-1: Y743, CVM-1: Y737) are indicated. PBCV-1 and CVM-1 topoisomerase II are naturally lacking the variable C-terminal domain of type II topoisomerases (yellow), and human topoisomerase IIαΔ1175 has been truncated at amino acid 1175 (the residue corresponding to the terminal residue of PBCV-1 topoisomerase II).

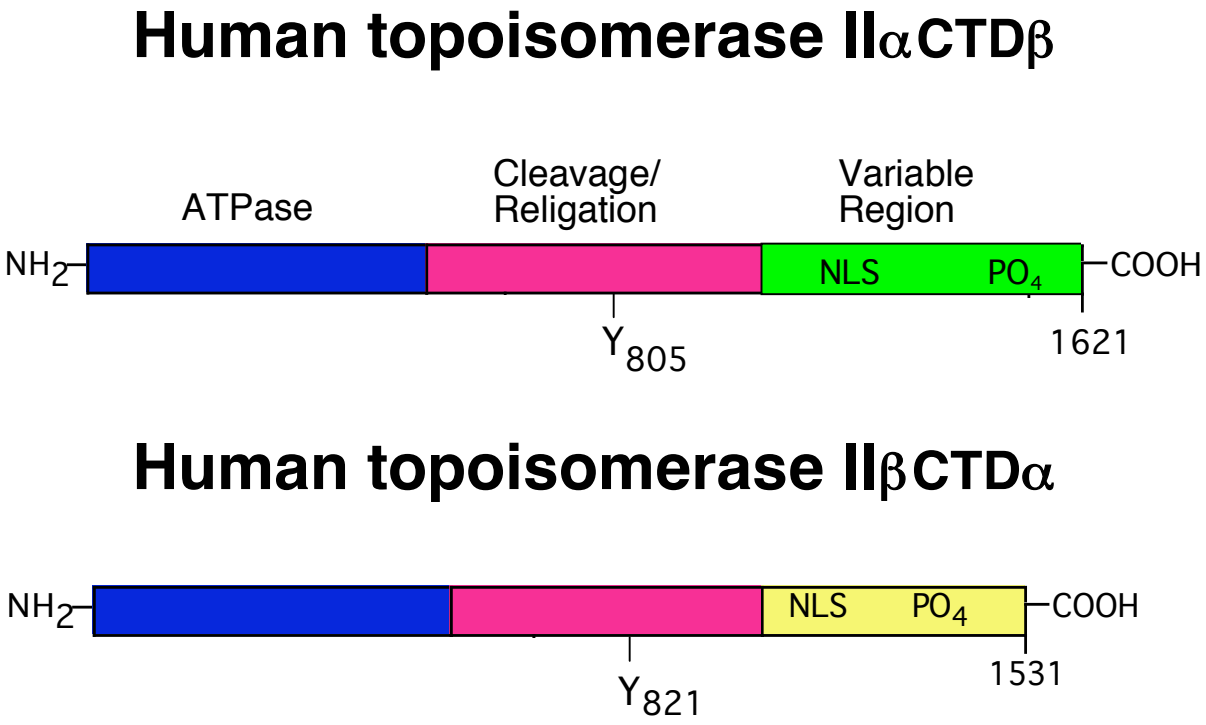

Figure 29. Domain structures of human topoisomerase IIαCTDβ and topoisomerase IIβCTDα. The domain structures of human topoisomerase IIαCTDβ and topoisomerase IIβCTDα are shown. The ATPase (blue) and cleavage/religation domains (pink) of the human isoforms are ~79% homologous. The variable C-terminal domain (yellow) is only ~31% homologous. The active site tyrosine residues (topoisomerase IIα: Y805, topoisomerase IIβ: Y821) are indicated, as well as the amino acid length of each mutant (hTop2αCTDβ: 1621 amino acids, hTop2βCTDα: 1531 amino acids).

during DNA cleavage and elements in the C-terminal domain to sense the handedness of supercoils during DNA relaxation.

Results

Relaxation of Negatively and Positively Supercoiled DNA by Chlorella Virus Type II Topoisomerases

It was proposed in chapter III of this dissertation that the C-terminal domain of topoisomerase IIα plays an important role in sensing DNA geometry during relaxation. As an initial test of this hypothesis, the ability of two viral enzymes, PBCV-1 and CVM-1 topoisomerase II, that naturally lack the C-terminal domain to relax negatively and positively supercoiled DNA was examined. Results of DNA relaxation assays are shown in Figures 30 (PBCV-1) and 31 (CVM-1). Both viral enzymes displayed high rates of DNA relaxation irrespective of the handedness of the initial plasmid substrates. As determined by two methods of quantitation (percent DNA relaxation from the gain of relaxed product or the loss of supercoiled substrate), PBCV-1 and CVM-1 topoisomerase II relaxed negatively and positively supercoiled DNA at similar rates. These findings support the hypothesis that the C-terminal domain of the protein is required for topoisomerase II to sense the handedness of DNA during relaxation.

Cleavage of Negatively and Positively Supercoiled DNA by Chlorella Virus Type II Topoisomerases

As a prerequisite to the strand passage event, topoisomerase II generates a transient double-stranded break in the genetic material (14-16,18,30). In contrast

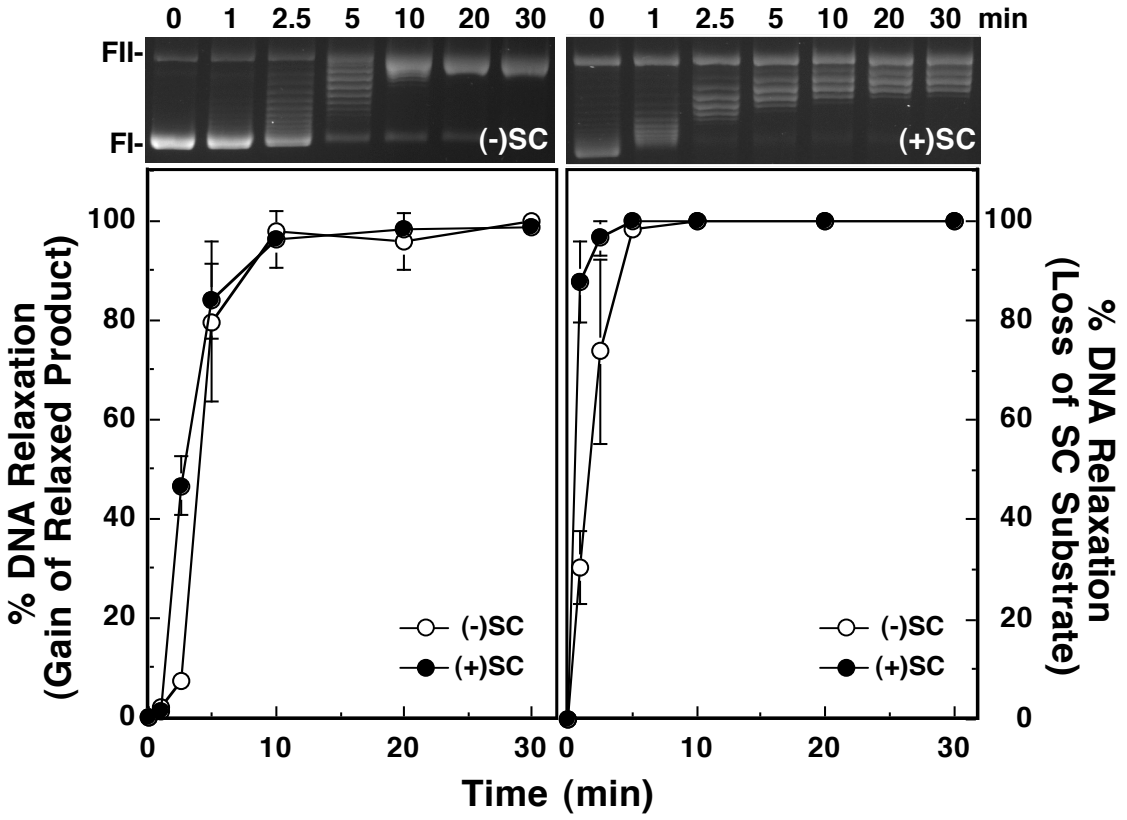

Figure 30. PBCV-1 topoisomerase II relaxes negatively and positively supercoiled plasmid DNA at similar rates. Ethidium bromide-stained gels (top) depict a time course for relaxation of negatively [(-)SC, *left gel*] or positively [(+)SC, *right gel*] supercoiled pBR322 plasmid DNA by PBCV-1 topoisomerase II. The positions of the supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Data for negatively supercoiled substrates are represented by open symbols and positively supercoiled substrates by closed symbols. Error bars represent the standard deviation of three independent experiments. This data was generated by Dr. Jennifer Dickey.

