THE DNA CLEAVAGE REACTION OF HUMAN TYPE II TOPOISOMERASES

By

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To my wife,

Elizabeth Askew Deweese

and

to our first child.

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4’Cl-2,5pQ	2-(4-chloro-phenyl)-[1,4]benzoquinone
Ala	alanine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
APP(NH)P	5’-adenylyl-beta,gamma-imidodiphosphate
Asp	aspartic acid
ATP	adenosine triphosphate
bp	base-pair
Cys	cysteine
ΔLk	linking difference
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallochatechin gallate
Glu	glutamic acid
kb	kilobase
kDa	kilodalton
Lk	linking number
Lk_0	linking number of relaxed DNA
MLL	mixed lineage leukemia gene
NAPQI	*N*-acetyl-*p*-benzoquinone imine

PCB	polychlorinated biphenyl
PML	promyelocytic leukemia gene
RARA	retinoic acid receptor α gene
SC	supercoiled
SDS	sodium dodecyl sulfate
σ	specific linking difference
THF	tetrahydrofuran
Tw	twist
Wr	writhe

CHAPTER I

INTRODUCTION

A number of enzymes that catalyze essential physiological processes also have the capacity to damage the genome during the course of their normal activities. For example, while the cell requires DNA polymerases to copy the genetic material, these enzymes insert an incorrect base approximately every 10^7 nucleotides (1). Consequently, in the absence of mismatch repair pathways, human DNA polymerases would generate several hundred mutations every round of cell division. Furthermore, while DNA glycosylases initiate base excision repair pathways, these enzymes can convert innocuous lesions to abasic sites with far greater mutagenic potential (2). Finally, while cytochrome P450 enzymes play critical roles in detoxification pathways, they sometimes convert inert xenobiotic chemicals to compounds with mutagenic properties (3).

Of all the enzymes required to sustain cellular growth, topoisomerase II is one of the most dangerous (4-8). As discussed below, this enzyme unwinds, unknots, and untangles the genetic material by generating transient double-stranded breaks in DNA (8-12). Although the cell cannot survive without topoisomerase II, the strand breaks that the enzyme generates have the potential to trigger cell death pathways or chromosomal translocations (8,13).

DNA Topology

The existence of topoisomerases is necessitated by the structure of the double helix. Each human cell contains ~2 meters of DNA that are compacted into a nucleus that is ~10 μm in diameter (14,15). Because the genetic material is anchored to the chromosome scaffold and the two strands of the double helix are plectonemically coiled, accessing the genome is a complex topological challenge (11,12,16-18).

Topological properties of DNA are those that can only be changed when the double helix is broken (12). The mathematical aspects of DNA topology involve three key concepts: twist (Tw), writhe (Wr), and linking number (Lk) (12,19-21). Twist represents the total number of double helical turns in a given segment of DNA. By convention, the right-handed twist of the Watson-Crick structure is assigned a positive value. Writhe is a property of the spatial course of the DNA and is defined as the number of times the double helix crosses itself if the molecule is projected in two dimensions (Figure 1). The helix-helix crossovers (*i.e.*, nodes) are assigned a positive or negative value based on the orientation (*i.e.*, handedness) of the DNA axis. The numerical term that describes the sum of the twist and the writhe is called the linking number, which represents the total linking within a DNA molecule. Mathematically, these properties of DNA can be expressed as:

$$Lk = Tw + Wr.$$

Two critical points need to be emphasized. First, as discussed above, as long as the ends of DNA are fixed in space, Lk is an invariant value that cannot be changed without opening the system (*i.e.*, breaking the DNA chain). Second, in the absence of knots or tangles, Tw and Wr are (at least mathematically) fluid values that can be interconverted.

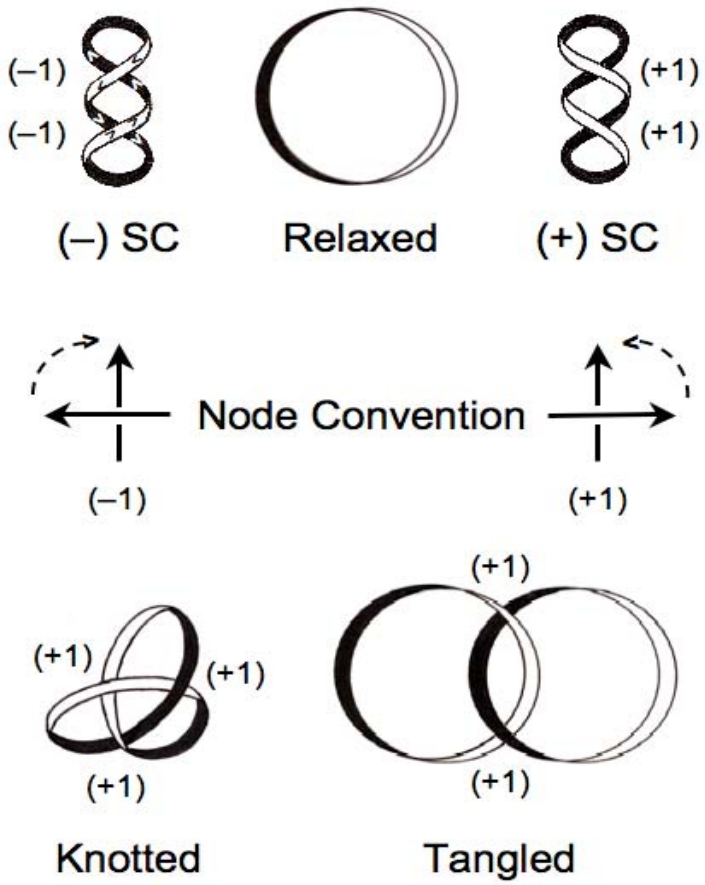

Figure 1: Topological relationships within DNA. DNA molecules are shown as circular ribbons for simplicity. Top: DNA with no torsional stress is referred to as "relaxed." Underwinding or overwinding DNA results in negative (–) or positive (+) supercoils, respectively. The directionality of the DNA is shown by internal arrowheads in the negatively supercoiled [(–)SC] molecule. Supercoils are shown as writhes (DNA crossovers or nodes) for visual ease, but it should be noted that supercoils can be interconverted from writhes to twists. By convention, each writhe (denoted by the crossing of one DNA segment over another segment) is given an integral value of –1 or +1. Middle: Tracing the direction of the DNA, the sign of the node is assigned based on the direction of movement required to align the front segment of DNA with the back segment using a rotation of <180º. Negative DNA writhes are represented by crossovers in which the front segment must be rotated in a clockwise manner to align it with the back segment, while positive writhes require a counterclockwise rotation. Bottom: Intramolecular knots and intermolecular tangles also form in DNA.

The classic example for this interconversion is a telephone cord (12,19-21). In its "native" unstretched configuration, the cord is coiled (*i.e.*, writhes about itself), but there is no visible twisting of the cord. If the cord is stretched, all of the coiling is lost and the cord is visibly twisted.

With the above mathematics in mind, think of the human genome as a series of 46 very long double-stranded ropes in which the two strands of each rope are plectonemically coiled about one another. Using this analogy, two aspects of topology can be envisioned. The first deals with topological relationships between the two strands of the double helix. DNA in the Watson-Crick structure is not under torsional stress. Hence, we refer to this DNA as "relaxed." By definition, a relaxed circular DNA molecule that is 1050 bp in length should have an Lk = +100 (1050 bp ÷ 10.5 bp/turn). However, relaxed DNA does not exist in nature. In all living systems, from bacteria to humans, DNA is globally underwound by ~6% (12,19-21). This means that for every ~1,050 base pairs, there are ~94 turns of the helix as opposed to the expected ~100 turns. The term ΔLk (linking difference) is used to describe this difference. ΔLk is equal to the actual linking number of a molecule minus the linking number of the DNA if it were completely relaxed (Lk_0). Hence for the above example, $\Delta Lk = Lk(94) - Lk_0(100) = -6$.

DNA underwinding or overwinding induces torsional stress within the molecule. If this stress is allowed to freely distribute along the nucleic acid, some of it will be converted to axial stress. This redistribution causes the molecule to writhe about itself forming superhelical twists (Figure 1). This is why DNA that is under torsional stress is referred to as supercoiled (12,19-21). Underwound and overwound molecules are called negatively and positively supercoiled, respectively.

The term ΔLk described above is dependent on the length of the DNA. In order to compare levels of supercoiling in molecules of different sizes, the term σ (specific linking difference or more commonly, superhelical density) is utilized. σ is independent of DNA length and can be calculated using the equation:

$$\sigma = \Delta Lk \div Lk_0.$$

Thus, for the example discussed above, $\sigma = \Delta Lk(-6) \div Lk_0(100) = -0.06$. The σ value for underwound DNA is always negative and for overwound DNA is always positive. However, negatively supercoiled DNA still has a positive Lk value.

The fact that DNA *in vivo* is globally underwound (*i.e.*, negatively supercoiled) is important because duplex DNA is merely the storage form for the genetic information (12,19-21). To replicate or express this information, the two strands of DNA must be separated. Since global underwinding of the genome imparts increased single-stranded character to the double helix, negative supercoiling greatly facilitates strand separation (12,16-18).

While negative supercoiling promotes many nucleic acid processes, DNA overwinding (*i.e.*, positive supercoiling) inhibits them. The linear movement of tracking enzymes, such as helicases and polymerases, compresses the turns of the double helix into a shorter region (Figure 2) (12,19-21). Consequently, the double helix becomes increasingly overwound ahead of tracking systems. The positive supercoiling that results makes it more difficult to open the two strands of the double helix and ultimately blocks essential nucleic acid processes (10,12,16-18).

The second aspect of DNA topology deals with relationships between separate DNA segments. Intramolecular knots (formed within the same DNA molecule) are generated

during recombination, and intermolecular tangles (formed between daughter DNA molecules) are produced during replication (Figure 2) (8,10,12,17). DNA knots block essential nucleic acid processes because they make it impossible to separate the two strands of the double helix. Moreover, tangled DNA molecules cannot be segregated during mitosis or meiosis (8,10,12,17). Consequently, DNA knots and tangles can be lethal to cells if they are not resolved.

DNA Topoisomerases

The topological state of the genetic material is regulated by enzymes known as topoisomerases (8,10,11,22,23). Topoisomerases are required for the survival of all organisms and alter DNA topology by generating transient breaks in the double helix (8,10,11,22,23). There are two major classes of topoisomerases, type I and type II, that are distinguished by the number of DNA strands that they cleave and the mechanism by which they alter the topological properties of the genetic material (8,10,11,22,23).

Eukaryotic type I topoisomerases are monomeric enzymes that require no high-energy cofactor (11,22,24). Type I enzymes are organized into two subclasses: type IA and type IB. These enzymes alter topology by creating transient single-stranded breaks in the DNA, followed by passage of the opposite intact strand through the break (type IA) or by controlled rotation of the helix around the break (type IB) (11,22,24). Type IA topoisomerases need divalent metal ions for DNA scission and attach covalently to the 5’-terminal phosphate of the DNA (11,22,24). In contrast, type IB enzymes do not require divalent metal ions and covalently link to the 3’-terminal phosphate (11,22,24).

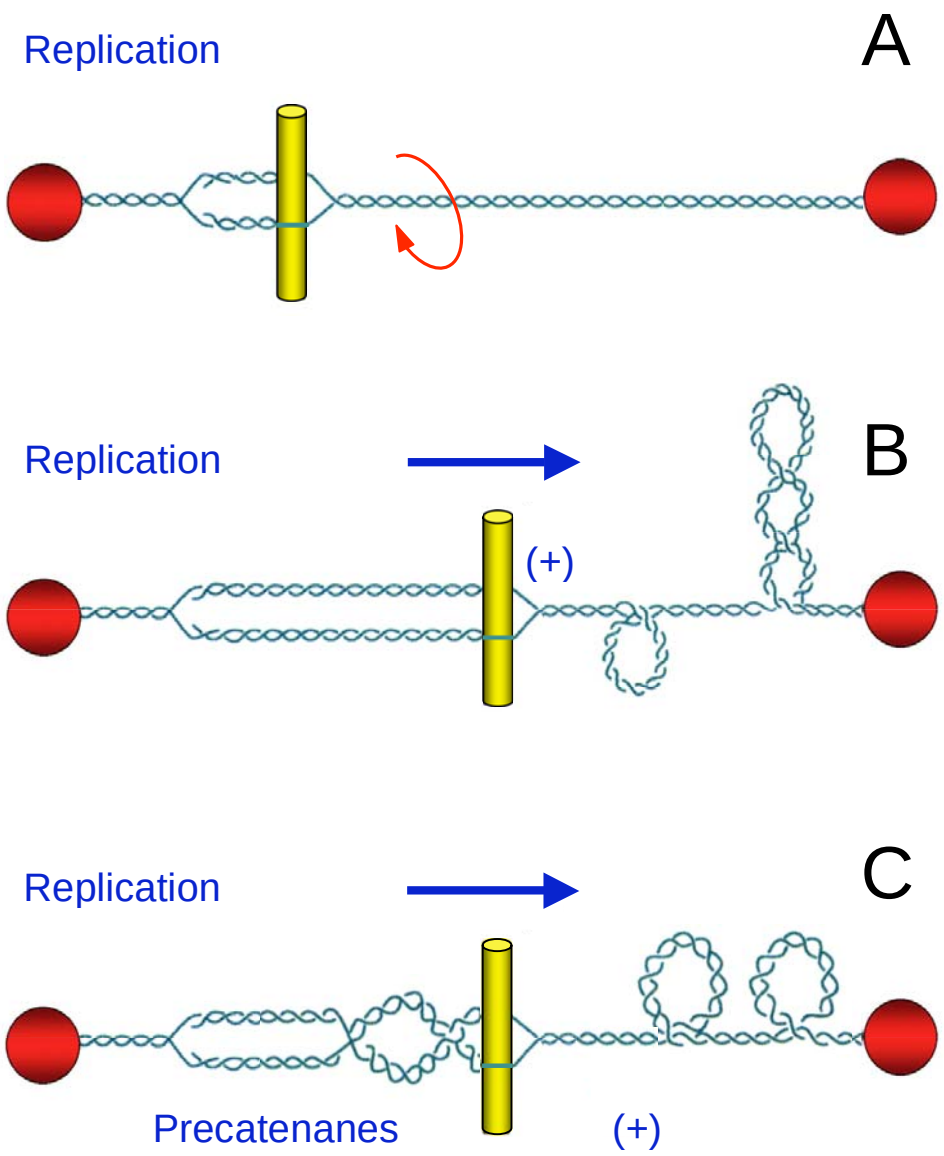

Figure 2: Nuclear processes induce changes in DNA topology. DNA replication is used as an example. Although chromosomal DNA is globally underwound in all cells, the movement of DNA tracking systems generates positive supercoils. As shown in A, chromosomal DNA ends are tethered to membranes or the chromosome scaffold (represented by the red spheres) and are unable to rotate. Therefore, the linear movement of tracking systems (such as the replication machinery represented by the yellow bars) through the immobilized double helix compresses the turns into a shorter segment of the genetic material and induces acute overwinding (*i.e.* positive supercoiling) ahead of the fork (B). In addition, the compensatory underwinding (*i.e.* negative supercoiling) behind the replication machinery allows some of the torsional stress that accumulates in the prereplicated DNA to be translated to the newly replicated daughter molecules in the form of precatenanes (C). If these precatenanes are not resolved, they ultimately lead to the formation of intertwined (*i.e.*, tangled) duplex daughter chromosomes. Adapted from Ref. (10).

As a result of their reaction mechanism, type I topoisomerases can modulate DNA under- and overwinding, but cannot remove knots or tangles from duplex DNA (22,24,25).

Eukaryotic type II topoisomerases function as homodimers and require divalent metal ions and ATP for complete catalytic activity (5,8,26-28). These enzymes interconvert different topological forms of DNA by a "double-stranded DNA passage reaction" that can be separated into a number of discrete steps (5,8,26-28). Briefly, type II topoisomerases 1) bind two separate segments of DNA, 2) create a double-stranded break in one of the segments, 3) translocate the second DNA segment through the cleaved nucleic acid "gate," 4) rejoin (*i.e.*, ligate) the cleaved DNA, 5) release the translocated segment through a gate in the protein, and 6) close the protein gate and regain the ability to start a new round of catalysis (5,26-33). Because of their double-stranded DNA passage mechanism, type II topoisomerases can modulate DNA supercoiling and also can resolve DNA knots and tangles.

Topoisomerase II

Lower eukaryotes and invertebrates encode only a single type II topoisomerase, known as topoisomerase II (34-37). In contrast, vertebrate species encode two closely related isoforms of the enzyme, topoisomerase IIα and topoisomerase IIβ. These isoforms differ in their protomer molecular masses (170 *vs.* 180 kDa, respectively) and are encoded by separate genes (8,10,22,28,38-44). Topoisomerase IIα and topoisomerase IIβ display a high degree (~70%) of amino acid sequence identity and similar enzymological characteristics. One notable difference between the two isoforms is that topoisomerase IIα relaxes (*i.e.*, removes) positive superhelical twists ~10 times faster than it does

negative *in vitro*, while the β isoform is unable to distinguish the geometry of DNA supercoils during DNA relaxation (45).

Topoisomerase IIα and topoisomerase IIβ have distinct patterns of expression and separate cellular functions. Topoisomerase IIα is essential for the survival of proliferating cells, and protein levels rise dramatically during periods of cell growth (46-49). The enzyme is further regulated over the cell cycle, with protein concentrations peaking in G2/M (48,50,51). Topoisomerase IIα is associated with replication forks and remains tightly bound to chromosomes during mitosis (9,49,52-54). Thus, it is believed to be the isoform that functions in growth-related processes, such as DNA replication and chromosome segregation (10,49).

Topoisomerase IIβ is dispensable at the cellular level (38,42) but appears to be required for proper neural development (52,55). Expression of topoisomerase IIβ is independent of proliferative status and cell cycle, and the enzyme dissociates from chromosomes during mitosis (42,52,56). Topoisomerase IIβ cannot compensate for the loss of topoisomerase IIα in mammalian cells, suggesting that these two isoforms do not play redundant roles in replicative processes (42,49,57,58). Although the physiological functions of topoisomerase IIβ have yet to be defined, recent evidence indicates involvement in the transcription of hormonally- or developmentally-regulated genes (58,59).