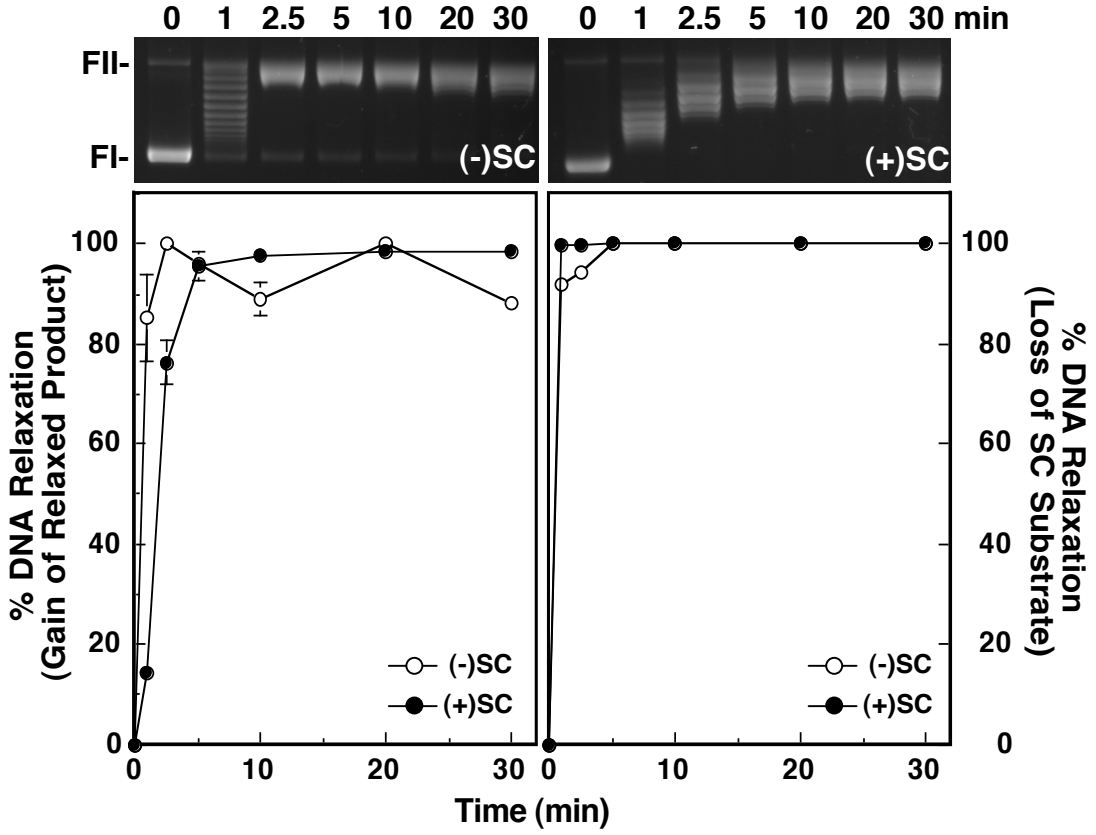

Figure 31. CVM-1 topoisomerase II relaxes negatively and positively supercoiled plasmid DNA at similar rates. Ethidium bromide-stained gels (top) depict a time course for relaxation of negatively [(-)SC, *left gel*] or positively [(+)SC, *right gel*] supercoiled pBR322 plasmid DNA by CVM-1 topoisomerase II. The positions of the supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Data for negatively supercoiled substrates are represented by open symbols and positively supercoiled substrates by closed symbols. Error bars represent the standard deviation of three independent experiments. This data was generated by Dr. Jennifer Dickey.

to results observed for DNA relaxation, both human topoisomerase IIα and β are able to discern the handedness of supercoils during the DNA cleavage event (chapter III, Figures 17 and 18). This finding demonstrates that topoisomerase II employs distinct mechanisms to recognize DNA geometry during different catalytic processes and suggests that the ability to distinguish the handedness of supercoils during the cleavage event resides in the conserved N-terminal or central domains of the enzyme. To address this issue, the effects of DNA under- and overwinding on the cleavage activities of PBCV-1 and CVM-1 topoisomerase II were examined (Figure 32).

Both viral enzymes were able to discern supercoil geometry during the DNA cleavage reaction. As found for the human enzymes, levels of DNA cleavage intermediates generated by PBCV-1 and CVM-1 topoisomerase II were ~2– to 4–fold lower when positively supercoiled plasmids were used as substrates (Figure 32). These results provide strong evidence that the elements that sense the handedness of DNA supercoils during the cleavage reaction of topoisomerase II must reside in the conserved N-terminal or central domain of the protein, rather than the C-terminal domain.

Three additional experiments were carried out to further investigate the recognition of DNA geometry during the cleavage event. First, the effects of DNA handedness on the site specificity of PBCV-1 and CVM-1 topoisomerase II were examined (Figure 33). Each enzyme cut negatively and positively supercoiled substrates at the same sites. However, levels of scission were reduced at all sites in the overwound molecules. Thus, the decrease in cleavage observed with positively supercoiled DNA does not result from a geometry-specific alteration in site specificity.

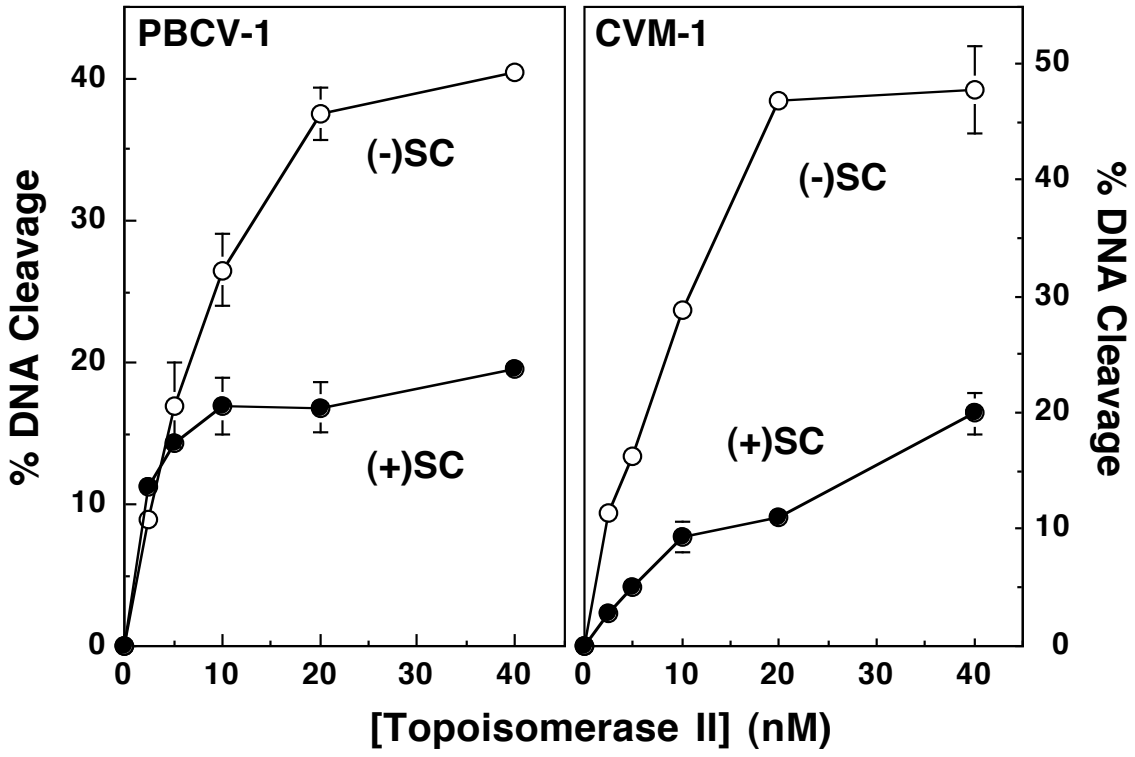

Figure 32. Type II topoisomerases from chlorella viruses maintain lower levels of DNA cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules. The ability of 0-40 nM PBCV-1 (*left panel*) or CVM-1 (*right panel*) topoisomerase II to cleave 10 nM negatively [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid DNA is shown. Error bars represent the standard deviation of three independent experiments. This data was generated by Dr. Jennifer Dickey.

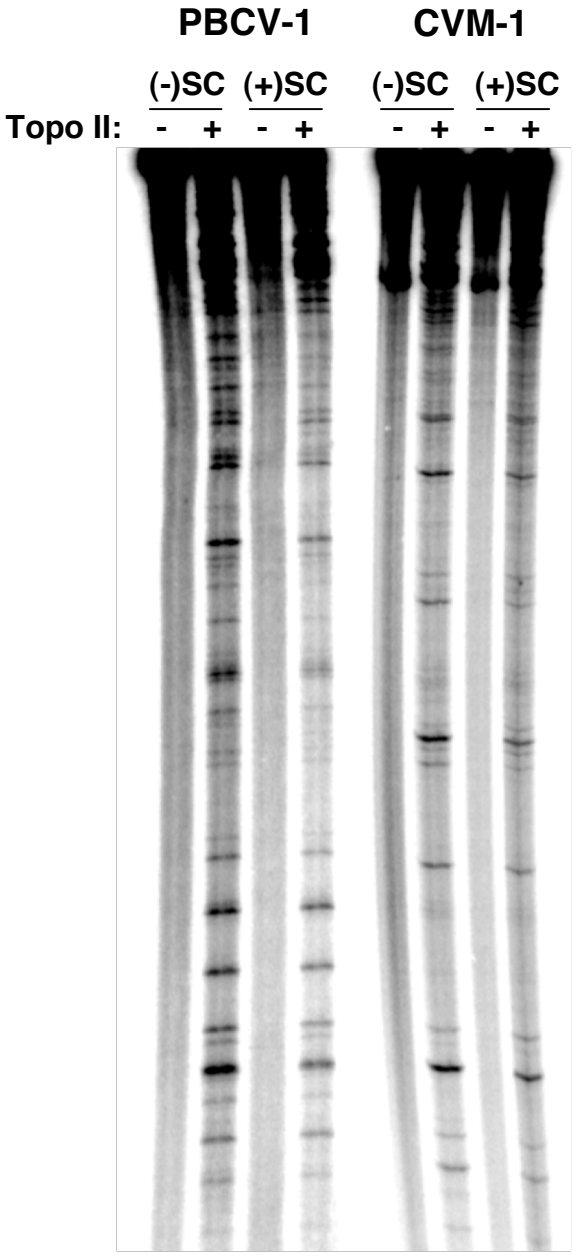

Figure 33. The DNA cleavage specificity of chlorella virus type II topoisomerases is not altered by the handedness of the supercoiled substrate. Sites cleaved by PBCV-1 or CVM-1 topoisomerase II were mapped in negatively [(-)SC] or positively [(+)SC] supercoiled pBR322 plasmid DNA. Products of DNA cleavage assays were linearized and singly-end labeled with [^{32}P]phosphate. The autoradiogram is representative of at least two independent assays.

Second, the effects of supercoil geometry on enzyme-DNA binding were characterized (Figure 34). As determined by competition binding experiments, PBCV-1 and CVM-1 topoisomerase II bound negatively and positively supercoiled plasmids with similar affinity. Therefore, the recognition of DNA handedness during cleavage does not reflect an increased affinity for negatively supercoiled substrates.

Third, the effects of supercoil handedness on enzyme-mediated DNA religation were examined (Figure 35). Similar rates of religation were observed for both viral enzymes with negatively and positively supercoiled substrates. Taken together, these results suggest that the effects of supercoil geometry must manifest themselves somewhere between the initial DNA binding and religation events. Most likely, the actual cleavage event is impacted by the handedness of the DNA substrate.

Relaxation and Cleavage of Negatively and Positively Supercoiled DNA by Human Topoisomerase IIαΔ1175

To further assess the potential role of the C-terminal domain of human topoisomerase IIα as a sensor of DNA geometry during relaxation and cleavage, and to confirm the results generated with the viral type II enzymes, a C-terminal deletion mutant of human topoisomerase IIα (hTop2αΔ1175) was generated. hTop2αΔ1175 allows a direct comparison between full length human topoisomerase IIα and an equivalent enzyme that contains only its ATPase and cleavage/ligation domains. Thus, the ability of hTop2αΔ1175 to relax and cleave negatively and positively supercoiled DNA was characterized. As seen in Figure 36, rates of DNA relaxation for negatively and positively supercoiled plasmids

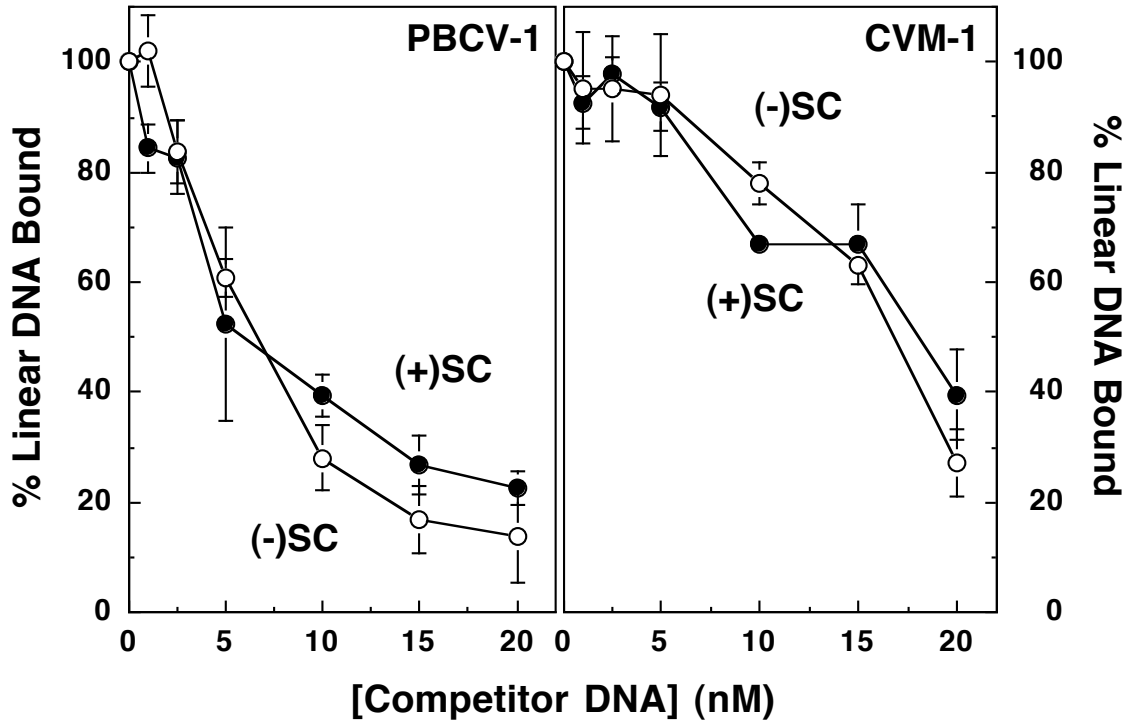

Figure 34. Type II topoisomerases from chlorella viruses display similar binding affinities for negatively and positively supercoiled DNA. The ability of 0–20 nM negatively supercoiled [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid DNA to compete with the binding of 5 nM [^{32}P]-labeled linear pBR322 DNA by PBCV-1 (*left panel*) or CVM-1 (*right panel*) topoisomerase II is shown. Percent linear DNA bound was determined by the ratio of cpm retained on a nitrocellulose filter *vs.* the input amount of radioactivity. Error bars represent the standard deviation of three independent assays.