Much of what we understand regarding the mechanism of action of type II enzymes comes from experiments with topoisomerase II from species that express only a single form of the protein. Consequently, eukaryotic type II topoisomerases will be referred to

collectively as topoisomerase II, unless the properties being discussed are specific to either the α or β isoform.

Topoisomerase II-Mediated DNA Cleavage and Ligation

The ability of topoisomerase II to cleave and ligate DNA is central to all of its catalytic functions (5,8,11,27). All topoisomerases utilize active site tyrosyl residues to mediate DNA cleavage and ligation. Since type II enzymes cut both strands of the double helix, each protomer subunit contains one of these residues (Tyr805 and Tyr821 in human topoisomerase IIα and topoisomerase IIβ, respectively).

Topoisomerase II initiates DNA cleavage by the nucleophilic attack of the active site tyrosine on the phosphate of the nucleic acid backbone (Figure 3) (11,23,26,27). The resulting transesterification reaction results in the formation of a covalent phosphotyrosyl bond that links the protein to the newly generated 5'-terminus of the DNA chain. It also generates a 3'-hydroxyl moiety on the opposite terminus of the cleaved strand. The scissile bonds on the two strands of the double helix are staggered and are located across the major groove from one another. Thus, topoisomerase II generates cleaved DNA molecules with 4-base 5'-single-stranded cohesive ends, each of which is covalently linked to a separate protomer subunit of the enzyme (60-62).

The covalent enzyme-DNA linkage plays two important roles in the topoisomerase II reaction mechanism. First, it conserves the bond energy of the sugar-phosphate DNA backbone. Second, because it does not allow the cleaved DNA chain to dissociate from the enzyme, the protein-DNA linkage maintains the integrity of the genetic material during the cleavage event (11,23,26,27). The covalent topoisomerase II-cleaved DNA

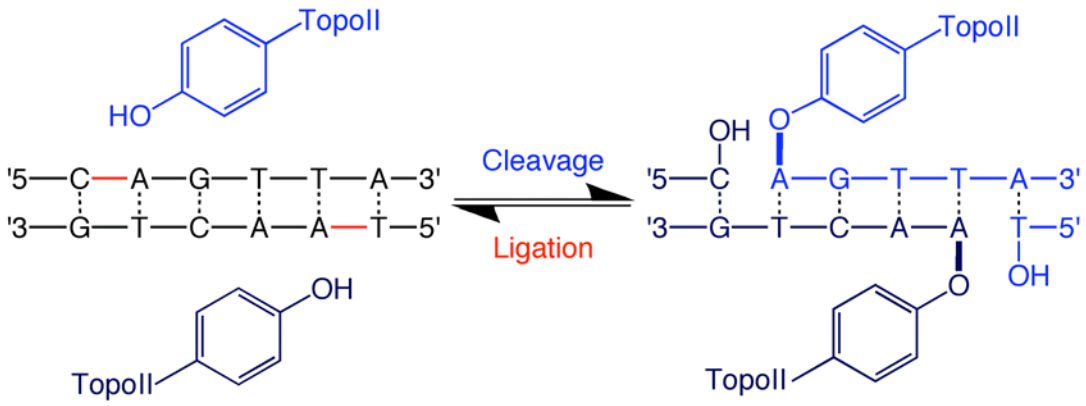

Figure 3: Double-stranded DNA cleavage mediated by topoisomerase II. Scissile bonds are located four bases apart on opposite strands of the double helix. During cleavage, the active site tyrosine residue of each topoisomerase II protomer subunit becomes covalently linked to the newly generated 5'-terminal phosphate moiety on each strand. This covalent linkage preserves the energy of the sugar-phosphate DNA backbone. The newly generated 3'-hydroxyl group interacts with topoisomerase II in a non-covalent fashion. Ligation represents the reverse of this process and leaves the DNA product chemically unchanged from the initial substrate.

reaction intermediate is referred to as the “cleavage complex” and is critical for the pharmacological activities of the enzyme, which are discussed later in this chapter.

Although topoisomerase II acts globally, it cleaves DNA at preferred sites (63). The consensus sequence for cleavage is weak, and many sites of action do not conform to it (63). Ultimately, the mechanism by which topoisomerase II selects DNA sites is not apparent, and it is nearly impossible to predict *de novo* whether a given DNA sequence will support scission. Most likely, the specificity of topoisomerase II-mediated cleavage is determined by the local structure, flexibility, or malleability of the DNA that accompanies the sequence, as opposed to a direct recognition of the bases that comprise that sequence (64).

Beyond the nucleophilic attack of the active site tyrosine on the DNA backbone, the details of topoisomerase II-mediated DNA cleavage are not well defined. However, information regarding the roles of specific amino acid residues comes from structural studies on the catalytic core of yeast topoisomerase II generated in the absence of DNA or in a non-covalent complex with its nucleic acid substrate (27,65).

The DNA cleavage reaction requires a divalent metal ion (5,30,66,67). Mg^{2+} appears to fulfill this function *in vivo* (5). A two-metal-ion mechanism for DNA cleavage mediated by the bacterial type II topoisomerase, DNA gyrase, has been proposed (68). This mechanism was proposed based on structural homology (to other enzymes) and limited biochemical evidence (68). However, it has not been demonstrated whether this mechanism also applies to the human type II topoisomerases.

Topoisomerase II-DNA cleavage complexes normally are short-lived and readily reversible (5,8,69), and the DNA cleavage/ligation equilibrium of the enzyme greatly

favors ligation (5,8,11,23,26,27,70). Under equilibrium conditions, ~0.5–1% of topoisomerase II in a DNA scission reaction mixture exists as a cleavage complex (60-62,71). Furthermore, when Mg^{2+} is utilized as the divalent metal ion, ~1/2–3/4 of the complexes contain double-stranded breaks with the remainder containing single-stranded DNA breaks. The fact that a significant proportion of cleavage complexes contain single-stranded breaks was initially taken as an indication for poor coordination between the two protomer subunits of topoisomerase II (72). However, in hindsight, if the protomers cut the two strands of the double helix in a completely non-coordinated fashion, virtually no double-stranded DNA breaks would be generated (1% x 1% ≈ 0.01% cleavage complexes). These findings suggest that there must be a relatively high degree of coordination between the two protomer active sites of the enzyme, even if they do not act in complete concert with one another. To this point, once topoisomerase II cleaves the first strand, it is estimated that the enzyme cuts the second strand ~20–fold faster (70).

Following strand passage, DNA ligation is initiated when a general acid extracts the hydrogen from the 3'-terminal hydroxyl group. The acid may be a water molecule or an unidentified amino acid in the active site of topoisomerase II. The conversion of the terminal hydroxyl moiety to an oxyanion induces a nucleophilic attack on the phosphotyrosyl bond (11,23,26,27). This action represents the reverse of the cleavage event and regenerates an intact DNA chain as well as the active site of topoisomerase II.

It should be noted that the chemical structure of the ligated DNA is identical to that of the original substrate. Only the topological properties of the double helix are altered by the actions of topoisomerase II.

Topoisomerase II as a Cellular Toxin

Proliferating cells cannot exist without type II topoisomerases (5,8,10). However, since these enzymes generate obligatory double-stranded DNA breaks as part of their reaction mechanism, they are intrinsically dangerous proteins. Thus, topoisomerase II assumes a Dr. Jekyll/Mr. Hyde character: while essential to cell viability, the enzyme also has the capacity to fragment the genome (Figure 4). Because of this dual persona, levels of cleavage complexes are maintained in a critical balance (5,8). When levels drop below threshold concentrations, daughter chromosomes remain entangled following replication. As a result, chromosomes cannot segregate properly during mitosis and cells die as a result of catastrophic mitotic failure (Figure 4).

When levels of cleavage complexes rise too high, cells also die, but for different reasons (Figure 4). Accumulated topoisomerase II-DNA cleavage intermediates are converted to permanent strand breaks when replication forks, transcription complexes, or DNA tracking enzymes such as helicases attempt to traverse the covalently bound protein "roadblock" in the genetic material (5,8,69,73). The resulting collision disrupts cleavage complexes and ultimately converts transient topoisomerase II-associated DNA breaks to permanent double-stranded breaks that are no longer tethered by proteinaceous bridges (5,8,69,73). The resulting damage and induction of recombination/repair pathways can trigger mutations, chromosomal translocations, and other aberrations. When these permanent DNA breaks are present in sufficient numbers, they can overwhelm the cell and initiate cell death pathways in eukaryotes (4,5,7,8,13,69,73).

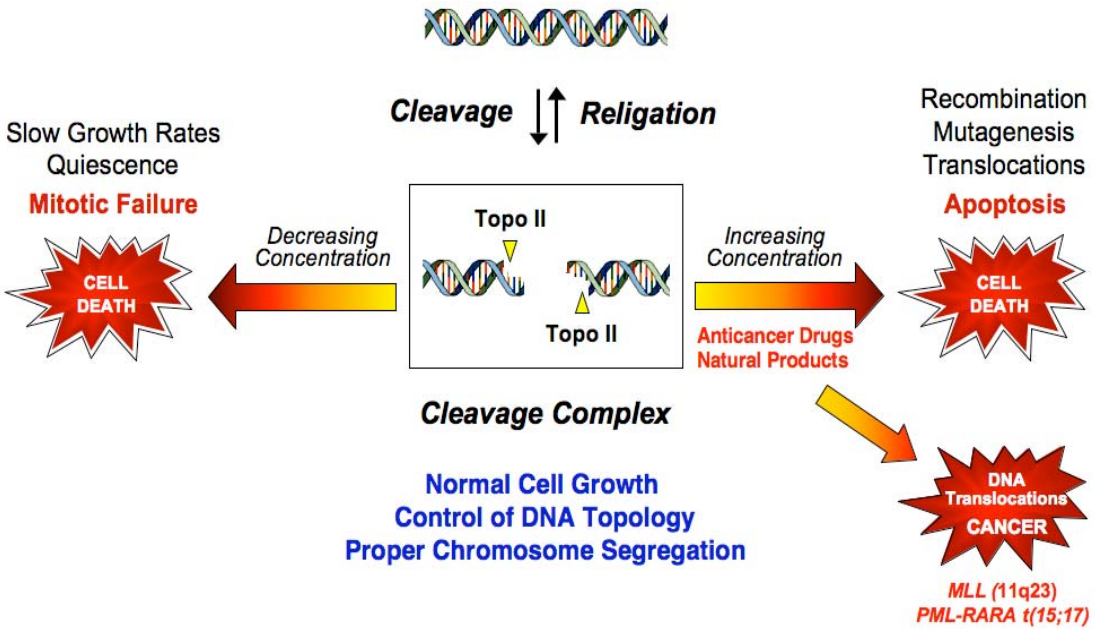

Figure 4: Topoisomerase II is an essential but genotoxic enzyme. The formation of topoisomerase II-DNA cleavage complexes is required for the enzyme to perform its critical cellular functions. If the level of topoisomerase II-DNA cleavage complexes falls too low (left arrow), cells are not able to untangle daughter chromosomes and ultimately die of mitotic failure. If the level of cleavage complexes becomes too high (right arrow) the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks. The resulting DNA breaks, as well as the inhibition of essential DNA processes, initiate recombination/repair pathways and generate chromosome translocations and other DNA aberrations. If the strand breaks overwhelm the cell, they can trigger apoptosis. This is the basis for the actions of several widely prescribed anticancer drugs. If the concentration of topoisomerase-mediated DNA strand breaks is too low to overwhelm the cell, mutations or chromosomal aberrations may be present in surviving populations. In some cases, exposure to topoisomerase II poisons has been associated with the formation of specific types of leukemia that involve the *MLL* (mixed lineage leukemia) gene at chromosome band 11q23 or the chromosome 15;17 translocation that joins the *PML* (promyelocytic leukemia) and *RARA* (retinoic acid receptor α) genes (lower right arrow) (74,75).

Topoisomerase II Poisons

Compounds that impact the catalytic activity of topoisomerase II can be separated into two categories. Chemicals in the first category decrease the overall activity of the enzyme and are known as catalytic inhibitors (5,8,69). Chemicals in the second category increase levels of topoisomerase II-DNA cleavage complexes. These latter compounds are said to "poison" the type II enzyme and convert it to a cellular toxin that initiates the mutagenic and lethal consequences described above (5,8,69). Because of their actions, these compounds are referred to as "topoisomerase II poisons" to distinguish them from inhibitors that do not affect enzyme mediated DNA cleavage/ligation (5,8,69). Although some topoisomerase II poisons also inhibit overall activity, the "gain of function" induced by these compounds in the cell (*i.e.*, increased levels of cleavage complexes), is the dominant phenotype (8,69).

Topoisomerase II poisons increase the concentration of cleavage complexes by two non-mutually exclusive pathways. Some compounds, such as the anticancer drug etoposide (see below), inhibit the ability of the enzyme to ligate cleaved DNA molecules (5,8,69). Other poisons, such as abasic sites and other forms of DNA damage (see below) have little effect on ligation and are believed to work primarily by enhancing the forward rate of scission (8,69). Because of the manner in which they act, abasic sites poison topoisomerase II without inhibiting overall catalytic activity.

Beyond their effects on DNA scission *vs.* ligation, topoisomerase II poisons (with the exception of DNA lesions) act by two distinct mechanisms. Compounds in the first group are referred to as traditional, non-covalent, interfacial, or redox-independent topoisomerase II poisons (8,44,69,76). These chemicals form non-covalent interactions

with topoisomerase II at the protein-DNA interface in the vicinity of the active site tyrosine (8,44,76-81). Because these compounds also interact with DNA within the ternary enzyme-DNA-poison complex, they generally alter the DNA cleavage site specificity of the enzyme (82). Finally, their actions against topoisomerase II are not affected by reducing agents, such as dithiothreitol, and these compounds induce similar levels of enzyme-mediated DNA cleavage whether they are added to the binary topoisomerase II-DNA complex or are incubated with the enzyme prior to the addition of nucleic acid substrates (8,76,77,83).

Unlike the traditional poisons, compounds that use the second mechanism require redox activity to facilitate their actions against topoisomerase II. The redox-dependent poisons covalently adduct to the enzyme at amino acid residues outside of the active site (8,69,83-90) and generally enhance DNA cleavage at sites that are intrinsically cut by the enzyme (83,88). Moreover, because these compounds require redox chemistry for activation, their ability to poison topoisomerase II is abrogated by reducing agents (83,85,88,91-93). Finally, compounds within this group enhance DNA cleavage when added to the protein-DNA complex but display the distinguishing feature of inhibiting topoisomerase II activity when incubated with the enzyme prior to the addition of DNA (83,85,88,91-93).

Topoisomerase II as a Target for Anticancer Drugs

Topoisomerase II poisons represent some of the most important and widely prescribed anticancer drugs currently in clinical use (Figure 5). These drugs encompass a diverse group of natural and synthetic compounds that are commonly used to treat a

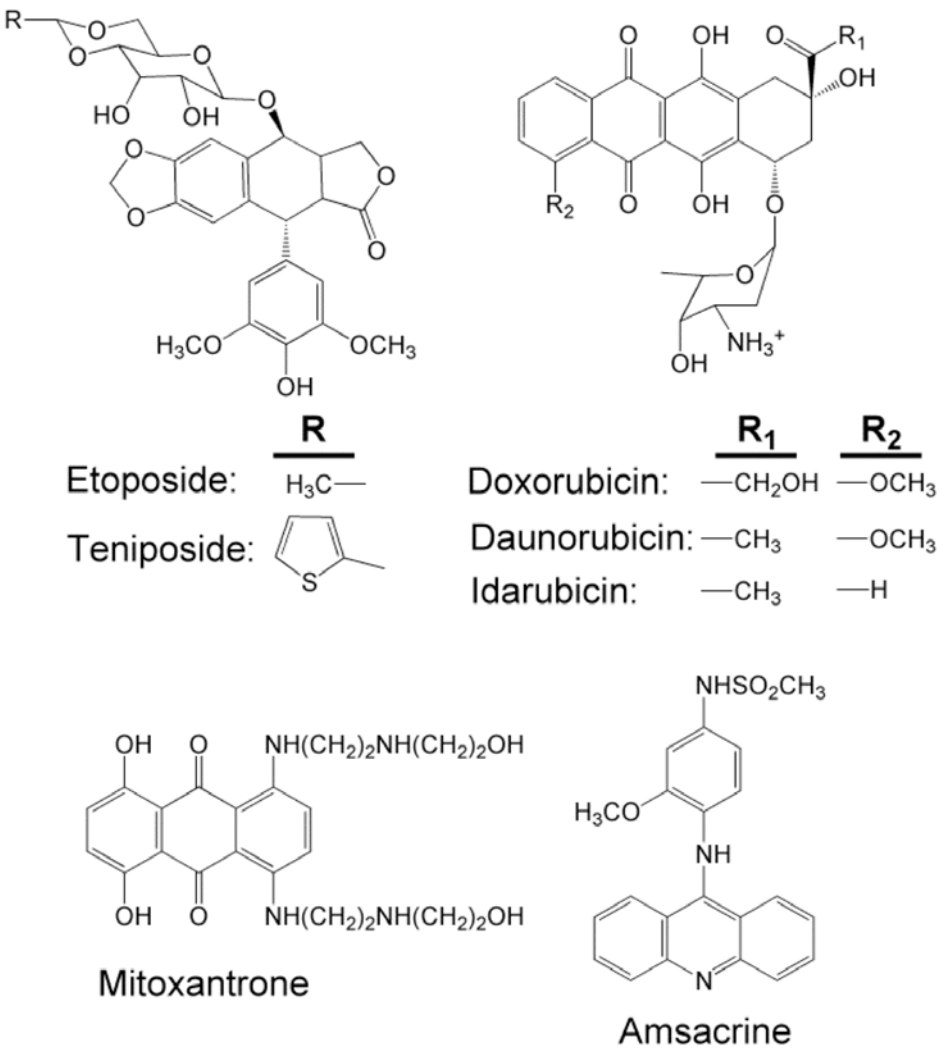

Figure 5: Topoisomerase II-targeted anticancer drugs. Structures of selected drugs are shown.

variety of human malignancies (5,8,69,76,94,95). At the present time, six topoisomerase II-targeted anticancer agents are approved for use in the United States, and additional drugs are prescribed elsewhere in the world (95). These agents all act as traditional topoisomerase II poisons and function primarily by inhibiting enzyme-mediated DNA ligation.