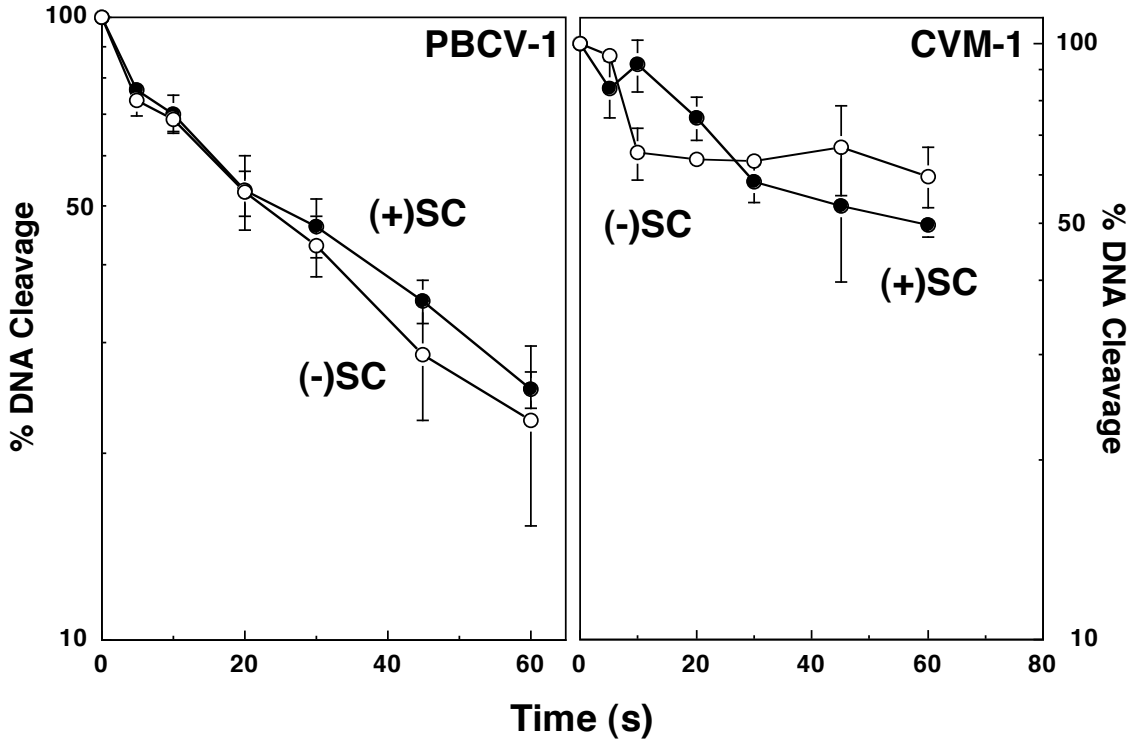

Figure 35. Type II topoisomerases from chlorella viruses display similar rates of religation with negatively and positively supercoiled DNA. A time course of religation in the presence of negatively [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid by PBCV-1 (*left panel*) or CVM-1 (*right panel*) is shown. The initial level of DNA cleavage was set to 100% and the rate of ligation was determined by quantifying the loss of the cleaved DNA over time. Error bars represent the standard deviation of three independent assays.

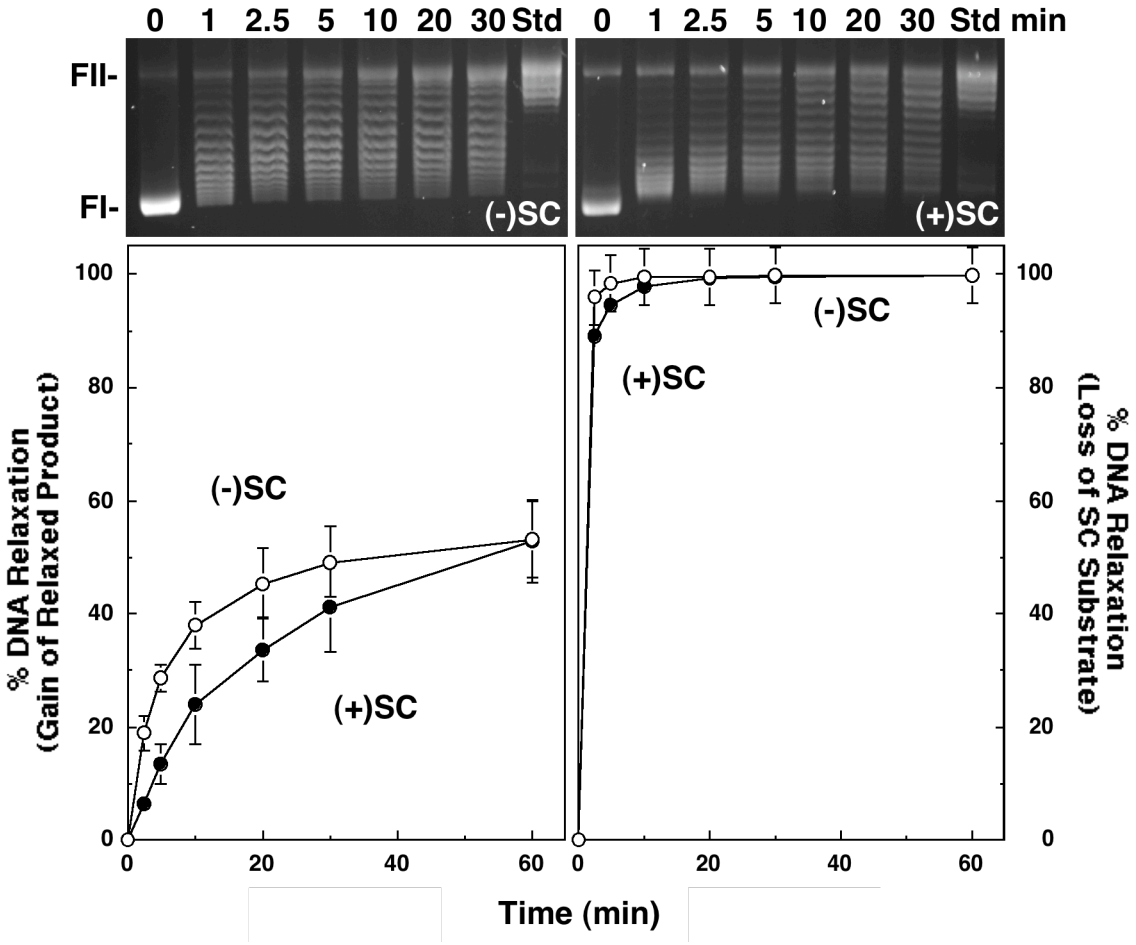

Figure 36. Human topoisomerase IIαΔ1175 does not preferentially relax positively supercoiled DNA. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *left gel*] or positively supercoiled [(+) SC; *right gel*] pBR322 plasmid DNA by human topoisomerase IIαΔ1175 are shown. The positions of supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Error bars represent the standard deviation at least three independent assays.

were equivalent with hTop2αΔ1175. Therefore, removal of the C-terminal domain of the human enzyme abrogated the ability of topoisomerase IIα to sense supercoil geometry during DNA relaxation.

In contrast, removal of the C-terminal domain of topoisomerase IIα did not affect the ability of the enzyme to recognize DNA geometry during the cleavage reaction. As seen in Figure 37, hTop2αΔ1175 maintained lower (~3-fold) levels of cleavage complexes with positively rather than negatively supercoiled substrates. Additionally, as observed with the chlorella virus type II enzymes, hTop2αΔ1175 displayed similar binding affinities (Figure 38) and religation rates (Figure 39) with negatively and positively supercoiled plasmids. Therefore, the recognition of DNA handedness during cleavage does not reflect an increased affinity for negatively supercoiled DNA or faster rates of religation with these substrates.

Taken together, the results obtained with PBCV-1 topoisomerase II, CVM-1 topoisomerase II, and hTop2αΔ1175 suggest that the elements of the protein that sense the handedness of supercoils during DNA relaxation must reside in the variable C-terminal domain of topoisomerase II. In contrast, the elements that sense the handedness of supercoils during DNA cleavage must be located in conserved N-terminal or central domain of the enzyme.

Relaxation of Negatively and Positively Supercoiled DNA by Human Topoisomerase IIαCTDβ and Topoisomerase IIβCTDα.

Results obtained with type II topoisomerases that lack the C-terminal domain suggest that this portion of the enzyme contains residues for facilitating the recognition and preferential relaxation of positively supercoiled DNA.