One of the first topoisomerase II-targeted agents to be discovered was etoposide, which is derived from podophyllotoxin (94,96). This natural product is found in *Podophyllum peltatum*, more commonly known as the mayapple or mandrake plant (94,96). Podophyllotoxin has been used as a folk remedy for over a thousand years (94,96). The clinical use of this compound as an antineoplastic agent was prevented by high toxicity, but two synthetic analogs, etoposide and teniposide, displayed increased antineoplastic activity and decreased toxicity (94,96). Etoposide was approved for clinical use against cancer in the mid-1980's and for several years was the most widely prescribed anticancer drug in the world (94,96).

Etoposide and other drugs such as doxorubicin (and its derivatives) are front-line therapy for a variety of systemic cancers and solid tumors, including leukemias, lymphomas, sarcomas, and breast, lung, and germline cancers (94-96). Mitoxantrone is used to treat breast cancer, and both it and amsacrine are used to treat relapsed acute myeloid leukemia (97,98). Every form of cancer that is considered to be curable by chemotherapy utilizes treatment regimens that include topoisomerase II-targeted drugs (94-96,99). In addition to the use of mitoxantrone in anticancer regimens, it is used as a treatment for autoimmune diseases, such as multiple sclerosis (100).

Although topoisomerase II is the cytotoxic target of the drugs shown in Figure 5, the relative contributions of topoisomerase IIα and topoisomerase IIβ to the chemotherapeutic effects of these agents has yet to be resolved. Some drugs appear to favor one isoform or the other (101); however, no truly "isoform-specific" agents have been identified. The issue of isoform specificity has potential clinical ramifications. For example, since topoisomerase IIα is not expressed appreciably in quiescent cells, the actions of topoisomerase II-targeted agents against the β isoform in differentiated tissues such as cardiac cells most likely are responsible for much of the off-target toxicity of these drugs (102-104). Alternatively, since topoisomerase IIα and topoisomerase IIβ are involved in different cellular processes, it may be that cleavage complexes formed with one or the other isoform are more likely to be converted to permanent DNA strand breaks.

Dietary Topoisomerase II Poisons

Many foods consumed in the human diet contain naturally occurring topoisomerase II poisons (Figure 6). The most prominent natural products with activity against the mammalian type II enzymes are the bioflavonoids (*i.e.*, phytoestrogens) (105-108). Bioflavonoids represent a broad group of polyphenolic compounds (including flavones, flavonols, isoflavones, and catechins) that are components of many fruits, vegetables, and plant leaves (109-112). These compounds affect human cells through a variety of pathways; they are strong antioxidants and efficient inhibitors of growth factor receptor tyrosine kinases (109-112). In addition, many bioflavonoids, especially genistein, are potent topoisomerase II poisons (84,105-108,113-115).

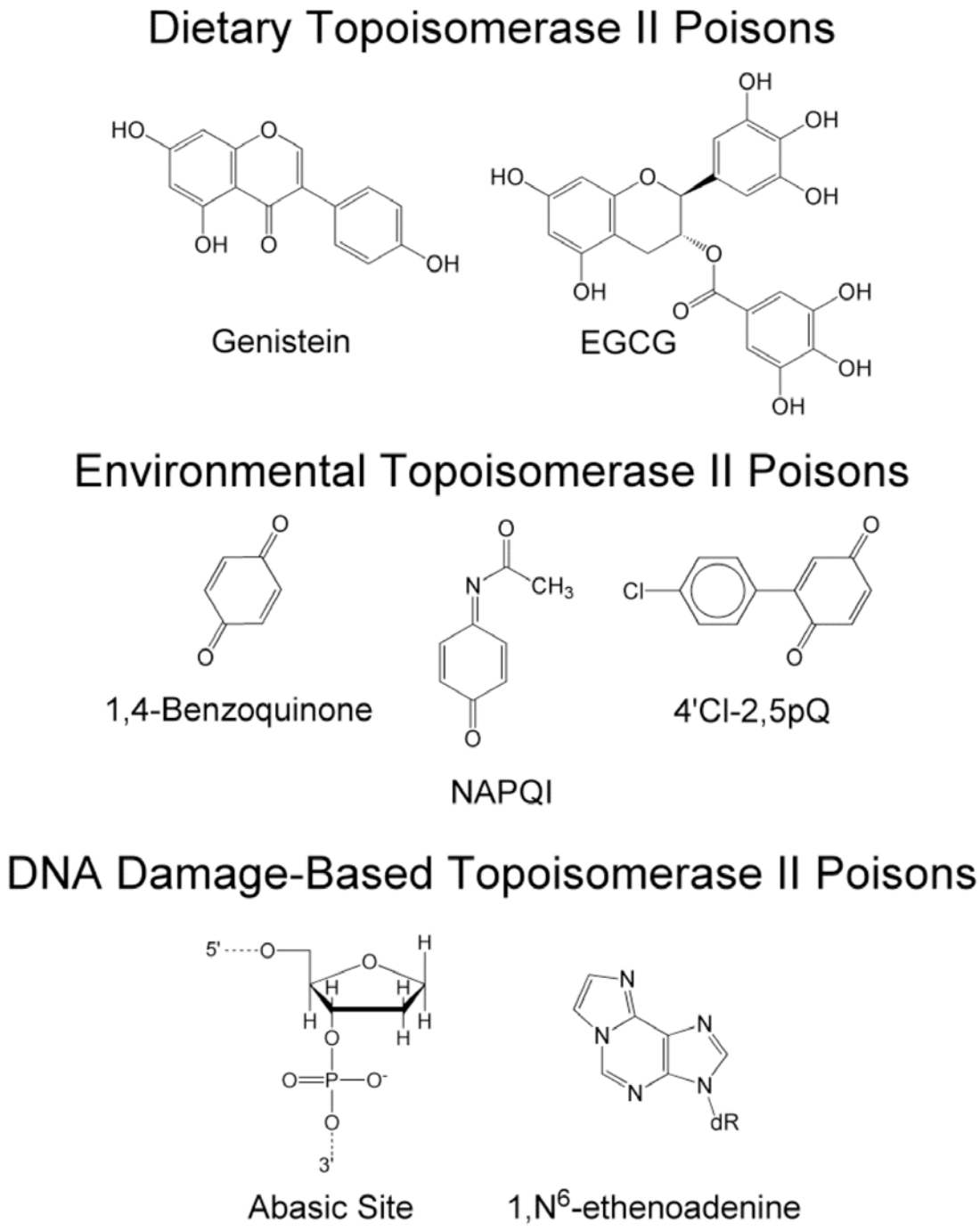

Figure 6: Dietary, environmental, and DNA-based topoisomerase II poisons. Abbreviations used are epigallocatechin gallate (EGCG), *N*-acetyl-*p*-benzoquinone imine (NAPQI), and 2-(4-chloro-phenyl)-[1,4]benzoquinone (4'Cl-2,5pQ).

Genistein, which is prominent in soy, is believed to be a chemopreventative agent in adults that contributes to the low incidence of breast and colorectal cancers in the Pacific Rim (Figure 6) (111,112). However, as discussed below, there also is evidence associating genistein consumption during pregnancy with the development of infant leukemias (106,116-119). In addition, (–)-epigallocatechin gallate (EGCG), the most abundant and biologically active polyphenol in green tea, is a topoisomerase II poison (Figure 6) (84,111,114). Many of the therapeutic benefits of green tea have been attributed to this compound.

The ring structure of genistein is remarkably similar to that of quinolones (115). These latter compounds, which target the prokaryotic type II topoisomerases DNA gyrase and topoisomerase IV, represent the most active and broad-spectrum antibacterials currently in clinical use (120-122). Like the quinolones and the anticancer drugs discussed above, genistein increases levels of topoisomerase II-DNA cleavage complexes as a traditional topoisomerase II poison (115). In contrast, since many bioflavonoids undergo redox chemistry, several members of the class act as redox-dependent topoisomerase II points. EGCG and some related catechins poison topoisomerase II by this latter mechanism (84,114).

Environmental Topoisomerase II Poisons

The toxic metabolites of some drugs and industrial chemicals are topoisomerase II poisons (Figure 6) (86,87,89,123,124). In all cases described to date, these chemicals include quinones (carbon rings that feature ketone groups) as part of their structures (125-128). Quinones commonly are produced in the body as a result of detoxification or

metabolism pathways (125-128). These compounds are highly reactive and often damage cells by generating oxidative radicals or by covalently modifying proteins and (to a lesser extent) nucleic acids (125-128).

Although acetaminophen is the most widely utilized analgesic in the world, overdosage (both accidental and intentional) of this drug represents the second most prevalent cause for toxic drug admissions to emergency departments in the United States (129). The toxic metabolite of acetaminophen, N-acetyl *p*-benzoquinone imine (NAPQI), is a potent topoisomerase II poison that produces liver failure (90). Benzene is an industrial solvent that is associated with the development of human leukemias (123,125-128). One of the most prevalent metabolites of benzene, 1,4-benzoquinone, is a highly reactive topoisomerase II poison (86,87,89,123,124). Finally, polychlorinated biphenyls (PCBs), which have multiple industrial uses, have been linked to a variety of human health issues (88). The quinone metabolites of these compounds all display activity against human type II topoisomerases. Consistent with their highly active redox chemistry, all quinone-based topoisomerase II poisons act in a redox-dependent manner that involves covalent attachment to the enzyme (85-87,91).

DNA Damage as Topoisomerase II Poisons

Several forms of nucleic acid damage enhance topoisomerase II-mediated DNA cleavage (Figure 6) (64,130-137). The type II enzymes are particularly sensitive to abasic sites, alkylated bases that contain exocyclic rings, and other lesions that distort the double helix.

DNA damage increases cleavage at naturally occurring sites of topoisomerase II action (64,130-137). To enhance cleavage, lesions must be located within the four-base stagger that separates the two scissile bonds (64,130-137).

The physiological benefits of DNA lesions as topoisomerase II poisons, if any, are unclear. However, human topoisomerase IIα and topoisomerase IIβ both appear to play roles in fragmenting genomic DNA and releasing chromosomal loops during apoptosis (138,139). It has been suggested that the apoptotic activities of topoisomerase II are enhanced (or perhaps triggered) by DNA lesions that are generated following the release of oxidative radicals from permeable mitochondria in apoptotic cells (138,139).

Topoisomerase II and Leukemia

Despite the importance of topoisomerase II in cancer chemotherapy, evidence suggests that DNA cleavage mediated by the enzyme can trigger chromosomal translocations that lead to specific types of leukemia (see Figure 4) (5-8,69,140,141). To this point, 2-3% of patients who receive regimens that include etoposide or other topoisomerase II-targeted drugs eventually develop acute myeloid leukemias (AMLs). Most of these leukemias are accompanied by translocations with breakpoints in the *MLL* (mixed lineage leukemia) gene at chromosomal band 11q23 (6,7,69). The MLL protein is a histone methyltransferase that regulates the *Hox* genes, which control proliferation in hematopoietic cells (6,7,69). Several breakpoints in *MLL* have been identified and are located in close proximity to topoisomerase II-DNA cleavage sites (6,7,69).

Recently, a link between topoisomerase II-targeted drugs and the development of acute promyelocytic leukemias (APLs) has been observed. Patients with these leukemias

display translocations between the *PML* (promyelocytic leukemia) gene on chromosome 15 and the *RARA* (retinoic acid receptor α) gene on chromosome 17 (74,75).

In addition to treatment-related leukemias, ~80% of infants with AML or acute lymphoblastic leukemia (ALL) display translocations that involve the *MLL* gene (6,7,106,116-118,140-142). The chromosomal translocations associated with these cancers have been observed *in utero*, indicating that infant leukemias are initiated during pregnancy. Epidemiological studies indicate that the risk of developing these infant leukemias is increased >3-fold by the maternal consumption of foods that are high in naturally occurring topoisomerase II poisons such as genistein or other bioflavonoids (106,116-119).

The ability of topoisomerase II poisons to *cause* rather than *cure* cancer may be related to cellular levels of topoisomerase II-mediated DNA cleavage complexes. If the concentration of enzyme-associated DNA breaks is sufficient, DNA recombination/repair pathways can be overwhelmed and cells will die (5,8,69). However, if the levels of breaks are not adequate to induce death, pathways that promote cell survival can lead to the formation of stable chromosomal translocations that ultimately lead to cancerous growth (Figure 4) (5,8,69).

Finally, the specific contributions of topoisomerase IIα and topoisomerase IIβ to cancer therapy *vs.* leukemogenesis are unclear. However, recent evidence suggests that (with at least some drugs) topoisomerase IIα may play a more important role in cytotoxicity (102,143), while topoisomerase IIβ may play a greater role in triggering drug-induced cancers (102).

Scope of the Dissertation

The DNA cleavage reaction of topoisomerase II is essential to the function of the enzyme and is required for the effects of topoisomerase II-targeted poisons. Therefore, it is necessary to have a clear understanding of the process of DNA cleavage by the enzyme. The goals of this dissertation are to establish a DNA cleavage system that separates enzyme-mediated cleavage from ligation, examine the coordination of the two protomers during cleavage, and determine the role of required metal ions during scission.

An overview of DNA topology and topoisomerases is found in Chapter I. The materials and methods used in this dissertation are located in Chapter II.

Chapter III of this dissertation describes the development of a system for examining topoisomerase II-mediated DNA cleavage in the absence of ligation using 3'-bridging phosphorothiolates. Findings made using this system have been published (144) and demonstrate that 3'-bridging phosphorothiolate substrates are cleaved by topoisomerase IIα in a covalent and metal-ion dependent manner. Further, enzyme-mediated cleavage of these substrates accumulates over time and cannot be reversed even in the presence of high salt or EDTA.

Chapter IV of this dissertation describes the coordination of the two protomer subunits of topoisomerase IIα and the ability of nicks to poison the type II enzyme. This work has been accepted for publication (145) and demonstrates that second-strand DNA cleavage occurs faster than first-strand DNA cleavage, allowing the enzyme to be suitably coordinated during scission. Based on the results, we believe that once the enzyme cuts the first strand, the DNA is more flexible and attains the transition state more readily in order for second strand cleavage to occur. These findings also show that

nicks poison topoisomerase II by enhancing the forward rate of DNA cleavage without inhibiting DNA ligation.

Chapter V of this dissertation describes the role of metal ions in the DNA scission reaction of human topoisomerase IIα. Results demonstrate that there is a critical interaction between a metal ion and the 3'-bridging atom of DNA that may stabilize a transition state during scission. This work has been published (146) and indicates that two metal ions are involved in DNA cleavage mediated by topoisomerase IIα. Moreover, point mutations of several conserved Asp residues (D541, D543, and D545) and a Glu residue (E461) in topoisomerase IIα were generated and characterized. Results suggest that these residues are required for DNA cleavage and are involved in coordination of metal ions during scission.

Chapter VI of this dissertation describes the involvement of metal ions during scission by human topoisomerase IIβ. Results demonstrate that the β isoform also uses a two metal ion mechanism similar to that of topoisomerase IIα. This work has been accepted for publication (147) and provides evidence that the mechanism of type II topoisomerases involve an interaction between a metal ion and a non-bridging phosphate oxygen. Although a non-bridging interaction is frequently postulated in models for metal ion usage by topoisomerases, this evidence from topoisomerase IIβ is the first biochemical demonstration that a metal ion does contact this position during scission.

Concluding remarks for the research presented in this dissertation are provided in Chapter VII.

CHAPTER II

METHODS

Materials

Enzymes

Human topoisomerase IIα and IIβ were expressed in *Saccharomyces cerevisiae* and purified as described previously (130,148,149). Yeast (*S. cerevisiae*) topoisomerase II was isolated as described previously (77,149).

Individual point mutations in human topoisomerase IIα were made using the PCR-based Lightning Mutagenesis Kit (Strategene). D541, D543, D545, and E461 were individually mutated to either Cys or Ala in the inducible overexpression YEpWOB6 plasmid. Primer sequences were as follows: D545C forward 5' – GGAAGATAATG ATTATGACAGATCAGGACCAATGTGGTTCCCACATC – 3'; reverse 5' – GATGT GGGAACCACATTGGTCCTGATCTGTCATAATCATTATCTTCC – 3'; D545A forward 5' – GGAAGATAATGATTATGACAGATCAGGACCAAGCTGGTTCCCAC ATC – 3'; reverse 5' – GATGTGGGAACCAGCTTGGTCCTGATCTGTCATAATCAT TATCTTCC – 3'; D543C forward 5' – GGAAGATAATGATTATGACA GATCAGTG CCAAGATGGTTCCCACATC – 3'; reverse 5' – GATGTGGGAACCATCTTGGC ACTGATCTGTCATAATCATTATCTTCC – 3'; D543A forward 5' – GGAAGATAA GATTATGACAGATCAGGCCCAAGATGGTTCCCACATC – 3'; reverse 5' – GATGT GGGAACCATCTTGGGCCTGATCTGTCATAATCATTATCTTCC – 3'; D541C

forward 5’ – GGAAGATAATGATTATGACATGTCAGGACCAAGATGGTTCCCA CATC – 3’; reverse 5' – GATGTGGGAACCATCTTGGTCCTGACATGTCATAATC ATTATCTTCC – 3'; D541A forward 5’ – GGAAGATAATGATTATGACAGCTCA GGACCAAGATGGTTCCCACATC – 3’; reverse 5' – GATGTGGGAACCATCTTGGT CCTGAGCTGTCATAATCATTATCTTCC – 3'; E461C forward 5’ – GAGTGTACGCT TATCCTGACTTGTGGAGATTCAGCCAAAACTTTGGCTG – 3’; reverse 5’ – CAG CCAAAGTTTTGGCTGAATCTCCACAAGTCAGGATAAGCGTACACTC – 3’; and E461A forward 5’ – GAGTGTACGCTTATCCTGACTGCGGGAGATTCAGCCAAAA CTTTGGCTG – 3’; reverse 5’ – CAGCCAAAGTTTTGGCTGAATCTCCCGCAGTC AGGATAAGCGTACACTC – 3’. Mutants were isolated, sequenced, and transformed back into JEL1Δtop1 yeast cells where they were expressed and purified as mentioned above (130,148,149).