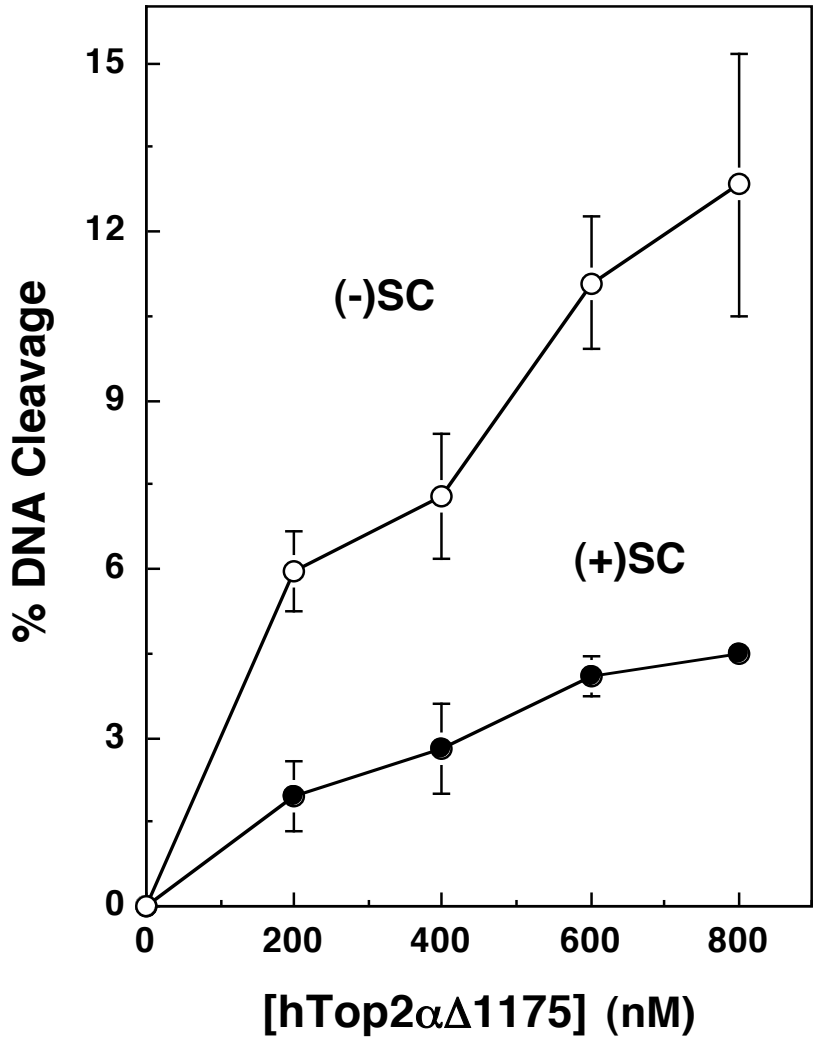

Figure 37. Human topoisomerase IIαΔ1175 maintains lower levels of DNA cleavage complexes with positively supercoiled DNA than with negatively supercoiled molecules. The ability of 0-800 nM hTop2αΔ1175 to cleave 10 nM negatively [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid DNA is shown. Error bars represent the standard deviation of three independent experiments.

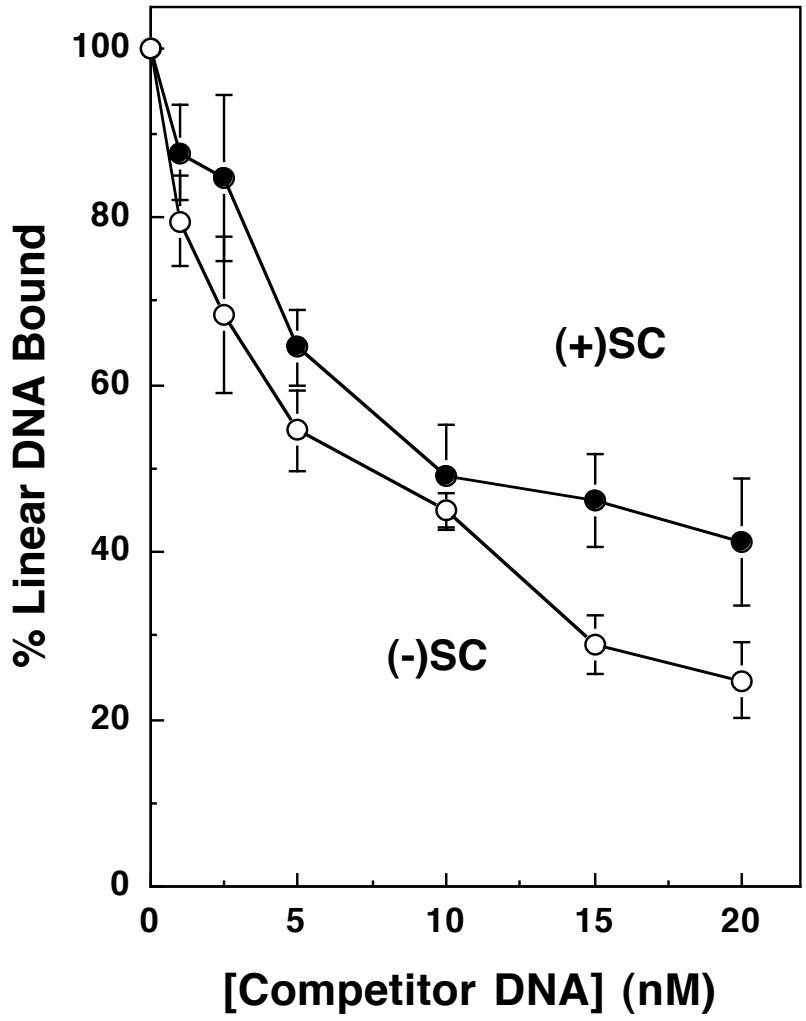

Figure 38. Human topoisomerase IIαΔ1175 displays similar binding affinities for negatively and positively supercoiled DNA. The ability of 0–20 nM negatively supercoiled [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid DNA to compete with the binding of 5 nM [^{32}P]-labeled linear pBR322 DNA by hTop2αΔ1175 is shown. Percent linear DNA bound was determined by the ratio of cpm retained on a nitrocellulose filter *vs.* the input amount of radioactivity. Error bars represent the standard deviation of three independent assays.