Preparation of Oligonucleotides

Two 50-bp oligonucleotide duplexes were designed using previously identified topoisomerase II cleavage sites from pBR322. These sites correspond to sequences designated as site 1 and site 2 by Fortune et al. (150). Wild-type oligonucleotide sequences were generated using an Applied Biosystems DNA synthesizer. The 50-mer site 1 sequence for the top and bottom strands were 5’–AGCGGTATCAGCTCACTCAAAGGC↓GGTAATACGGTTATCCACAGAATCAG-3’ and 5’–CTGATTCTGTGGATAACCGTAT↓TACCGCCTTTGAGTGAGCTGAT-ACCGCT-3’, respectively (arrow denotes point of cleavage). The 50-mer site 2 top and bottom sequences were 5’-TTGGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTT-

CGGAAAAAGAGTTGGT-3’ and 5’-ACCAACTCTTTTTCCGAAGGT↓AACTGGCT-TCAGCAGAGCGCAGATACCAA-3’, respectively. Nested 40-, 30-, 25-, and 20-mer oligonucleotides were generated by incrementally shortening the 50-mer site 2 sequence from each end and maintaining the central cleavage site. The sequences used for the bottom strand of these shortened oligonucleotides were 5’–AACTCTTTTTCCGAAGG-T↓AACTGGCTTCAGCAGAGCGCAG-3’, 5’–TTTTTCC-GAAGGT↓AACTGGCTT-CAGCAGAG-3’, 5’-TTTTTCCGAAGGT↓AACTGGCTTC-AG-3’, and 5’-TTCCGA-AGGT↓AACTGGCTTC-3’, respectively. Depending on the experiment, the top strand was either the complementary sequence of the same length or the full-length 50-mer site 2 top strand sequence.

The top and bottom sequences for the 50-mer substrate that did not contain a native cleavage site were 5’-ACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAA CAAACCACCGC-3’ and 5’-GCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAAC TCTTTTTCCGAAGGT-3’, respectively. The “non-cleavage” sequence that was selected is located proximal to the site 2 sequence shown above. In the absence of anticancer drugs or other poisons, no cleavage by human topoisomerase IIα was observed anywhere within the oligonucleotide. A few sites of enzyme-mediated scission in the “non-cleavage” substrate were seen in the presence of 100 μM amsacrine or etoposide (data not shown). However, neither drug generated cleavage complexes at the positions induced by nick 1, 2, or 3, with the following exception: etoposide produced ~0.13% cleavage at the position induced by nick 3. This level of scission is ~1/3 that generated by the presence of nick 3.

Oligonucleotide duplexes with site-specific nicks were generated by annealing the full length (50-mer) bottom strand with two complementary fragments generated from the top strand sequence. The lengths of the two fragments were altered to move the position of the nick on the top strand. In some cases, oligonucleotides contained a tetrahydrofuran abasic site analog in place of a specific nucleotide. The positions of these alterations (*i.e.*, nicks or abasic sites) are denoted in the appropriate experiments. Substrates containing a racemic phosphorothioate in place of the non-bridging scissile bond oxygens of the bottom strand of site 1 and site 2 were synthesized by Operon.

Preparation of Phosphorothiolate Oligonucleotides

DNA containing a single 3'-bridging phosphorothiolate linkage at the site of topoisomerase II-mediated cleavage was synthesized using 3'-*S*-(2-cyanoethyl-*N*,*N*-diisopropylphosphorothioamidite)-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)thymidine (151). The synthesis of the DNA oligonucleotide was performed on an ABI392 DNA synthesizer (Applied Biosystems) similar to published protocols (152) except that standard activator (0.45 M tetrazole in CH_3CN) and low iodine containing oxidizing solution (0.02 M I_2 in tetrahydrofuran/pyridine/water) were used during all coupling and oxidation steps. All reagents for DNA synthesis were purchased from Glen Research. DNA oligonucleotides were deprotected in concentrated ammonia (Aldrich) containing 0.1% β-mercaptoethanol (Aldrich) at room temperature for 48 h, and partially purified by reverse phase HPLC chromatography as described previously (153). Unless stated otherwise, the location of the phosphorothiolate was always on the bottom strand at the scissile bond (denoted by the arrow). Confirmation of the presence of the bridging sulfur

was accomplished by treating oligonucleotides with $AgNO_3$ (154) and resolving fragments by gel electrophoresis.

Radioactive Labeling of Oligonucleotides

[γ-^{32}P]ATP (~5000 Ci/mmol) was obtained from ICN. Single-stranded oligonucleotides were labeled on their 5' termini using T4 polynucleotide kinase (New England Biolabs). Labeling of 3' termini with [α-^{32}P]cordycepin (ICN) was performed using terminal deoxynucleotidyl transferase (New England Biolabs). Following labeling and gel purification, complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

DNA Cleavage Mediated by Topoisomerase II

Oligonucleotide DNA Cleavage Assays

DNA cleavage assays were carried out by a modification of the procedure of Fortune *et al.* (150). Unless indicated, oligonucleotide substrates were 5'-end-labeled. DNA cleavage reactions with human type II topoisomerases contained 200 nM human topoisomerase IIα or IIβ and 100 nM double-stranded oligonucleotide in a total of 20 μL of cleavage buffer: 10 mM Tris-HCl (pH 7.9), 135 mM KCl, 5 mM divalent cation ($MgCl_2$, unless otherwise stated), 0.1 mM EDTA, and 2.5% glycerol (v/v). Reactions with yeast topoisomerase II contained 200 nM enzyme and 100 nM double-stranded oligonucleotide in a total of 20 μL of 10 mM Tris-HCl (pH 7.9), 35 mM KCl, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 2.5% glycerol (v/v). Reactions were

initiated by the addition of enzyme and were incubated at 37 °C (human) or 28 °C (yeast) for 0 to 30 min. DNA cleavage products were trapped by the addition of 2 μL of 10% SDS followed by 2 μL of 250 mM Na_2EDTA (pH 8.0). Proteinase K (2 μl of 0.8 mg/mL) was added to digest the enzyme, and cleavage products were resolved by electrophoresis in a 14% denaturing polyacrylamide gel. DNA cleavage products were visualized and quantified using a Bio-Rad Molecular Imager.

As a test of the reversibility of topoisomerase II-mediated cleavage, 2 μL of 250 mM Na_2EDTA (pH 8.0) or 2 μL of 5 M NaCl were added and samples were incubated at 37 °C for 5 min prior to the addition of SDS. Reactions with ATP contained a final concentration of 1 mM. In some cases, assay mixtures included 50 μM etoposide or amsacrine. In some cases, the concentration of divalent cation ($MgCl_2$, $MnCl_2$, $CaCl_2$, or $CoCl_2$) was varied and/or combinations of cations were used. Studies incorporating experiments that monitored DNA cleavage over a range that included divalent cation concentrations below 1 mM utilized cleavage buffer that lacked EDTA. To inhibit oxidation of cleaved oligonucleotides containing 3'-terminal –SH moieties and the formation of multimers in the gel, 100 mM DTT was added to the sample loading buffer.

Pre-equilibrium Oligonucleotide DNA Cleavage

In order to determine rate constants for DNA cleavage, pre-equilibrium reactions were monitored for 5 ms to 3 s using a KinTek (Austin, TX) model RQF-3 chemical quench flow apparatus. Cleavage was initiated by rapidly mixing nearly equal volumes of two independent solutions. The first contained a noncovalent complex formed between human topoisomerase IIα and ^{32}P-labeled oligonucleotide in cleavage buffer that lacked

divalent cation. The second solution contained cleavage buffer in which the divalent cation concentration was 2 times higher than normal (10 mM). The two solutions were mixed at 37 ºC, and DNA cleavage was quenched with 1% SDS (v/v final concentration). Products were processed and analyzed as described above. The observed rate constant of DNA cleavage was determined by a fit of the data to the double exponential equation $y = b_1(1 - e^{-k_1 t}) + b_2(1 - e^{-k_2 t})$, where y is the percent of DNA cleavage, b is the amplitude, k is the observed rate constant, and t is the time.

Since assays were initiated by the addition of a divalent metal ion to a pre-formed topoisomerase IIα-DNA complex, in the simplest case, cleavage should follow first order reaction kinetics. However, time courses for pre-equilibrium DNA cleavage reactions were best fitted to a double exponential equation. Two previous studies that examined pre-equilibrium DNA cleavage by topoisomerase II, one with the yeast type II enzyme (70) and the other with human topoisomerase IIα (see chapter V), also found that the best fit for the reaction was to a double exponential equation.

The mechanistic basis underlying this fit is not obvious. However, the most likely possibility is that there is a conformational change in the enzyme-DNA complex that occurs between the DNA binding and cleavage steps. This change may represent either a requisite conversion from a non-productive to a productive state or an isomerization from a less active to a more active state. Such a proposed conformational change is suggested by kinetic analysis of the DNA binding, cleavage, and ligation events mediated by yeast topoisomerase II (70). In addition, it is supported by the fact that the orientation of the active site tyrosyl residue in the crystal structure of the non-covalent complex between DNA and the catalytic core of yeast topoisomerase II is too far away from the scissile

bond to initiate scission (65). This finding implies that the enzyme active site in the structure "has not yet attained a cleavage competent configuration."

Topoisomerase II-mediated Plasmid DNA Cleavage

Plasmid DNA cleavage reactions were performed using the procedure of Fortune and Osheroff (155). Reaction mixtures contained 200 nM human topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in 20 μL of DNA cleavage buffer as defined above with 5 mM $MgCl_2$. DNA cleavage mixtures were incubated for 10 min at 37 °C. Enzyme−DNA cleavage complexes were trapped by adding 2 μL of 5% SDS followed by 2 μL of 250 mM Na_2EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 37 °C to digest topoisomerase II. Samples were mixed with 2 μL of 60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue (w/v), and 0.5% xylene cyanol FF (w/v), heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Single-stranded and double-stranded DNA cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to nicked and linear molecules, respectively. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

Oligonucleotide DNA Ligation Assays

DNA ligation assays were carried out by a modification of the procedure of Kingma et al. (156). DNA cleavage/ligation equilibria were established as described for human

topoisomerase IIα in the oligonucleotide DNA cleavage section except that 5 mM $CaCl_2$ replaced the $MgCl_2$ in the cleavage buffer. Topoisomerase IIα-DNA cleavage complexes were trapped by the addition of Na_2EDTA (pH 8.0) to a final concentration of 6 mM, followed by NaCl to a final concentration of 0.5 M to prevent re-cleavage of the DNA. Ligation was initiated by the addition of $MgCl_2$ to a final concentration of 0.1 mM and terminated by the addition of 2 μL of 10% SDS. Samples were processed and analyzed, and levels of cleavage were quantified as described above. The percent DNA cleavage at time zero was set to 100%, and the rate of ligation was determined by quantifying the loss of cleaved DNA over time.

CHAPTER III

USING 3'-BRIDGING PHOSPHOROTHIOLATES TO ISOLATE THE FORWARD DNA CLEAVAGE REACTION OF HUMAN TOPOISOMERASE IIα

Introduction

To fully understand the basis for topoisomerase II action and the role of the type II enzyme in curing and causing cancer, it is essential to characterize the mechanism by which they cleave and ligate DNA. Unfortunately, these reactions have been difficult to study for two major reasons. First, the DNA cleavage/ligation equilibrium of mammalian type II topoisomerases lies heavily toward ligation (5,8,44,60-62,71). Consequently, in unperturbed systems, levels of cleavage complexes generated by type II enzymes, including human topoisomerase IIα, are extremely low (<1%). To overcome this challenge, it is common to include topoisomerase II poisons, such as anticancer drugs (5,8,44) or DNA substrates that contain damaged or missing bases, in reaction mixtures (33,133,136,156). The use of non-physiological divalent cations such as Ca(II) or Co(II) also has been reported (157,158). Although all of these conditions shift the cleavage/ligation equilibrium of topoisomerase II toward the cleaved state, they often perturb the specificity of the enzyme. Furthermore, since they do not uncouple the two reaction steps from one another (see below), in most cases they cannot be used to reveal intrinsic properties of the DNA cleavage or ligation reactions.

Second, the DNA cleavage and ligation reactions of type II topoisomerases are tightly coupled (5,8,44,61,62,150,157). Because these reactions take place in a highly concerted fashion, it often is difficult to specifically ascribe a given effect to either

catalytic event. Several approaches have been employed to separate the DNA cleavage and ligation reactions and study them independently. “Suicide substrates” represent the primary approach that has been used in an attempt to isolate the forward DNA cleavage reaction of topoisomerase II (159-164). These DNA substrates all contain perturbations or non-B-form structures, such as nicks, double strand/single strand junctions, or hairpins. With “normal” intact B-form substrates, both halves of the cleaved DNA intermediate are covalently attached to topoisomerase II. In contrast, when suicide substrates are cut by the enzyme, one of the DNA halves is held in place by non-covalent, rather than covalent, interactions. If the “non-covalent” portion of the cleaved DNA dissociates from topoisomerase II, the enzyme is unable to rejoin the original substrate. This process results in a time-dependent accumulation of cleavage complexes (159-164). Although results with suicide substrates often are interpreted as representative of a unidirectional forward cleavage reaction, evidence suggests that topoisomerase II actually establishes a short-lived cleavage/ligation equilibrium with these substrates prior to the dissociation event (162). As a result, some conditions that impair the ability of topoisomerase II to reseal DNA breaks could increase the rate of accumulation of “trapped” cleavage complexes, even though they have no direct effect on the DNA scission reaction. This could lead to erroneous mechanistic conclusions regarding the actions of anticancer drugs or other topoisomerase II poisons.

In contrast to DNA cleavage, a number of approaches have been utilized to isolate the ligation reaction of topoisomerase II (33,60,165-169). These include ligation of cleavage complexes generated with suicide substrates to acceptor oligonucleotides, manipulation of the divalent cation, shifting reactions to suboptimal temperatures, or the

use of oligonucleotide substrates with activated 5'-terminal phosphates. The last approach has the advantage that it does not require the enzyme to cleave the substrate prior to ligation (168,169).

The goal of the work described in this chapter was to uncouple the DNA cleavage reaction of topoisomerase II from ligation. To this end, a novel system was developed that employed oligonucleotide substrates containing a 3'-bridging phosphorothiolate at the scissile bond. Similar DNA substrates containing 5'-bridging phosphorothiolates have been used to provide important mechanistic information about human, bovine, and pox virus type IB topoisomerases as well as bacteriophage lambda integrase (153,170-173). [5'-Bridging phosphorothiolates were used in these studies because type IB topoisomerases and lambda integrase form covalent links to the 3'-terminal phosphates of cleaved DNA (11,22,24,174), as opposed to the 5'-linkage formed with eukaryotic topoisomerase II (5,8,22,26,27,44,61,62).] 5'-Bridging phosphorothiolate oligonucleotides ligate extremely poorly (rates of ligation mediated by bovine topoisomerase I were <1% those observed for corresponding wild-type oligonucleotides) (171), and have been used in structural studies to trap the cleavage complex formed with human topoisomerase I (175-180).

The use of 3'-bridging phosphorothiolate oligonucleotides to study topoisomerase II mechanism has significant advantages over previous systems. In contrast to suicide substrates, phosphorothiolate-containing oligonucleotides are fully double-stranded B-form DNA and display no evidence of being ligated by topoisomerase II. Results with human topoisomerase IIα indicate that phosphorothiolate-containing oligonucleotides can be utilized effectively to examine the DNA cleavage reaction of the enzyme in isolation

and provide information regarding the mechanism of action of type II topoisomerases and topoisomerase II poisons.

Results and Discussion

3'-Bridging Phosphorothiolates

The forward DNA cleavage reaction of type II topoisomerases has been difficult to study because it exists in a tightly coupled equilibrium with the reverse ligation reaction (5,8,44,61,62,150,157). Furthermore, equilibrium levels of cleavage are low (5,8,44,60-62,71). Therefore, in order to establish a system that isolates topoisomerase II-mediated DNA scission from ligation and allows the unidirectional accumulation of cleavage complexes, double-stranded oligonucleotide substrates that contained a 3'-bridging phosphorothiolate at the scissile bond were synthesized (Figure 7).

In contrast to cleavage of wild-type DNA, which generates a 3'-terminal –OH moiety, cleavage of substrates that contain a 3'-bridging phosphorothiolate generates a 3'-terminal –SH group. While the terminal –OH group is a good nucleophile that readily facilitates ligation, the terminal –SH group is very poor nucleophile that does not efficiently attack the phosphotyrosyl protein-DNA bond (153) (171). Consequently, once phosphorothiolate substrates are cleaved, they should not be able to support appreciable levels of topoisomerase II-mediated DNA ligation.

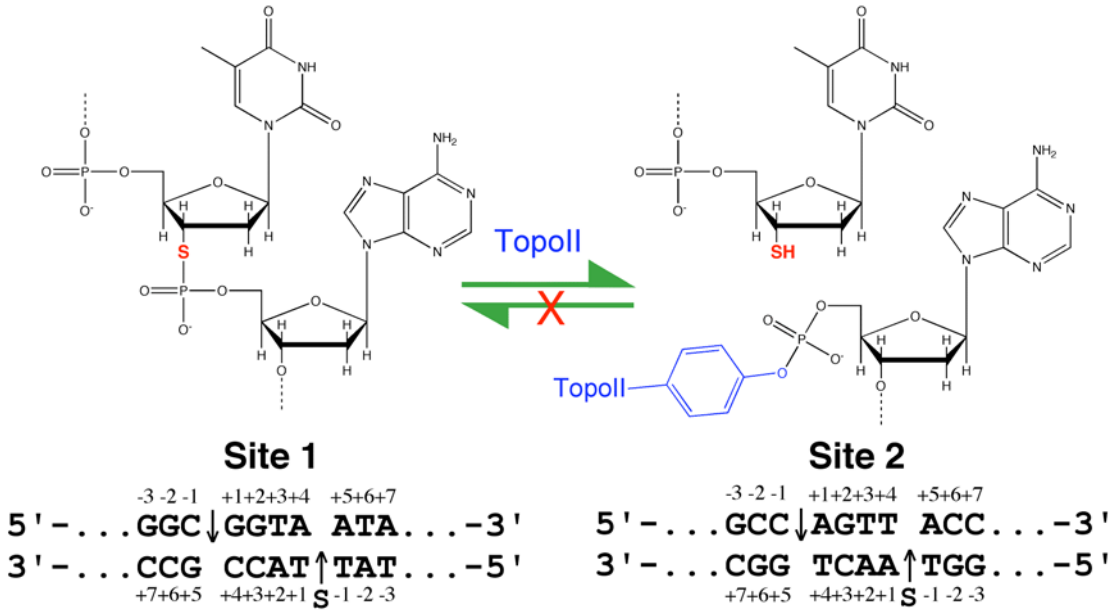

Figure 7: Schematic of topoisomerase II-mediated cleavage of DNA containing a 3'-bridging phosphorothiolate. As diagrammed in the top of the figure, the oxygen at the 3'-bridging position of the scissile bond is replaced with sulfur. Cleavage of this substrate generates a 5'-terminal phosphate that is covalently attached to the enzyme and a 3'-terminal –SH moiety (instead of the –OH group seen with wild-type DNA). In contrast to –OH, the –SH moiety is a poor nucleophile in this context and cannot readily support ligation. The sequence of the central portions of site 1 and site 2 are shown at the bottom. The phosphorothiolate modification (denoted "S") is on the bottom strand of these sequences at the scissile bond (–1/+1 position). Scissile bonds are denoted by arrows.