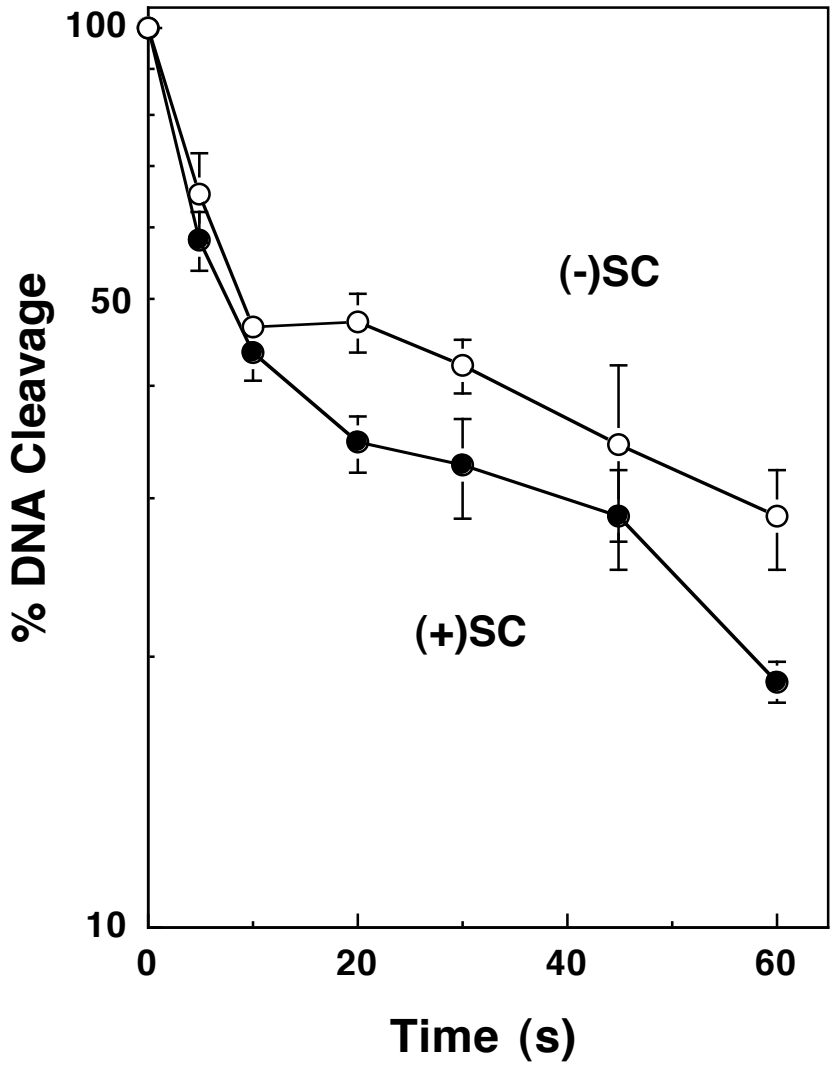

Figure 39. Human topoisomerase IIαΔ1175 displays similar rates of religation with negatively and positively supercoiled DNA. A time course of religation in the presence of negatively [(-)SC, open symbols] or positively [(+)SC, closed symbols] supercoiled pBR322 plasmid by hTop2αΔ1175 is shown. The initial level of DNA cleavage was set to 100% and the rate of ligation was determined by quantifying the loss of the cleaved DNA over time. Error bars represent the standard deviation of three independent assays.

However, it is possible that the C-terminal domain does not interact directly with the DNA substrate. Instead, its presence may alter the structure of topoisomerase II and allow other regions of the enzyme to recognize DNA topology. Therefore, to more fully examine the role of the C-terminal domain of human topoisomerase IIα in the recognition of supercoil geometry during DNA relaxation, two additional mutants enzymes were generated in which the C-terminal domains of human topoisomerase IIα and β were switched (Figure 29).

The ability of human topoisomerase IIα that carries the C-terminal domain of topoisomerase IIβ (hTop2αCTDβ) and topoisomerase IIβ that carries the C-terminal domain of topoisomerase IIα (hTop2βCTDα) to relax negatively and positively supercoiled DNA was examined. While topoisomerase IIα displays preferential relaxation of positively supercoiled DNA, addition of the C-terminal domain of topoisomerase IIβ results in an enzyme that no longer recognizes supercoil geometry during relaxation. As seen in Figure 40, hTop2αCTDβ relaxed negatively and positively supercoiled substrates with similar rates. Furthermore, topoisomerase IIβ, which does not preferentially relax positively supercoiled DNA, gained this characteristic with the addition the C-terminal domain of topoisomerase IIα. As seen in Figure 41, hTop2βCTDα relaxes positively supercoiled substrates >10-fold faster than negatively supercoiled molecules. These results strongly support the hypothesis that the recognition and rapid relaxation of positively supercoiled DNA by human topoisomerase IIα is modulated by the C-terminal domain of the enzyme.

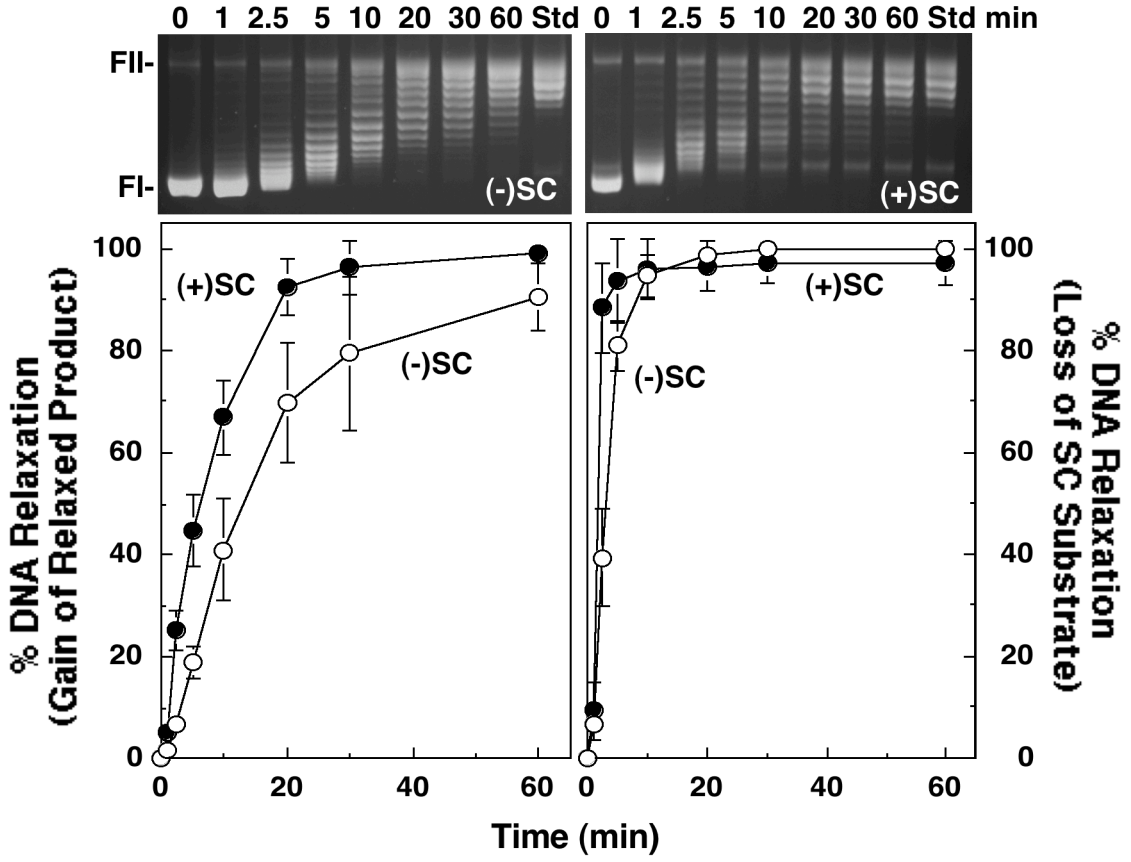

Figure 40. Human topoisomerase IIαCTDβ displays similar relaxation rates with negatively and positively supercoiled DNA. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *left gel*] or positively supercoiled [(+) SC; *right gel*] pBR322 plasmid DNA by human topoisomerase IIαCTDβ are shown. The positions of supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Error bars represent the standard deviation at least three independent assays.