Cleavage of Oligonucleotide Substrates Containing 3'-Bridging Phosphorothiolates by Human Topoisomerase IIα

The ability of human topoisomerase IIα to cleave a 5' end-labeled oligonucleotide substrate (denoted site 2 in Figure 7) containing a 3'-bridging phosphorothiolate at the scissile bond is shown in Figure 8. Scission took place at the predicted scissile bond (lane 5), generating the expected 21-mer product (lane 2). Characteristic of a topoisomerase II-mediated DNA reaction, cleavage required both the human enzyme and a divalent cation (compare lane 5 to lanes 3 and 4). Following a 30-min incubation, cleavage of the phosphorothiolate-containing substrate was dramatically higher than observed with the wild-type phosphate-containing oligonucleotide (compare lanes 5 and 6). Similar results were seen for the site 1 phosphorothiolate oligonucleotide (not shown). Finally, when the site 2 phosphorothiolate oligonucleotide was incubated with $AgNO_3$, which hydrolyzes S-P bonds (154), a 21-mer product resulted (lane 8). This last control demonstrates both the presence of the 3'-bridging phosphorothiolate and its location at the scissile bond.

Although cleavage at the 3'-bridging phosphorothiolate requires topoisomerase IIα and Mg(II), it is possible that the environment of the enzyme active site promotes a cleavage event that is independent of the active site tyrosyl residues. A hallmark of topoisomerase II-mediated scission is a covalent linkage between the cleaved DNA and the protein. Therefore, the 3'-bridging phosphorothiolate site 2 substrate was labeled on its 3'-terminus to determine whether the product was covalently linked to topoisomerase IIα (Figure 9). Once again, cleavage was observed only in the presence of enzyme. Following treatment with proteinase K, cleavage products were seen as a diffuse series of bands that ran with a mobility that was faster than that of the substrate band (lane 3).

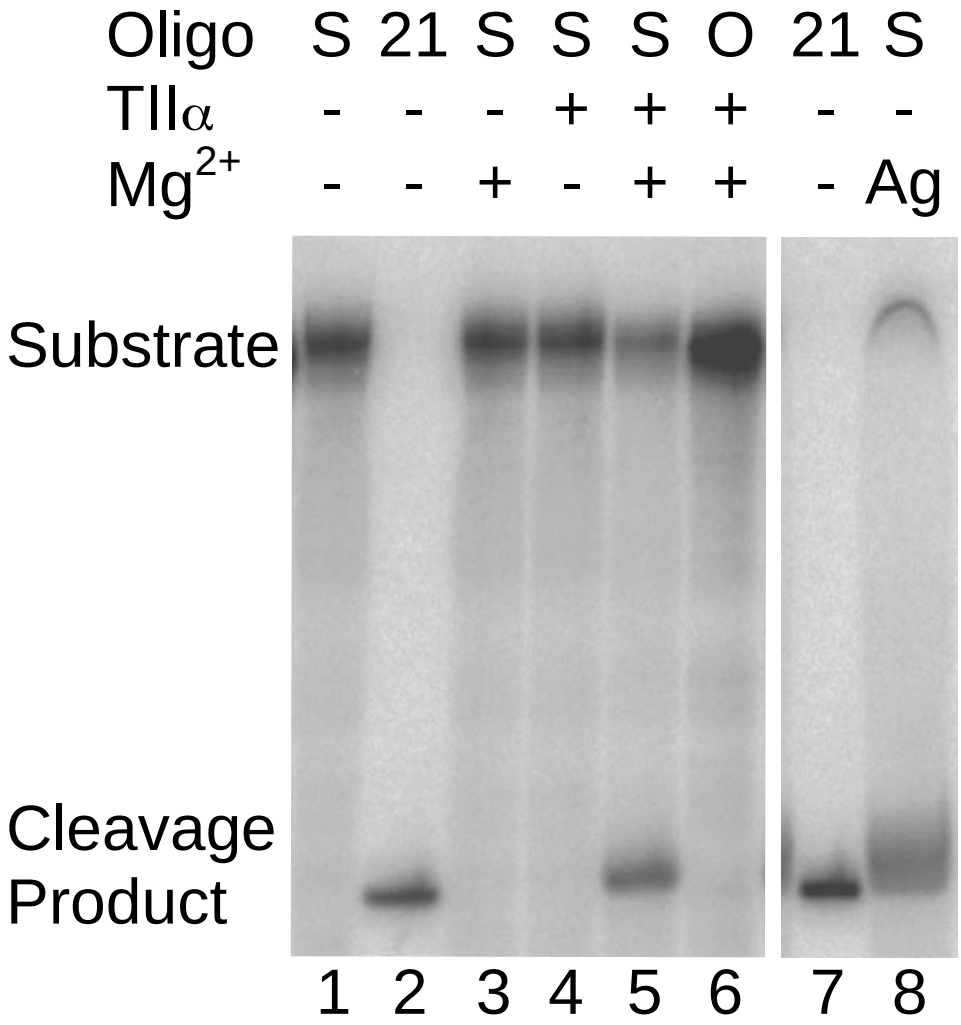

Figure 8: Topoisomerase IIα-mediated cleavage of the site 2 oligonucleotide containing a 3'-bridging phosphorothiolate at the scissile bond. An autoradiogram of a polyacrylamide gel is shown. The substrate is denoted at the top (Oligo): S, 3'-bridging phosphorothiolate substrate; O, wild-type substrate; 21, 21-mer marker. The presence of enzyme (TIIα) or divalent cation (Mg^{2+}) is denoted by a plus (+) and the absence by a minus (–). The migration of the 50-mer substrate and the 21-mer cleavage product are denoted on the left side. It is notable that the presence of the 3'-terminal –SH moiety slightly retards the electrophoretic mobility of phosphorothiolate cleavage products as compared to wild-type standards with 3'-terminal –OH groups. The addition of $AgNO_3$ to cleave the S-P bond is denoted by Ag in lane 8. For this and all subsequent figures, the concentrations of topoisomerase IIα and oligonucleotide substrate were 200 nM and 100 nM, respectively. Data are representative of four independent experiments.

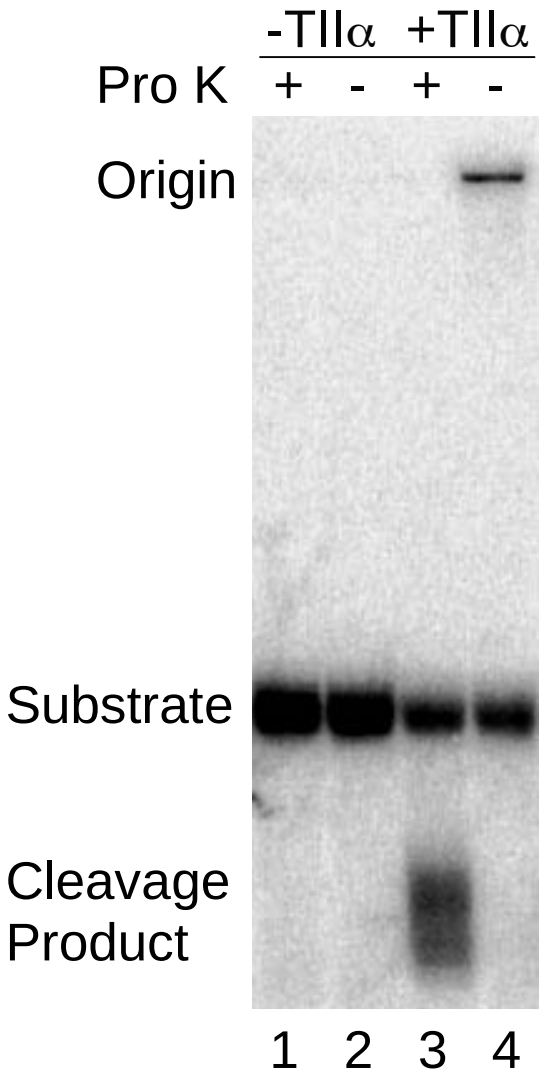

Figure 9: Covalent protein-DNA linkage occurs when the site 2 oligonucleotide containing a 3'-bridging phosphorothiolate is cleaved by topoisomerase IIα. An autoradiogram of a polyacrylamide gel is shown. Substrates were 3' end-labeled and reacted with topoisomerase IIα. Lanes 1 and 2 were in the absence of enzyme (–TIIα) with (+) or without (–) proteinase K (Pro K). Lane 3 and 4, were in the presence of enzyme (+TIIα) with or without proteinase K. Data are representative of three independent experiments.

These product bands are indicative of cleaved oligonucleotides that are linked to small peptides that are not completely digested by proteinase K. To confirm the protein-DNA linkage, proteinase K was omitted from the reaction (lane 4). The cleavage product bands disappeared and were replaced by a DNA band that remained at the origin. These results demonstrate that cleavage products generated with a 3'-bridging phosphorothiolate at the scissile bond are covalently attached to topoisomerase IIα. Together with the data shown in Figure 8, these findings provide strong evidence that topoisomerase IIα cleaves the phosphorothiolate oligonucleotide in a manner that is consistent with the normal scission reaction of the enzyme.

Time courses for the cleavage of site 1 and site 2 by human topoisomerase IIα are shown in Figure 10. Comparable results were observed with both sequences. Two striking features were seen. First, levels of cleavage of the phosphorothiolate substrate were dramatically higher (~10– to 100–fold at 30 min) than were generated with the comparable wild-type oligonucleotides. Second, while a cleavage/ligation equilibrium was reached rapidly with both wild-type substrates, levels of cleaved phosphorothiolate oligonucleotides continued to accumulate until 20–30 min. Both of these features are characteristic of reactions in which the ability of topoisomerase IIα to ligate cleaved DNA molecules is either blocked or severely compromised. Similar results were seen when the site 2 phosphorothiolate substrate was cleaved by yeast topoisomerase II or human topoisomerase IIβ (Figure 11). Albeit, levels of cleaved products were somewhat lower with these latter two enzymes. Taken together, these findings indicate that 3'-bridging phosphorothiolates can be used with a variety of type II topoisomerases and DNA cleavage sites.

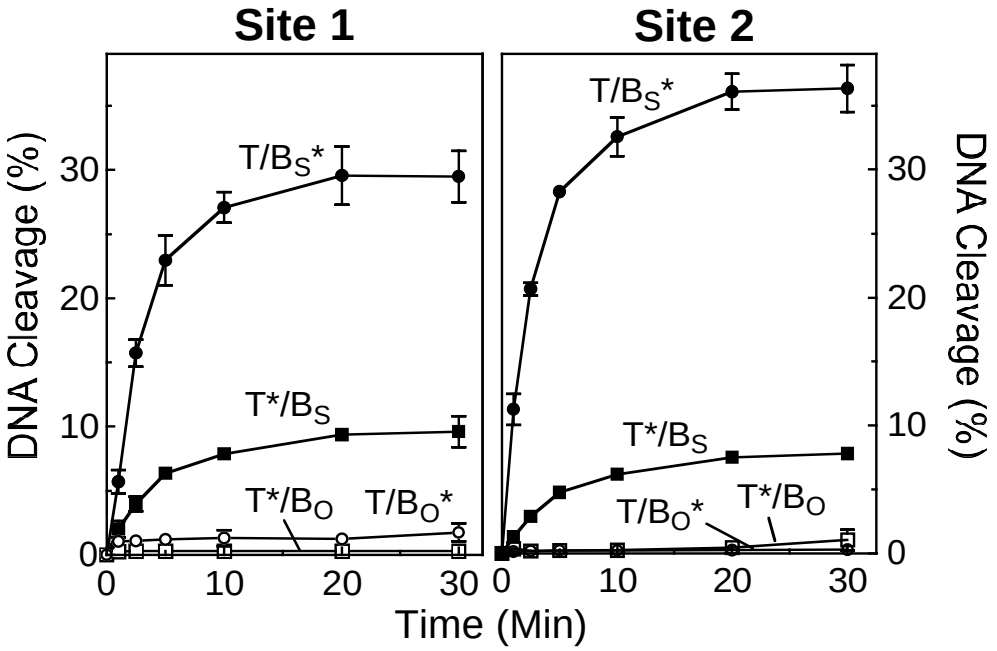

Figure 10: Time-courses for cleavage of oligonucleotide substrates by topoisomerase IIα. Results for site 1 (*left panel*) and site 2 (*right panel*) are shown. The asterisk (*) denotes the 5' end-labeled strand (top, T; or bottom, B) on which cleavage was monitored. The wild-type duplex containing an O-P scissile bond (subscript O) is denoted by T/B_O* for the labeled bottom strand (open circles) and T^*/B_O for the labeled top strand (open squares). The phosphorothiolate substrate containing an S-P scissile bond (subscript S) is denoted by T/B_S* for the labeled phosphorothiolate bottom strand (closed circles) and T^*/B_S for the labeled wild-type top strand (closed squares). Error bars represent the standard deviation of at least three independent experiments.

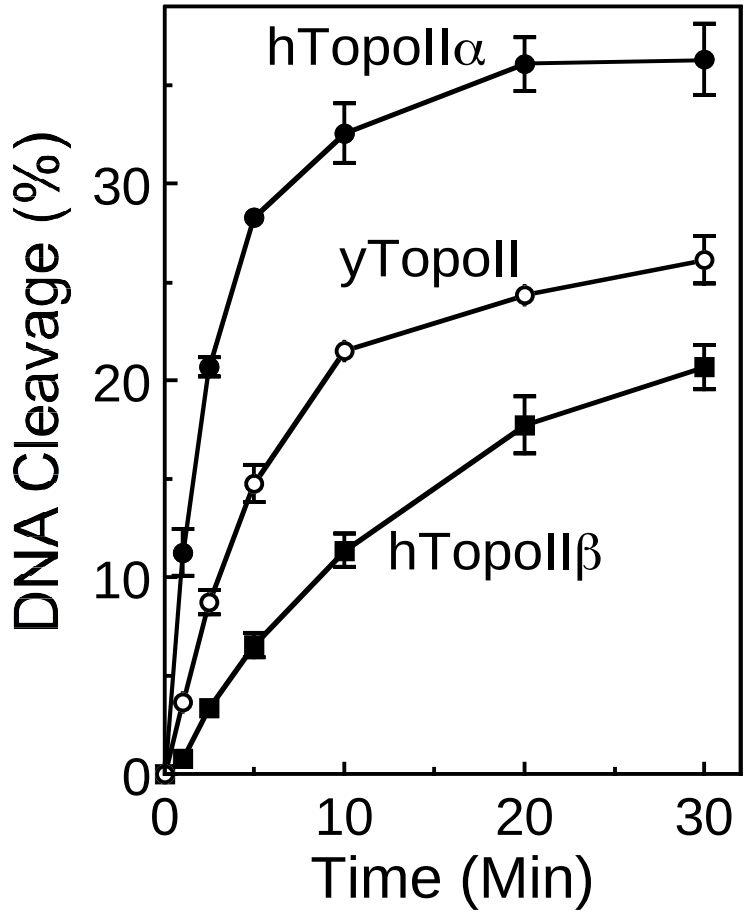

Figure 11: Ability of eukaryotic type II topoisomerases to cleave the phosphorothiolate substrate. Time courses are shown for cleavage of the bottom strand of a site 2 oligonucleotide containing a phosphorothiolate S-P linkage at the scissile bond of the bottom strand by human topoisomerase IIα (hTopoIIα, closed circles), human topoisomerase IIβ (hTopoIIβ, closed squares), and yeast topoisomerase II (yTopoII, open circles). Error bars represent the standard deviation of at least three independent experiments.

Cleavage of 3'-Bridging Phosphorothiolate Substrates is Sequence Specific

Type II topoisomerases cleave DNA with an intrinsic specificity (63). The above experiments demonstrate that human topoisomerase IIα cleaves an S-P linkage when it is situated at the scissile bond of an established topoisomerase II-DNA recognition sequence. However, it is not known whether the ability of the enzyme to cut the S-P linkage requires the bond to be situated at a "normal" cleavage site or is independent of the intrinsic sequence specificity of the protein.

To address this critical issue, the phosphorothiolate of site 1 was moved one position from the natural scissile bond, bridging the -1/+1 nucleotides, to a location bridging the +1/+2 nucleotides (Figure 12). In contrast to the high levels of scission generated when the S-P linkage was situated at the normal -1/+1 position (compare lanes 1 and 2), virtually no cleavage was seen at the S-P bond when it was moved to the +1/+2 position (compare lanes 4 and 6). As a control, to ensure that the oligonucleotide with the +1/+2 S-P bond was capable of being recognized and cleaved by topoisomerase IIα, scission at the -1/+1 position was monitored. While cleavage at the normal scissile bond was decreased somewhat compared to that seen with the completely wild-type oligonucleotide (compare lanes 1 and 3), a significant level of scission still was observed. Taken together, these data demonstrate that the specificity of topoisomerase IIα is not altered by the presence of the 3'-bridging phosphorothiolate.

Cleavage of 3'-Bridging Phosphorothiolates by Topoisomerase IIα Is Not Reversible

Topoisomerase II-mediated cleavage of oligonucleotides that contain a 3'-bridging phosphorothiolate at the scissile bond generate products with a 3'-terminal –SH group

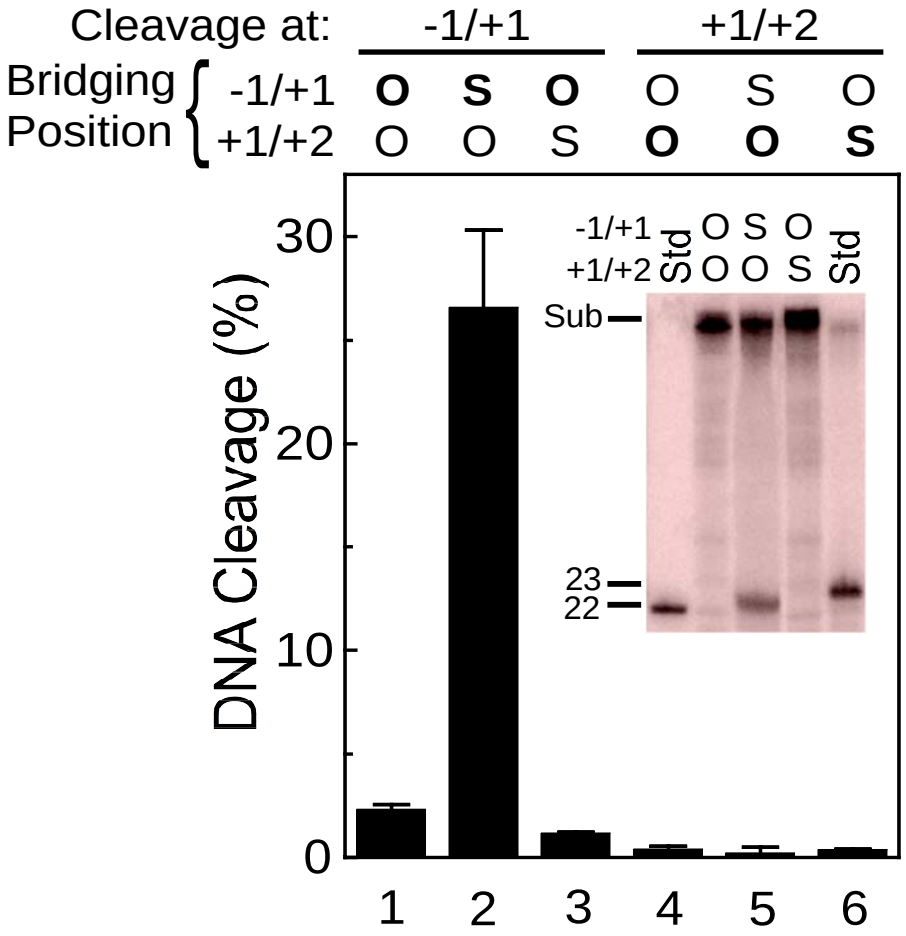

Figure 12: Site-specificity of DNA cleavage mediated by topoisomerase IIα is not altered by the location of the 3'-bridging phosphorothiolate. Cleavage was monitored on three versions of the site 1 substrate: wild-type duplex containing an O-P linkage at both the –1/+1 and +1/+2 positions; duplex containing an S-P linkage at the normal scissile bond (–1/+1 position) and an O-P linkage at the +1/+2 position; and duplex containing an O-P linkage at the normal scissile bond (–1/+1 position) and an S-P linkage at the +1/+2 position. Cleavage was quantified at the –1/+1 position (lanes 1-3, 22-mer product) and the +1/+2 position (lanes 4-6, 23-mer product). The nature of the linkage at the bridging position (O-P linkage, O; S-P linkage, S: bolded when being monitored) is denoted at the top of the figure. A representative gel is shown in the *inset*. The positions of the intact 50-mer substrate (Sub) as well as 22-mer and 23-mer standards (Std) representing cleavage products at the -1/+1 and +1/+2 sites, respectively, are indicated. As noted in Figure 2, the presence of the 3'-terminal –SH moiety slightly retards the electrophoretic mobility of phosphorothiolate cleavage products as compared to wild-type standards with 3'-terminal –OH groups. Error bars represent the standard deviation of three independent experiments.

(see Figure 7). Previous studies with type I topoisomerases and 5'-bridging phosphorothiolate substrates suggest that the –SH group should not be able to attack the covalent enzyme-DNA phosphotyrosine bond with any level of efficiency (153,171).

Therefore, to determine whether topoisomerase IIα is able to rejoin cleaved phosphorothiolate oligonucleotides, reactions were terminated with either EDTA or 0.5 M NaCl prior to the addition of SDS. It is believed that Mg(II), which is essential for both cleavage and ligation, can be chelated by EDTA only after ligation has taken place. Consequently, treatment with EDTA allows ligation to take place but prevents re-cleavage (62,157). Similarly, treatment with high salt allows ligation, but promotes dissociation of topoisomerase II from the DNA after it is no longer covalently attached to the nucleotide substrate. Once again, this leads to a unidirectional closure of the cleaved oligonucleotide (61,157). As expected, when wild-type site 1 (not shown) or site 2 (Figure 13, *left panel inset*) oligonucleotide was used as substrate, termination of a 30 min DNA scission reaction with EDTA or NaCl led to a significant reduction in the level of cleaved products. For example, greater than 90% of the cleaved oligonucleotides were ligated following a 5-min incubation with 0.5 M NaCl.

In marked contrast, no decrease in cleaved products was seen in parallel reactions that employed either site 1 (not shown) or site 2 (Figure 13, *left panel*) phosphorothiolate oligonucleotide, even when incubation periods in EDTA or NaCl were extended to 30 min (not shown). Furthermore, time courses for 30-min DNA cleavage reactions terminated with SDS, EDTA, or NaCl were essentially superimposable. These findings provide strong evidence that human topoisomerase IIα is incapable of ligating 3'-bridging phosphorothiolate cleavage products with any appreciable level of efficiency. Thus, 3'-

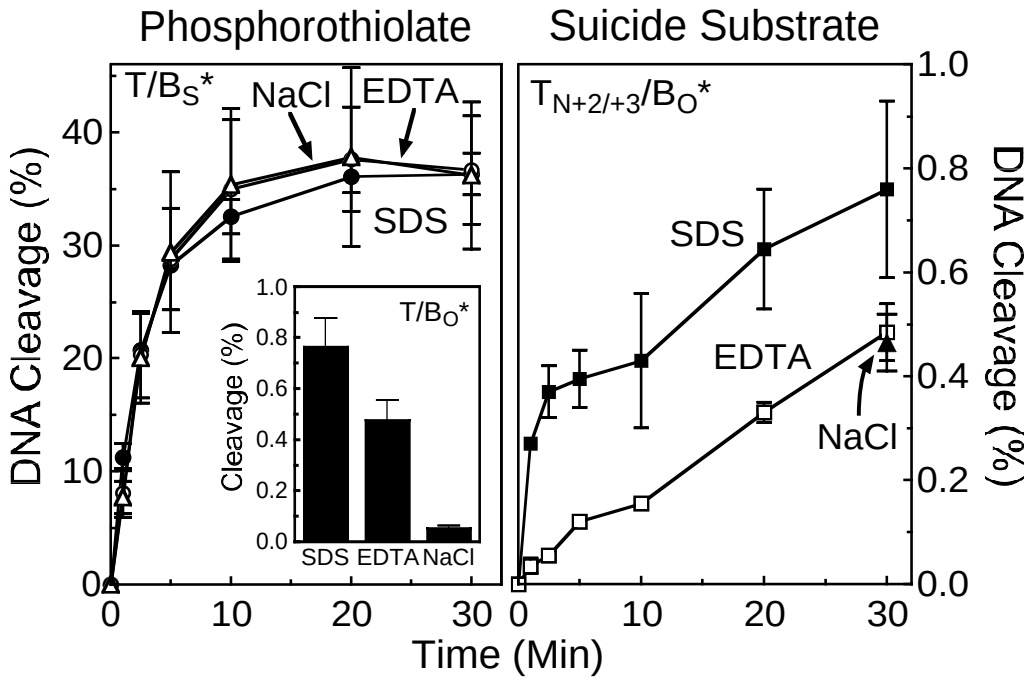

Figure 13: Reversibility of DNA cleavage mediated by topoisomerase IIα. *Left panel*, a time course is shown for cleavage of the site 2 oligonucleotide containing a 3'-bridging phosphorothiolate at the scissile bond (T/B$_S$*; see Figure 10 for nomenclature). Reactions were terminated with SDS (closed circles), EDTA (open circles), or NaCl (open triangles). *Left panel inset,* cleavage of the wild-type site 2 oligonucleotide containing an O-P linkage at the scissile bond (T/B$_O$*). A 30-min reaction terminated with SDS, EDTA, or NaCl is shown. *Right panel*, a time course is shown for cleavage of the site 2 suicide substrate containing a nick (N) between the +2 and +3 positions on the top strand (T$_{N+2/+3}$/B$_O$*). Reactions were terminated with SDS (closed squares), EDTA (open squares), or NaCl (30-min time point, closed triangle). Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.

-bridging phosphorothiolates allow the forward DNA scission reaction of topoisomerase IIα to be studied in isolation from the ligation event.

Cleavage of Suicide Substrates by Topoisomerase IIα Is Reversible

The only other substrates that are believed to promote a unidirectional topoisomerase II-mediated DNA cleavage reaction are known collectively as "suicide substrates." These systems trap cleavage complexes by utilizing nucleic acid molecules that contain non-contiguous (*i.e.*, nicked) DNA (164), double strand/single strand junctions (160,161,163), or unusual secondary structure (*i.e.*, hairpins) (159,162). When suicide substrates are cut by topoisomerase II, a segment of the cleaved DNA is able to dissociate from the active site, rendering the enzyme unable to ligate the original substrate (161). This process results in a time-dependent accumulation of cleavage complexes.

One obvious advantage of 3'-bridging phosphorothiolates over "traditional" suicide substrates is that the former are composed of intact duplex B-form DNA molecules. More importantly, evidence with a hairpin molecule suggests that topoisomerase II actually establishes a short-lived cleavage/ligation equilibrium with suicide substrates prior to the dissociation event (162). If this is the case, then the use of these substrates to monitor the forward scission reaction may not be appropriate.

Therefore, to compare cleavage of an S-P linkage to that of a traditional suicide substrate, a site 2 oligonucleotide containing a nick between the +2 and +3 positions of the top strand was synthesized. In contrast to scission of the intact wild-type substrate, which reached cleavage/ligation equilibrium within 5 min, cleaved products of the nicked oligonucleotide accumulated throughout the 30 min time course when the reaction was

terminated with SDS (Figure 13, *right panel*). This finding demonstrates that the nicked oligonucleotide acts as a suicide substrate for topoisomerase IIα.

To examine the ability of the human enzyme to ligate the nicked suicide substrate, a parallel time course for scission was terminated with EDTA. As seen in Figure 13 (*right panel*) cleavage levels were significantly lower in the EDTA-treated reactions. A similar result was observed when a 30 min reaction was terminated by the addition of 0.5 M NaCl (see indicated data point in Figure 13, *right panel*). These findings indicate that a significant proportion of the initial cleavage complexes formed with the suicide substrate are reversible.

It is notable that the relative fraction of cleavage complexes that was irreversible (*i.e.*, not ligated following treatment with EDTA) rose over time. While levels of irreversible cleavage with the suicide substrate represented only ~10% of the total scission observed at 1 min, they increased to ~50% by 20-30 min. This result provides further evidence that suicide substrates (at least the nicked substrate employed in the present study) require a time-dependent conversion from a reversible cleavage complex to an irreversible DNA strand break. Presumably, this conversion reflects the point at which the non-covalent cleavage product dissociates from topoisomerase II.

Thus, while traditional suicide substrates represent an important system to accumulate topoisomerase II-DNA cleavage complexes and to provide starting material for unidirectional ligation assays, they do not segregate the forward enzyme-mediated DNA scission reaction from the cleavage/ligation equilibrium. Consequently, the use of these substrates to analyze the forward cleavage reaction of topoisomerase II may lead to erroneous mechanistic conclusions.

Coordination Between the Two Protomer Subunits of Human Topoisomerase IIα During DNA Cleavage

Although topoisomerase II must generate a double-stranded nucleic acid break in order to carry out the DNA passage reaction, results from numerous laboratories indicate that the two protomer subunits of eukaryotic type II enzymes do not cleave and ligate DNA in a totally concerted fashion. For example, it has long been known that equilibrium levels of DNA strand breaks at the two scissile bonds of a cleavage site (60,61,71,181) and rates of ligation of the two strands are often different (60). Furthermore, alterations of base sequence (or the inclusion of abasic sites) that have a direct affect on rates of ligation of one strand have little effect on rates of ligation of the other strand (33,168). Finally, changes in base sequence that enhance the ability of etoposide to inhibit ligation at one scissile bond have no significant effect on rates of ligation at the opposite scissile bond (72).

The above notwithstanding, the same alterations in DNA sequence that specifically enhance the ability of etoposide to inhibit ligation at one scissile bond lead to a modest increase in topoisomerase IIα-generated double-stranded breaks at that cleavage site (72). This finding implies that there may be some communication between the protomer subunits of the human enzyme during the DNA cleavage event, even though there appears to be little coordination during ligation.

To address the issue of subunit communication, levels of cleavage on the top strand of site 1 and site 2 were monitored in the absence or presence of a 3'-bridging phosphorothiolate on the bottom strand (see Figure 10). As expected, when situated opposite a wild-type O-P scissile bond, rapid DNA cleavage/ligation equilibria

characterized by low levels of scission were established on the top strand of both sites. However, when situated opposite an S-P scissile bond that did not allow ligation, levels of cleavage on the top strand of both sites were ~5– to 7–fold higher as compared to comparable wild-type substrates and rose over the course of the assay. Moreover, the time courses for the accumulation of DNA breaks on the top strands paralleled those seen for the bottom S-P strands.

These results indicate that there is at least some level of coordination between the two protomer active sites of topoisomerase IIα. However, since levels of cleavage on the strand opposite the S-P bond were several–fold lower than observed on the phosphorothiolate-containing strand, it is clear that DNA cleavage by the two protomer subunits does not take place in a completely concerted fashion.

The partial coordination between the protomer subunits of topoisomerase IIα may be explained by at least three independent mechanisms that are not mutually exclusive. First, cleavage of one scissile bond may decrease the ability of the enzyme to ligate the opposite scissile bond. This possibility seems unlikely, as it would provide no benefit to the catalytic reaction of topoisomerase II. In addition, previous studies have found little coordination between the protomer subunits of topoisomerase IIα during ligation (33,60,72). However, as a control, the rate of ligation of the top strand of site 2 was determined when it was situated opposite an O-P or an S-P scissile bond (Figure 14). As predicted, rates of ligation of the two substrates were comparable.

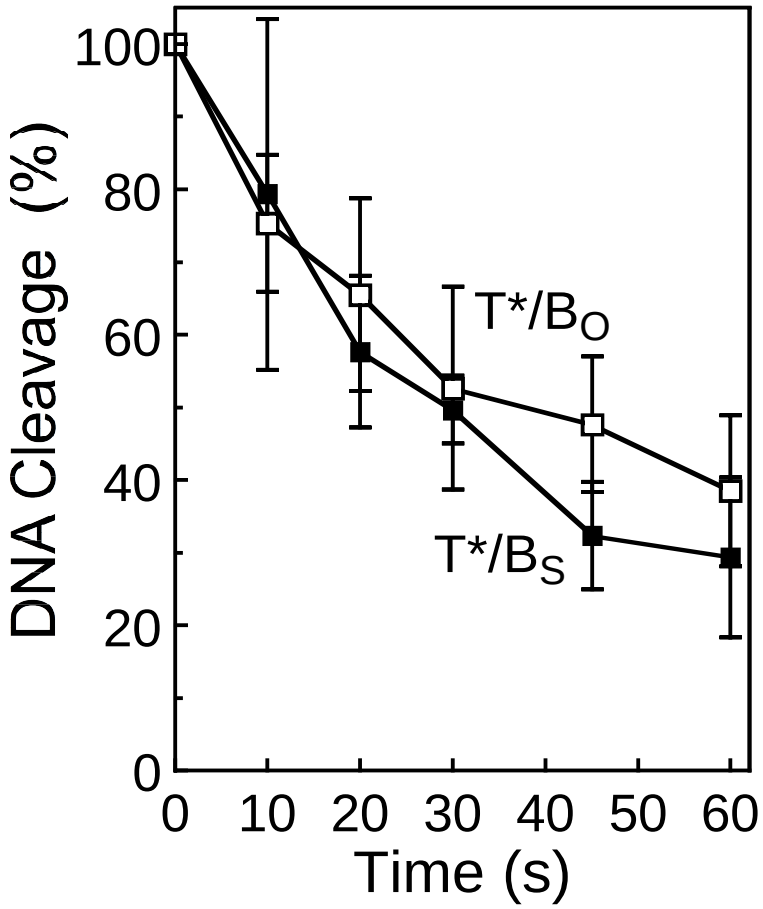

Figure 14: Time course for topoisomerase IIα-mediated ligation of the wild-type top strand of the site 2 oligonucleotide. Site 2 substrates containing either a wild-type O-P linkage (T*/B_O, open squares) or phosphorothiolate S-P linkage (T*/B_S, closed squares) at the scissile bond of the bottom strand were employed. The total percent cleavage prior to initiation of ligation was set to 100%. Error bars represent the standard deviation of at least three independent experiments.

Second, trapping a cleavage complex at one scissile bond should increase the concentration of enzyme that contains the opposite scissile bond in its active site. Since levels of cleavage are directly proportional to levels of topoisomerase II-DNA binding, this effect could account for the enhanced scission on the opposite strand. This mechanism requires no alteration in the forward rate of scission and is consistent with the finding that the accumulation of breaks on the top strand of site 1 and site 2 paralleled that seen for the bottom S-P strands (Figure 10).

Third, cleavage at one scissile bond may increase the rate of cleavage at the opposite scissile bond. As a first attempt to address this hypothesis, a site 2 oligonucleotide that contained a nick at the scissile bond (-1/+1 position) of the top strand was employed as a model of a DNA substrate that had already been cut on one of its two strands. It should be noted, however, that the nicked oligonucleotide is not covalently attached to topoisomerase II. Thus, it is unclear whether this substrate adequately mimics an enzyme-DNA cleavage complex in which one of the two strands has been cut by topoisomerase II.