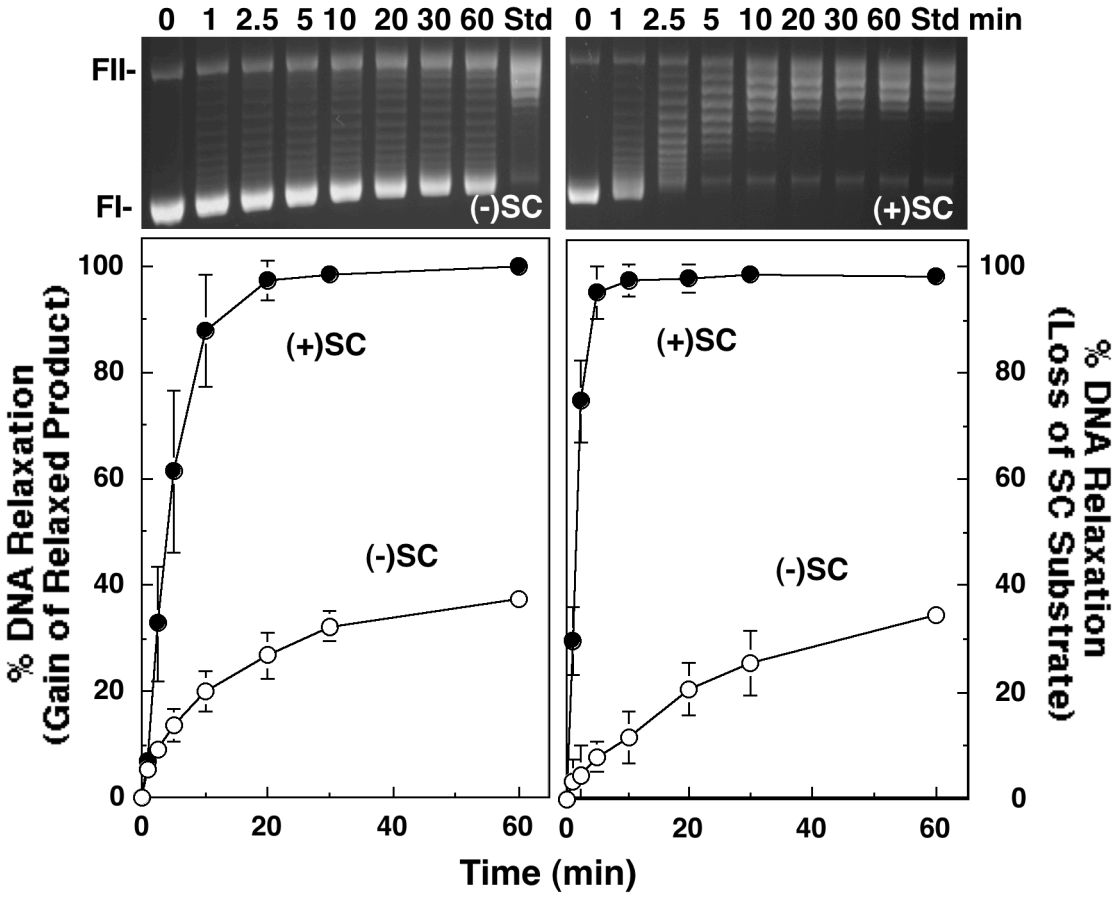

Figure 41. Human topoisomerase IIβCTDα relaxes positively supercoiled DNA faster than negatively supercoiled DNA. Ethidium bromide-stained agarose gels displaying representative time courses for relaxation of negatively supercoiled [(-) SC; *left gel*] or positively supercoiled [(+) SC; *right gel*] pBR322 plasmid DNA by human topoisomerase IIβCTDα are shown. The positions of supercoiled DNA (form I, FI) and relaxed DNA (form II, FII) are indicated. DNA relaxation was quantified from either the gain of fully relaxed product (*left panel*) or the loss of supercoiled substrate (*right panel*). Error bars represent the standard deviation at least three independent assays.

Discussion

Bimodal Recognition of DNA Supercoil Geometry by Topoisomerase II

Previous studies with human and bacterial topoisomerases (62-64,213,214) suggest that the type II enzyme utilizes two distinct mechanisms to recognize the handedness of DNA supercoils. It has been proposed that the ability of some type II enzymes, such as human topoisomerase IIα and *E. coli* topoisomerase IV, to distinguish supercoil geometry during DNA relaxation is mediated by elements in the variable C-terminal domain (62-64). In contrast, the ability of human topoisomerase IIα and β to discern the handedness of DNA during cleavage suggests that residues in the conserved N-terminal or central domain of the protein are involved in this process.

As a first test of this hypothesis, the ability of three enzymes that lack the C-terminal domain, PBCV-1 topoisomerase II, CVM-1 topoisomerase II (201,204), and hTop2αΔ1175, a C-terminal truncation mutant of human topoisomerase IIα, to relax and cleave negatively and positively supercoiled plasmids was compared. While these enzymes relaxed under- and overwound substrates at similar rates, they were able to discern the handedness of supercoils during the cleavage reaction and preferentially cut negatively supercoiled DNA. Preferential cleavage was not due to a change in site specificity, DNA binding, or DNA religation. These findings are consistent with a role for the C-terminal domain of topoisomerase II in sensing the geometry of superhelical twists during relaxation. Furthermore, they demonstrate that the intrinsic ability to discern supercoil geometry during DNA cleavage must reside within the conserved N-terminal or central domain of the type II enzyme.

To determine whether the recognition of supercoil geometry during DNA relaxation is actually carried within the C-terminal domain of human topoisomerase IIα, two additional mutants, hTop2αCTDβ and hTop2βCTDα were generated. While topoisomerase IIα displayed preferential relaxation of positively supercoiled DNA, hTop2αCTDβ no longer displays this preferential relaxation. Additionally, while topoisomerase IIβ did not preferentially relax positively supercoiled DNA, hTop2βCTDα gained this characteristic. These results provide strong evidence that the C-terminal domain of topoisomerase IIα modulates the recognition and rapid relaxation of positively supercoiled substrates.

It is not clear how topoisomerase II utilizes elements in different protein domains to sense DNA geometry during different catalytic processes. However, based on our studies with human and chlorella virus type II topoisomerases, as well as models described for geometry sensing in bacterial topoisomerase IV (62-64), we propose the following models for the actions of eukaryotic topoisomerase II.

Recognition of DNA Supercoil Geometry by the N-terminal or Central Domain of Topoisomerase II

The conserved N-terminal or central domain of the eukaryotic enzyme possesses an intrinsic ability to sense the geometry of its DNA substrate. This postulate is supported by the fact that type II topoisomerases that lack the C-terminal domain are still able to differentiate between under- and overwound substrates during DNA cleavage. The elements in the conserved domains of topoisomerase II that discern DNA geometry during cleavage are likely to be

located within (or proximal to) the active site of the protein. Two segments of DNA, the T- and G-segments, intersect as a crossover in the active site, irrespective of the supercoiled state of the nucleic acid substrate. However, the geometry of the crossover formed by negative or positive supercoils differs significantly. While DNA nodes formed with positively supercoiled molecules are left-handed and have acute angles (~60°), those formed with negatively supercoiled molecules are right-handed and have obtuse angles (~120°) (64,65). Therefore, elements in the conserved domains to topoisomerase II may be in direct contact with both the T- and the G-segments and provide the catalytic core of the enzyme with an innate ability to recognize the angle of the DNA crossover. Since the presence of the T-segment has been shown to increase scission of the G-segment (245), it is possible that the geometry of the crossover may also affect the ability of topoisomerase II to generate breaks in the DNA backbone.

Alternatively, changes in DNA twist associated with under- and overwinding profoundly alter the properties of the double helix. Since the catalytic core of topoisomerase II is in intimate contact with the G-segment (30), the enzyme may recognize the twist of the G-segment rather than the angle of the DNA crossover and maintain lower levels of cleavage complexes with overwound substrates.

It should be noted that although elements in the conserved N-terminal or central domain of topoisomerase II can distinguish negative and positive superhelical twists, they promote strand passage of substrates containing either without prejudice. This is evidenced by the finding that enzymes that lack their C-terminal domains relax under- and overwound plasmids at similar rates.

Recognition of DNA Supercoil Geometry by the C-terminal Domain of Topoisomerase II

Although the C-terminal domain of the type II enzyme is distal to the active site of the protein, it is believed to have specific interactions with the T-segment during relaxation (62-64). In the case of positively supercoiled DNA, interactions with the C-terminal domain facilitate the strand passage reaction, resulting in faster rates of DNA relaxation. This point is supported by the fact that full-length human topoisomerase IIα and hTop2βCTDα preferentially relax overwound DNA. Thus, recognition of DNA geometry during relaxation most likely is related to the path of the T-segment into and out of the active site of the enzyme that is imposed by handedness of the DNA crossover.

Physiological Implications for the Bimodal Recognition of DNA Supercoil Geometry by Type II Topoisomerases

The variable C-terminal domain of bacterial type II topoisomerases appears to play important roles in determining the specific functions catalyzed by individual enzymes. For example, this region of the protein allows DNA gyrase to introduce negative supercoils into the bacterial chromosome (246,247) and topoisomerase IV to preferentially remove positive supercoils (213,214). Results of the present study suggest that the C-terminal domain may also endow specific eukaryotic type II topoisomerases with unique catalytic properties. For example, by using the variable C-terminal domain to distinguish positive from negative supercoils during relaxation, specific type II topoisomerases, such as human topoisomerase IIα, are better suited to actively participate in the removal of torsional stress ahead of replication forks or transcription complexes.

In contrast, by using the conserved portions of the protein to discern DNA geometry during cleavage, all type II topoisomerases may be able to maintain lower levels of cleavage complexes with positively supercoiled DNA. This latter characteristic lessens the probability of collisions between DNA tracking systems and covalent topoisomerase II-cleaved DNA complexes, thereby decreasing the chance that the enzyme will introduce permanent strand breaks into the genome during its normal physiological activities.