With the above caveat in mind, the ability of topoisomerase IIα to cleave the bottom strand of site 2 was monitored when the top scissile bond was either intact or nicked (Figure 15). The initial experiment utilized a substrate that contained a wild-type O-P linkage at the scissile bond of the bottom strand. Two observations were noted. First, levels of cleavage of the bottom strand increased ~20–fold when situated opposite a nicked top strand. Second, the concentration of cleavage complexes reached a rapid equilibrium and did not rise over the time course of the experiment. This result indicates

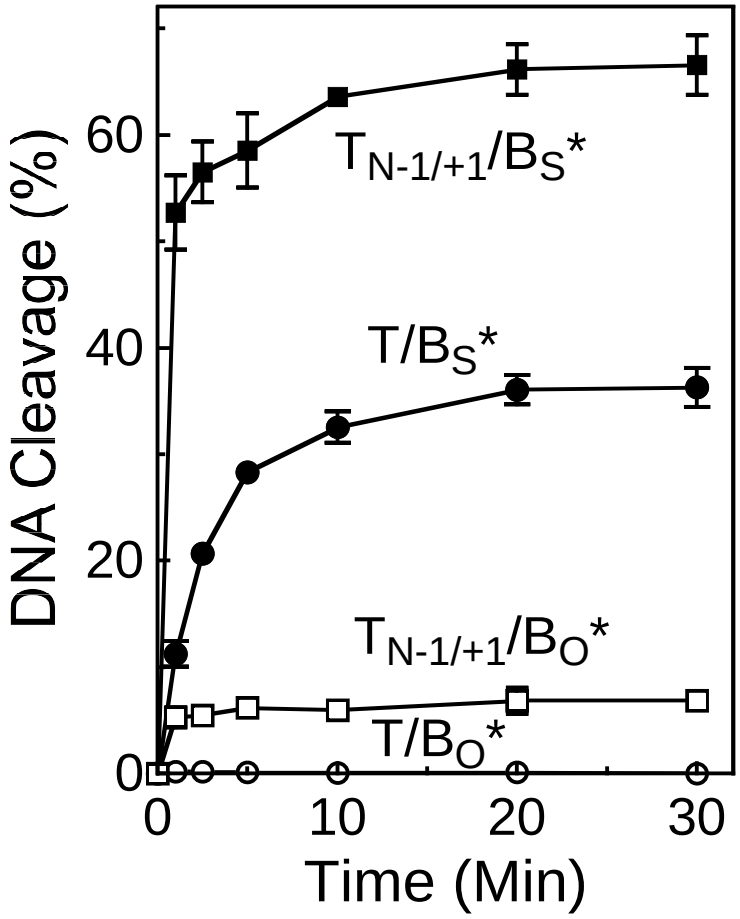

Figure 15: Topoisomerase IIα–mediated cleavage of a substrate containing a nick at one scissile bond. Time courses are shown for the following site 2 substrates: oligonucleotide containing an intact top strand and a wild-type O-P linkage at the scissile bond of the bottom strand (T/B_O*, open circles); oligonucleotide containing a nick at the scissile bond (–1/+1 position) of the top strand and a wild-type O-P linkage at the scissile bond of the bottom strand ($T_{N-1/+1}/B_O$*, open squares); oligonucleotide containing an intact top strand and a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T/B_S*, closed circles); and oligonucleotide containing a nick at the scissile bond of the top strand and a phosphorothiolate S-P linkage at the scissile bond of the bottom strand ($T_{N-1/+1}/B_S$*, closed squares). Error bars represent the standard deviation of at least three independent experiments.

that unlike the nicked +2/+3 substrate utilized in Figure 13, the nicked scissile bond (-1/+1) oligonucleotide did not act as a suicide substrate for topoisomerase IIα.

To determine whether the enhanced cleavage observed when a wild-type strand was situated opposite a nicked scissile bond reflected an increase in the forward rate of DNA cleavage, the experiment was repeated using a site 2 substrate that contained a 3'-bridging phosphorothiolate at the scissile bond of the bottom strand (Figure 15). When situated opposite the nicked strand, the rate of cleavage of the bottom S-P scissile bond rose considerably. Although rates were too rapid to measure accurately, it appears that they increased several–fold. These data imply that cleavage on one strand by topoisomerase II has the capacity to increase the rate of cleavage on the opposite strand. Whether the increased rate of scission reflects a change in the conformation of the DNA, the enzyme, or a combination of both remains to be determined.

Taken together, the above studies suggest that at least two mechanisms may contribute to the partial coordination between the two subunits of topoisomerase IIα: increased concentration of the second scissile bond in the active site of the enzyme and an enhanced rate of scission of the second strand following cleavage of the first. This issue is addressed in greater detail in Chapter IV.

Effect of ATP on the Forward DNA Scission Reaction of Human Topoisomerase IIα

For topoisomerase II-mediated DNA strand passage to occur, both strands of the "gate" segment must be cleaved (5,8,22,26,27,44). ATP binding (one molecule per topoisomerase II protomer) induces opening of the DNA gate (5,8,22,26,27,29,44,182,183), and it is believed that hydrolysis of one of the ATP molecules enhances the rate of strand

passage (184). Several laboratories have demonstrated that levels of topoisomerase II-DNA cleavage complexes rise in the presence of the high-energy cofactor (5,8,22,26,27,44). Although the mechanistic basis for this observation is not known, one study reported that rates of DNA ligation were slowed in the presence of the non-hydrolyzable ATP analog APP(NH)P (166). To determine whether ATP has an effect on the forward DNA scission reaction of human topoisomerase IIα, the ability of the enzyme to cleave the 3'-bridging phosphorothiolate substrate (site 2) in the presence and absence of the nucleotide triphosphate was compared. As seen in Figure 16, ATP had virtually no effect on the rate of DNA scission. Therefore, it is concluded that the increased concentration of cleavage complexes seen in the presence of ATP is not due to an enhanced forward rate of cleavage.

Dependence of the Forward Scission Reaction of Topoisomerase IIα on DNA Length

A previous study demonstrated that the ability of human topoisomerase IIα and chlorella virus topoisomerase II to form cleavage complexes was dependent on the length of the DNA substrate (150). Consistent with the footprint of topoisomerase II on DNA (~28 bp) (185), a minimal requirement for a 20-bp oligonucleotide was noted and substantial scission was not seen until a substrate of ~40 bp was employed. Levels of cleavage complexes did not correlate with changes in binding affinity (150). Consequently, they must be due to a change in the ability of topoisomerase II to either cleave or ligate short substrates.

To determine whether the effect of DNA length on cleavage complex formation was due to a decreased ability to cleave short substrates, a series of nested site 2

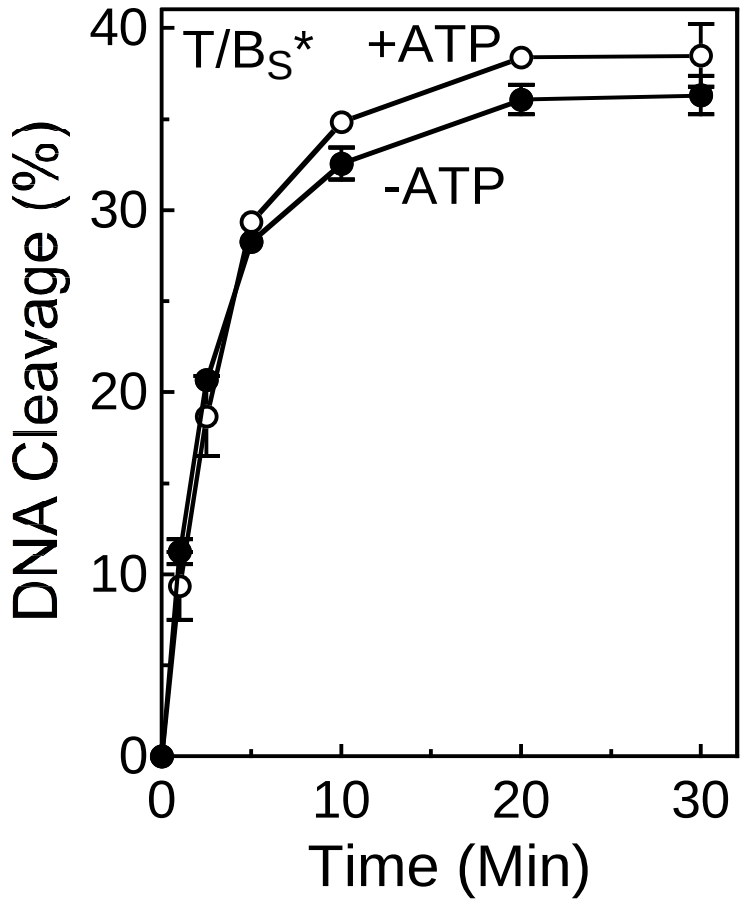

Figure 16: Topoisomerase IIα-mediated DNA cleavage in the presence of ATP. A time course is shown for cleavage of the site 2 substrate containing a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T/B_S*) in the absence (-ATP, closed circles) or presence of ATP (+ATP, open circles). Error bars represent the standard deviation of at least three independent experiments.

oligonucleotides with 3'-bridging phosphorothiolates at the scissile bond of the bottom strand was synthesized (Figure 17, *left panel*). As found previously with wild-type substrates, phosphorothiolate substrates displayed virtually no cleavage with the 20-mer, and low levels were seen with the 25- and 30-mer. A modest rise in cleavage was observed when substrate length was increased to 40 bp, followed by a dramatic rise when it was increased to 50 bp (the length of the parent oligonucleotide). These data indicate that the DNA length dependence of topoisomerase IIα is due to an effect on the forward scission reaction and imply that protein-DNA contacts outside of the active site of the enzyme may be required to promote cleavage.

Although topoisomerase IIα can sense the length of its substrate, only the central portion of the DNA needs to be duplex (Figure 17, *right panel*). When the nested oligonucleotides containing the S-P linkage at the scissile bond were annealed with the parental wild-type 50-mer top strand, a substantial increase in cleavage was observed as the length of the bottom strand was increased.

Effects of Topoisomerase II Poisons on the Forward DNA Cleavage Reaction of Human Topoisomerase IIα

Topoisomerase II poisons increase the concentration of cleavage complexes by two non-mutually exclusive mechanisms. Anticancer drugs, such as etoposide and amsacrine, strongly inhibit the ability of the enzyme to ligate cleaved DNA substrates (72,94,165,166,186,187). Because of their affect on ligation, it has been assumed that these agents have no significant effect on the forward scission reaction. In contrast, DNA lesions that poison topoisomerase II, such as abasic sites, have little effect on rates of ligation (64,135,136,156,163). Consequently, it has been assumed that they act primarily

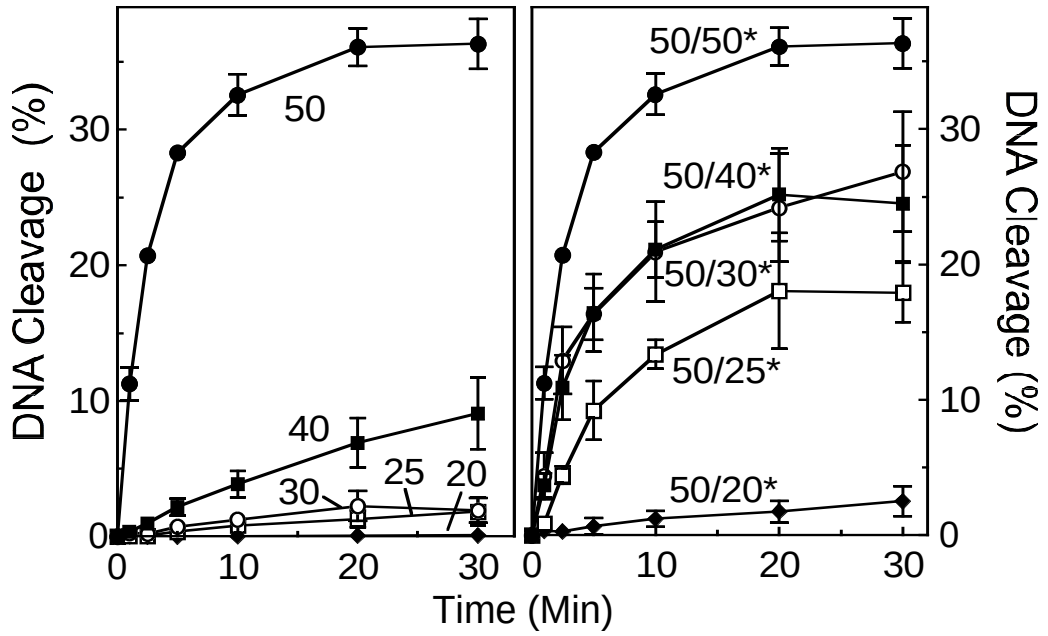

Figure 17: Effect of oligonucleotide length on DNA cleavage mediated by topoisomerase IIα. *Left panel*, time courses are shown for cleavage of the phosphorothiolate-containing bottom strand of nested site 2 substrates. Reactions were monitored with 50-mer (closed circles), 40-mer (closed squares), 30-mer (open circles), 25-mer (open squares), or 20-mer (closed diamonds) duplex substrates. *Right panel*, time courses are shown for cleavage of the phosphorothiolate-containing bottom strand of nested site 2 substrates containing a 50-mer top strand and a 50-mer (closed circles), 40-mer (closed squares), 30-mer (open circles), 25-mer (open squares), or 20-mer (closed diamonds) bottom strand. Error bars represent the standard deviation of at least three independent experiments.

by enhancing the forward rate of DNA scission. Unfortunately, it has not been possible to test these assumptions directly. Therefore, the ability of these topoisomerase II poisons to alter the rate of DNA scission was characterized using the phosphorothiolate substrates.

The effects of etoposide and amsacrine on the forward rate of cleavage of site 1 and site 2 are shown in Figures 18 and 19, respectively. In all cases, the drugs increased the concentration of cleavage complexes when wild-type oligonucleotides were employed. However, virtually no increase in the rate of scission was observed when 3'-bridging phosphorothiolate substrates were examined. These data strongly suggest that etoposide and amsacrine increase levels of topoisomerase IIα-DNA cleavage complexes primarily by inhibiting enzyme-mediated ligation.

As discussed above, suicide substrates establish a short-lived DNA cleavage/ligation equilibrium before irreversibly trapping cleavage complexes. Consequently, their use as a model for the forward topoisomerase II DNA cleavage reaction was questioned. For example, if etoposide inhibits enzyme-mediated ligation prior to the "suicidal" dissociation of the non-covalent DNA cleavage product, the drug could increase levels of trapped cleavage complexes independent of any effect on the forward rate of scission. To address this critical point, the effect of etoposide on the ability of topoisomerase IIα to cut the nicked suicide substrate described in Figure 13 was characterized (Figure 18, *lower panel*). Despite the fact that etoposide does not increase the forward rate of DNA scission, a dramatic rise in cleavage complexes was observed when the drug was included in reactions with the suicide substrate. This finding underscores the need to exercise

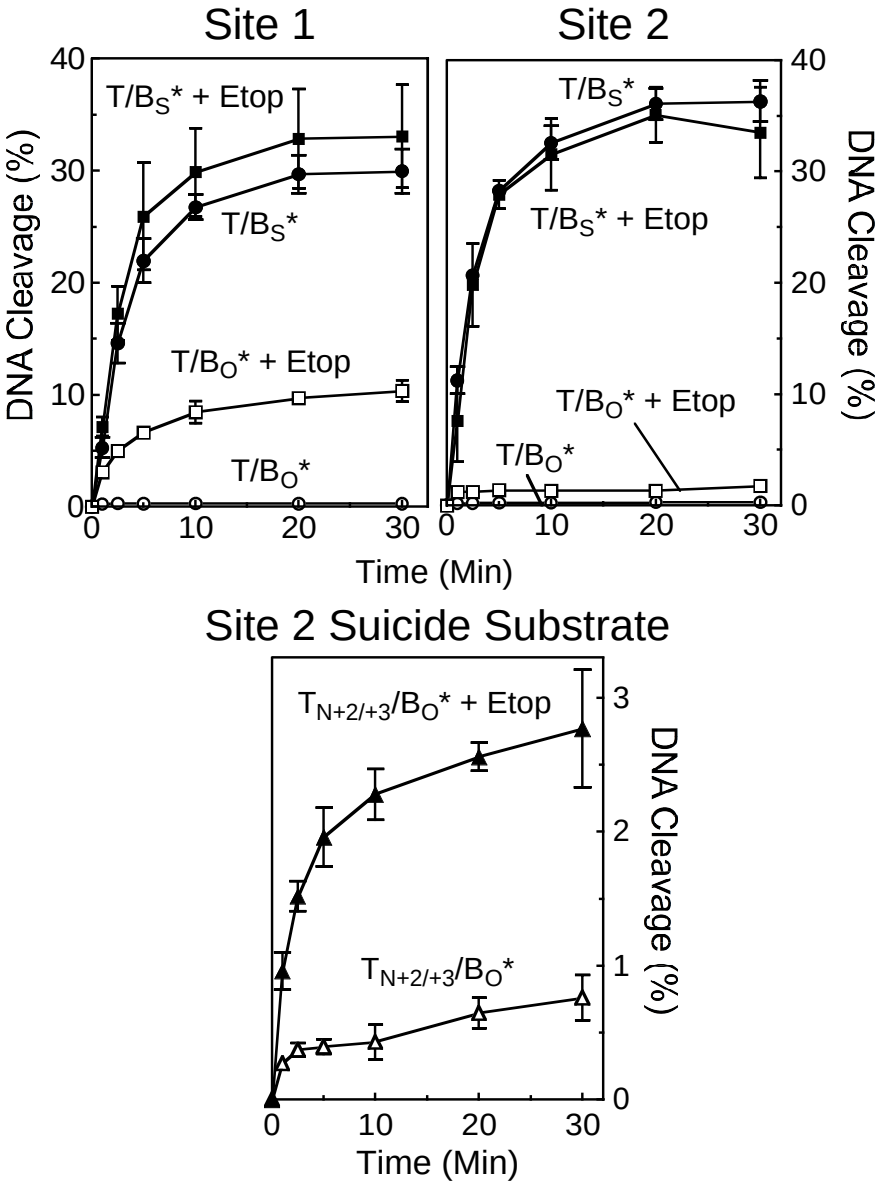

Figure 18: Effect of etoposide on the forward rate of topoisomerase IIα-mediated DNA scission. Time courses for cleavage of the bottom strand of the site 1 (*left panel*) and site 2 (*right panel*) substrates are shown for oligonucleotides containing a wild-type O-P linkage at the scissile bond of the bottom strand (T/B_O*) and oligonucleotides containing a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T/B_S*) in the absence (T/B_O*, open circles; T/B_S*, closed circles) or presence (T/B_O* + Etop, open squares; T/B_S* + Etop, closed squares) of 50 μM etoposide. The *bottom panel* shows time courses for cleavage of the bottom strand of the site 2 suicide substrate containing a nick at the +2/+3 position of the top strand in the absence ($T_{N+2/+3}/B_O$*, open triangles) or presence ($T_{N+2/+3}/B_O$* + Etop, closed triangles) of 50 μM etoposide. Error bars represent the standard error of the mean of two independent experiments or the standard deviations of at least three independent experiments.