CHAPTER VI

CONCLUSIONS

DNA in all species ranging from bacteria to humans is globally negatively supercoiled (3-6). However, DNA ahead of tracking systems, such as replication or transcription complexes, is positively supercoiled (3,5-7). Since topoisomerase I can relax positive or negative supercoils (14,19,195), it has been assumed to act primarily ahead of DNA tracking systems to alleviate torsional stress. Conversely, because topoisomerase II can untangle duplex DNA molecules (12,14-16,30), it is assumed to act primarily behind DNA tracking systems to resolve knots and links in the DNA. In marked contrast to this proposed segregation of function, models for anticancer drug action always place topoisomerase II ahead of approaching DNA tracking enzymes, such as the replication machinery (15,16,90,196-198). This discrepancy raises the question of whether eukaryotic type II topoisomerases have normal physiological functions ahead of DNA tracking systems and was the starting point for this dissertation.

Overall Conclusions

A number of conclusions can be drawn from the results presented in this dissertation. First, human topoisomerase IIα, which is involved in replicative processes, displays preferential relaxation of positively supercoiled DNA, the substrate generated ahead of DNA tracking complexes such as the replication machinery. This suggests that topoisomerase IIα can function efficiently ahead of DNA tracking enzymes to alleviate accumulating torsional stress. Topoisomerase

IIβ, which is not thought to be involved in DNA replication, does not preferentially relax positively supercoiled substrates. These results demonstrate a major enzymological difference between the two human isoforms of topoisomerase II and is consistent their proposed functions in the cell.

Second, both topoisomerase IIα and β maintain lower levels of DNA cleavage complexes with positively supercoiled substrates. This decreases the probability that a collision with DNA tracking enzymes would result in the formation of a topoisomerase II-associated double-stranded break in the genetic material, suggesting that the human type II enzymes can function safely, under normal physiological conditions, ahead of DNA tracking systems.

Lower levels of DNA cleavage are also observed in the presence of non-intercalative topoisomerase II-targeting drugs. However, in the presence of intercalative compounds, higher levels of DNA cleavage complexes were maintained with positively supercoiled substrates at increasing concentrations of drug. These results suggest that intercalative drugs are better suited to target topoisomerases on positive supercoils generated ahead of DNA tracking complexes, because they do not accumulate in these substrates (as they accumulate in negatively supercoiled molecules) and inhibit enzyme activity.

Additionally, topoisomerase I maintains higher levels of cleavage complexes with positively supercoiled DNA. Thus, this enzyme may be an intrinsically more lethal target for anticancer drugs than either topoisomerase IIα or β.

Finally, topoisomerase IIα and β distinguish supercoil geometry differently during DNA relaxation and cleavage assays. This suggests that the human type II enzymes utilize a bimodal mechanism for the recognition of DNA supercoils. Taken together, the results observed for the mutant enzymes described in

chapter V of this dissertation suggest that while the C-terminal domain appears to be necessary for the recognition of supercoil geometry during DNA relaxation, the elements of topoisomerase II involved in recognizing supercoil geometry during DNA cleavage most likely reside in the conserved N-terminal or central domains of the enzyme.

Ramifications

The results described in this dissertation lead to interesting proposals not only for the normal cellular functions of human topoisomerases, but also for these enzymes as important drug targets. While human topoisomerase IIα has been believed to work primarily behind DNA tracking systems, such as replication complexes, this work demonstrates that this enzyme actually preferentially relaxes the substrate generated ahead of replication forks. In addition, the ability to maintain lower levels of cleavage complexes with positively supercoiled DNA suggests that topoisomerase IIα can work both efficiently and safely ahead of DNA tracking systems.

Until the publication of the work described in chapter III, human topoisomerase IIα and β were thought to be enzymologically similar. However, the differential relaxation activities observed with supercoiled substrates indicates a major enzymological distinction between the two human isoforms and supports the proposed functions of each enzyme in the cell. Additionally, the work described in this dissertation demonstrates a catalytic function for the variable C-terminal domain of the human type II topoisomerases. Although this region is believed to have physiological roles, it was shown to be dispensable for

catalysis and previously was thought to play no role in the enzymological activities of type II topoisomerases.

Finally, the work described in chapter IV of this dissertation demonstrates that the supercoil geometry of a DNA substrate can have a profound impact on topoisomerase-targeting drugs. It appears that intercalative drugs preferentially target topoisomerase II-DNA cleavage complexes formed ahead of DNA tracking systems. Additionally, human topoisomerase I seems to be a potentially more lethal target than either human type II enzyme, providing a possible reason why camptothecin-based drugs are active against cancers that are refractory to other established anticancer agents.

Future Directions

As described in chapter I of this dissertation, the C-terminal domains of DNA gyrase (GyrA) and topoisomerase IV (ParC) adopt a unique fold called a β-pinwheel (chapter I, Figure 5, *left panel*) (62-64). The outer rim of this fold was observed to be positively charged (chapter I, Figure 5, *right panel*) and is thought to both bind and bend DNA substrates. Computational studies were attempted with human topoisomerase IIα to determine whether the human isoform also forms a distinct structure for interacting with DNA substrates. Unfortunately, homology between the C-terminal domains of the different species of topoisomerase II was too low to generate a confident predicted structure. However, amino acid sequence alignments comparing the C-terminal domains of the bacterial type II topoisomerases and human topoisomerase IIα did reveal some promising similarities. The clusters of positively charged residues located in the C-terminal region of DNA gyrase and topoisomerase IV and that are

responsible for protein-DNA interactions also are found in the C-terminal domain of the human type II enzymes. This provides a strong indication that this region of topoisomerase IIα may be involved in interacting with the DNA substrate.

Studies currently are underway to narrow down the region(s) of the C-terminal domain of topoisomerase IIα responsible for the recognition and preferential relaxation of positively supercoiled DNA. This work will include additional C-terminal domain truncation mutants of topoisomerase IIα, as well as mutations in certain positively charged regions of the C-terminal domain. In addition, isolation and purification of the C-terminal domain of topoisomerase IIα currently is underway to determine whether this portion of the enzyme can independently bind a DNA substrate. Ultimately, these studies could potentially lead to a crystal structure of this domain.

In an attempt to carry the *in vitro* results described in this dissertation into a cellular setting, it will be of interest to examine the human topoisomerase IIα and β C-terminal tail-switched mutants in a mammalian cell system. Human topoisomerase IIα and β display different patterns of cellular localization throughout the cell cycle, and the C-terminal domains of these enzymes are the locations for both nuclear localization sequences and phosphorylation sites. It will be interesting to determine whether switching the C-terminal domains of human topoisomerase IIα and β has any impact on the localization of either enzyme during DNA replication and mitosis, and possibly on any protein-protein interactions involving these enzymes. It is quite possible that the recognition of DNA supercoil geometry observed *in vitro* will correlate with the different functional roles of human topoisomerase IIα and β in cells.

Studies also are underway to more fully assess the interactions between positively supercoiled DNA, intercalative drugs, and topoisomerase IIα. Figures displayed in this dissertation (see gels in Figures 12, 17, 20, 25 and 27) suggest that intercalative compounds, such as ethidium bromide, bind positively supercoiled DNA more poorly than negatively supercoiled molecules. We proposed in chapter IV that intercalative drugs, such as amsacrine and TAS-103, induce higher levels of topoisomerase II-mediated DNA cleavage with positively supercoiled substrates at increasing drug concentrations (Figure 24), because they do not accumulate on the DNA substrate. To address this hypothesis, we currently are designing quantitative binding studies to more fully examine the interactions between intercalative topoisomerase II-targeting compounds and positively supercoiled substrates.

Lastly, in light of the unexpected DNA cleavage results obtained with human topoisomerase I on positively supercoiled DNA, studies currently are underway to more thoroughly examine the interactions between this human enzyme and supercoiled DNA substrates. As discussed in chapter IV, human topoisomerase I (unlike the type II enzymes) maintained higher levels of cleavage complexes with positively supercoiled substrates. Due to the fact that topoisomerase I characteristically functions to alleviate positive supercoiling ahead of DNA tracking systems, the cellular ramifications of this enzyme feature are unclear. Therefore, it is of interest to our lab to examine how positively supercoiled DNA affects the enzymatic activity of human topoisomerase I, how this substrate affects the response of the enzyme to different anticancer agents, and what elements of topoisomerase I are responsible for discerning DNA supercoil geometry.

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