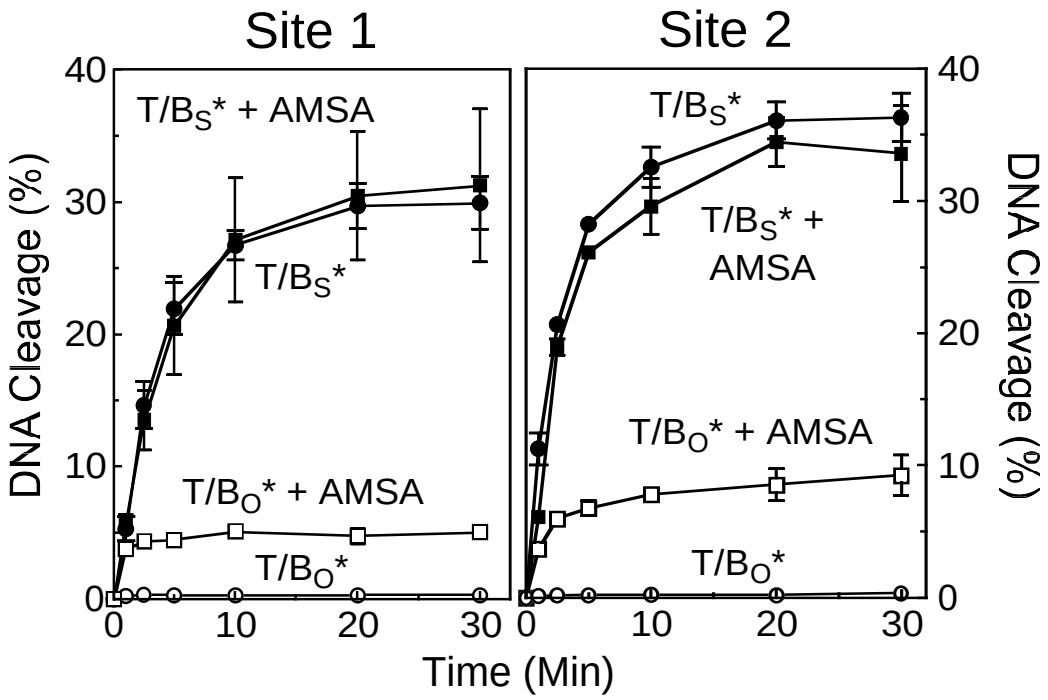

Figure 19: Effect of amsacrine on the forward rate of topoisomerase IIα-mediated DNA scission. Time courses for cleavage of the bottom strand of the site 1 (*left panel*) and site 2 (*right panel*) substrates are shown for oligonucleotides containing a wild-type O-P linkage at the scissile bond of the bottom strand (T/B_O*) and oligonucleotides containing a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T/B_S*) in the absence (T/B_O*, open circles; T/B_S*, closed circles) or presence (T/B_O* + AMSA, open squares; T/B_S* + AMSA, closed squares) of 50 μM amsacrine. Error bars represent the standard deviation of at least three independent experiments.

caution when drawing mechanistic conclusions about the forward rate of DNA cleavage based on the results obtained with suicide substrates.

Finally, when abasic sites are situated within the 4-bp region located between the two scissile bonds, their presence raises the concentration of topoisomerase II-DNA cleavage complexes (64,136,156,164). As seen in Figure 20, the inclusion of an abasic site at the +1 position of the top strand of site 1 or site 2 increased levels of cleavage complexes several–fold when substrates with wild-type O-P scissile bonds were used. In contrast to the anticancer drugs, which had little effect on the forward cleavage reaction, the abasic site stimulated the rate of DNA scission of substrates with S-P scissile bonds ~2– to 3–fold (Figure 20). Similar results were seen with substrates that contained an abasic site at the +2 or +4 position (not shown). These findings confirm the assumption that abasic sites act by stimulating the forward rate of topoisomerase II-mediated DNA scission.

Conclusions

Despite the importance of DNA cleavage to the cellular functions of type II topoisomerases and their role as targets for anticancer drugs, the tight coupling of the cleavage/ligation equilibrium has made it difficult to characterize the mechanism by which these enzymes cut the genetic material. To uncouple the forward DNA scission reaction of human topoisomerase IIα from the ligation event, a novel system that utilized 3'-bridging phosphorothiolates at the scissile bonds of well-defined topoisomerase II-DNA cleavage sites was developed. Cleavage of these substrates appeared to be irreversible, resulting in a unidirectional enzyme-mediated DNA scission

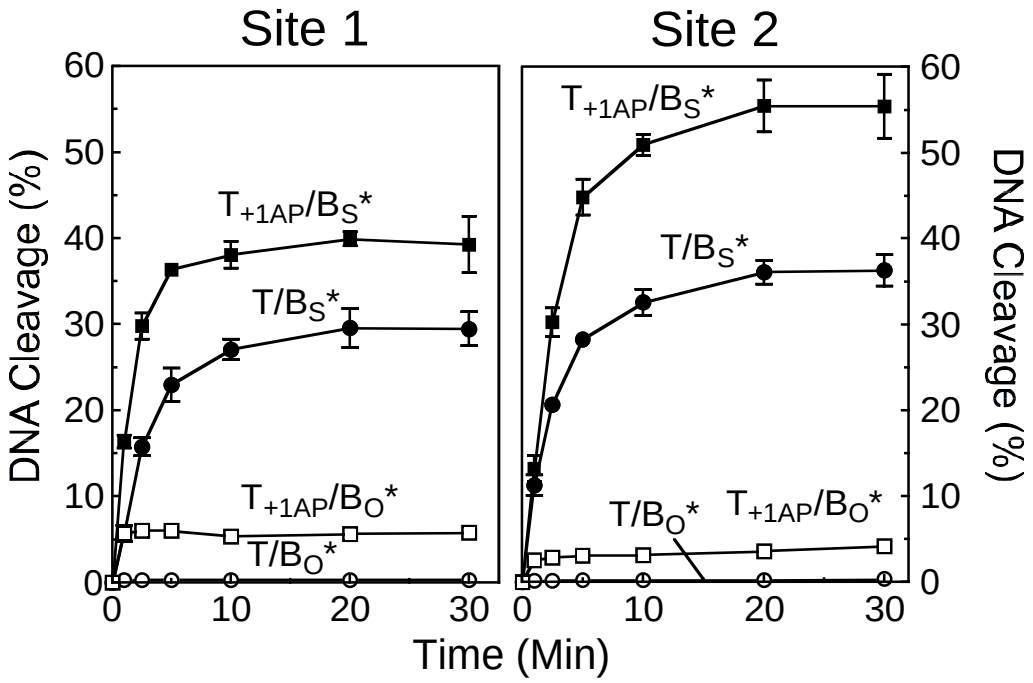

Figure 20: Effect of an abasic site on the forward rate of topoisomerase IIα-mediated DNA scission. Time courses are shown for the following site 1 (*left panel*) and site 2 (*right panel*) substrates: oligonucleotide containing a wild-type top strand and a wild-type O-P linkage at the scissile bond of the bottom strand (T/B_O*, open circles); oligonucleotide containing an abasic (AP) site at the +1 position of the top strand and a wild-type O-P linkage at the scissile bond of the bottom strand (T_{+1AP}/B_O*, open squares); oligonucleotide containing a wild-type top strand and a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T/B_S*, closed circles); and oligonucleotide containing an abasic site at the +1 position of the top strand and a phosphorothiolate S-P linkage at the scissile bond of the bottom strand (T_{+1AP}/B_S*, closed squares). Error bars represent the standard deviation of at least three independent experiments.

reaction. The use of oligonucleotides with 3'-bridging phosphorothiolates provided critical information on the coordination of the protomer subunits of topoisomerase IIα and the mechanism of action of topoisomerase II poisons. These substrates will be a valuable resource for future studies on DNA scission and the topoisomerase II-DNA cleavage complex.

CHAPTER IV

COORDINATING THE TWO PROTOMER ACTIVE SITES OF HUMAN TOPOISOMERASE IIα: NICKS AS TOPOISOMERASE II POISONS

Introduction

Given the central nature of the DNA cleavage reaction to the physiological and pharmacological functions of topoisomerase II, it is important to understand the mechanistic details of this process. In this regard, it has long been known that type II topoisomerases generate two "coordinated" nicks on the opposite strands of the double helix as opposed to one "unified" double-stranded DNA break. Evidence for a coordinated mechanism stems from the findings that levels of scission on the opposite strands of a cleavage site are often non-equal (61) and that the enzyme religates the two strands of cleaved molecules in a sequential fashion (60). Under equilibrium conditions, ~0.5–1% of human topoisomerase IIα in a scission reaction mixture exists as a covalent enzyme-cleaved DNA complex (188) [Figure 21]. Approximately half of these complexes contain double-stranded breaks with the remainder containing single-stranded DNA breaks. This finding suggests that every time the enzyme cuts one strand of the double helix, it goes on to cut the second strand a high proportion of the time. If the enzyme cut the two strands of DNA in a completely non-coordinated fashion, virtually no double-stranded breaks would be generated (1% cleavage of the first strand x 1% cleavage of the second strand ≈ 0.01% double-stranded breaks). Thus, there must be a relatively high degree of coordination between the two protomer active sites of

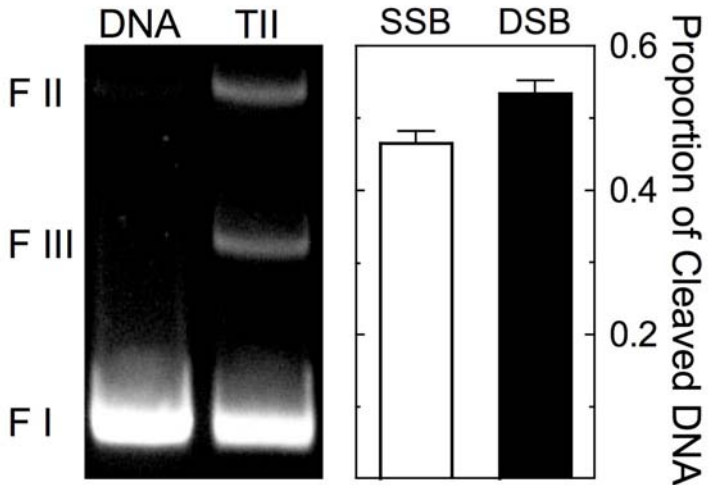

Figure 21: Coordination between protomer halves during plasmid DNA cleavage. An agarose gel from a topoisomerase II-DNA cleavage reaction in the absence (DNA) or presence (TII) of topoisomerase IIα is shown at left. The enzyme:plasmid ratio used for this experiment was 40:1. Supercoiled (F I), nicked (F II), and linear (F III) plasmid bands are denoted. The proportion of nicked plasmids (containing single-stranded breaks, SSB) and linear plasmids (containing double-stranded breaks, DSB) generated by topoisomerase II-mediated DNA cleavage (out of a total of 1.0) is shown at right. Error bars represent the standard deviation three or more independent experiments.

topoisomerase IIα, even if they do not act in complete concert. Consistent with the above conclusion, once topoisomerase II cleaves the first strand, it cuts the second strand at a rate that is >10–fold faster (70). However, the mechanism that underlies the enhanced cleavage of the second strand is not known.

It is notable that the nucleic acid substrates for first and second strand cleavage are not equivalent. Unlike the former substrate, which is an intact duplex, the latter contains a nick at one of the scissile bonds. Consequently, the previous chapter proposed that human topoisomerase IIα generates a high proportion of double-stranded DNA breaks because nicked molecules are a more efficient substrate for the enzyme as shown in Figure 15. To this point, equilibrium levels of cleavage on one strand of an oligonucleotide that contains a nick at the scissile bond on the opposite strand are >20–fold higher than those generated by the enzyme with the equivalent intact duplex (see Figures 24 and 25 discussed below). The present chapter explores the possible role of nicks in coordinating DNA cleavage by the two protomer subunits of topoisomerase IIα.

Results and Discussion

Coordination of the Two Protomer Subunits of Topoisomerase IIα

To more fully assess the potential of nicks to coordinate the two protomer active sites of topoisomerase IIα during DNA scission, the kinetics of cleavage of an intact substrate and an equivalent oligonucleotide that contained a nick at one of the scissile bonds were studied by rapid chemical quench. Reactions followed cleavage of the strand opposite the nick over a time course from 5 ms to 3 s (Figure 22, *left panel*). Mg^{2+} or

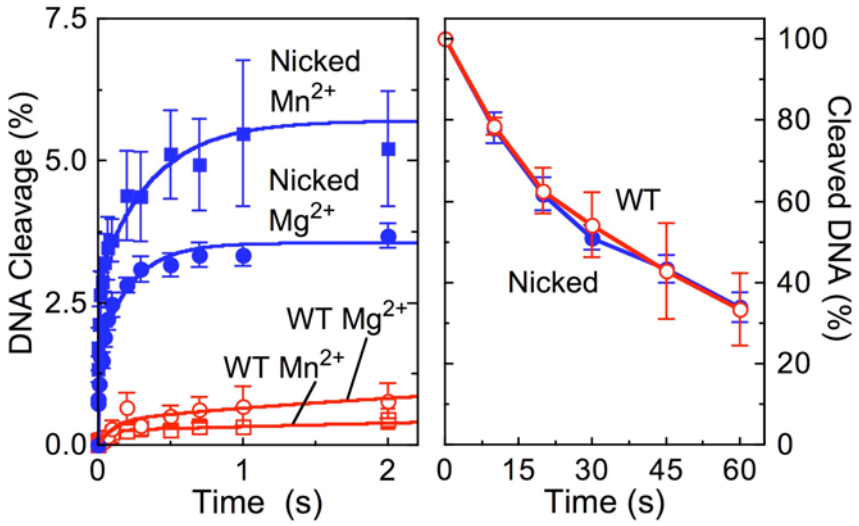

Figure 22: A nick at the scissile bond enhances the forward rate of DNA cleavage of topoisomerase IIα. *Left panel*, rapid time courses for reactions in the presence of topoisomerase IIα. Reactions were followed up to 3 s (plots are shown to 2 s) with un-nicked duplex (wild-type, WT; red) substrates in the presence of Mg^{2+} (open circles) or Mn^{2+} (open squares) or scissile bond nicked (-1/+1 position, blue) substrates in the presence of Mg^{2+} (closed circles) or Mn^{2+} (closed squares). Data were best fitted by double exponential curves (see Chapter III). *Right panel*, topoisomerase II ligation reactions were performed with un-nicked duplex (wild-type, WT; open circles, red) or scissile bond nicked (closed circles, blue) substrates. Total percent DNA cleavage was set to 100% at time zero. Error bars represent the standard error of the mean of two independent experiments or the standard deviation three or more independent experiments.

Mn^{2+} was used as the required divalent cation in these experiments. Although Mg^{2+} most likely represents the physiological divalent metal ion, higher rates and levels of cleavage are observed in the presence of Mn^{2+} (see Chapter V). With either metal ion, initial velocities of cleavage mediated by topoisomerase IIα were 10– to 20–fold higher with the nicked as compared to the duplex substrate. Initial velocities for cleavage of the nicked and duplex substrates in the presence of Mg^{2+} were 110 ± 5.6 and 7.8 ± 2.4 s^{-1}, respectively, and in the presence of Mn^{2+} were 230 ± 12 and 9.4 ± 1.3 s^{-1}, respectively. Taken together, these data suggest that the rate of cleavage increases dramatically (~15– to 25–fold) in the presence of a nick.

Levels of topoisomerase II-generated DNA strand breaks can be raised either by increasing the forward rate of cleavage or by inhibiting the backward rate of ligation (8,94). Most clinically relevant topoisomerase II-targeted drugs act by the latter mechanism (8). However, if DNA nicks are being used to coordinate the two protomer active sites of human topoisomerase IIα during the cleavage reaction, they should act primarily on the forward rate of scission. To determine whether this is the case, the effect of a nicked scissile bond on the rate of enzyme-mediated DNA ligation of the opposite strand was assessed. As seen in Figure 22 *right panel*, time courses for ligation of the duplex and nicked oligonucleotides were indistinguishable. The apparent first order rate constants for the duplex and nicked substrates were 0.031 ± 0.0079 and 0.036 ± 0.0074 s^{-1}, respectively. Together with the kinetic data discussed above, these results indicate that the introduction of a nick at one scissile bond greatly stimulates the forward rate of scission of the second strand by topoisomerase IIα.

Why does topoisomerase IIα preferentially cleave nicked DNA? Structural studies provide insight into this important mechanistic question. Recently, the crystal structure of the catalytic core of yeast topoisomerase II complexed with DNA was solved (65). The complex was non-covalent in nature, and the substrate contained nicks at both scissile bonds. Surprisingly, DNA in the active site of the enzyme was markedly reshaped, with its trajectory being bent by ~150º (65) (Figure 23). Considerable distortion was observed within the double helix, such that the bases between the scissile bonds existed as A-form rather than B-form DNA. Strain was observed over ~16 base pairs of the DNA cleavage site, and the highest degree of strain, as determined by the degree of deviation of twist from B-form DNA, occurred at the scissile bonds on each strand of the double helix.

It is reasonable to assume that the acute bending of the double helix seen in the crystal structure approximates the transition state of the nucleic acid substrate during cleavage by topoisomerase II. Given the high degree of distortion and strain required for the transition state, it is difficult for intact duplex DNA to attain this structure. Therefore, we propose that once the enzyme has cut the first strand of the double helix, the increased conformational flexibility that results from the nick greatly facilitates the conversion to the cleavage transition state. As a result, scission of the second strand by topoisomerase II occurs at a considerably faster rate, effectively coordinating the two protomer subunits of the enzyme.

Consistent with this proposal, DNA lesions (such as abasic sites or alkylated bases) that distort, bend, or increase the flexibility of the double helix are highly effective topoisomerase II poisons when located between the scissile bonds. To this point, DNA lesions often increase enzyme-mediated DNA cleavage >10–fold (64,133,136).

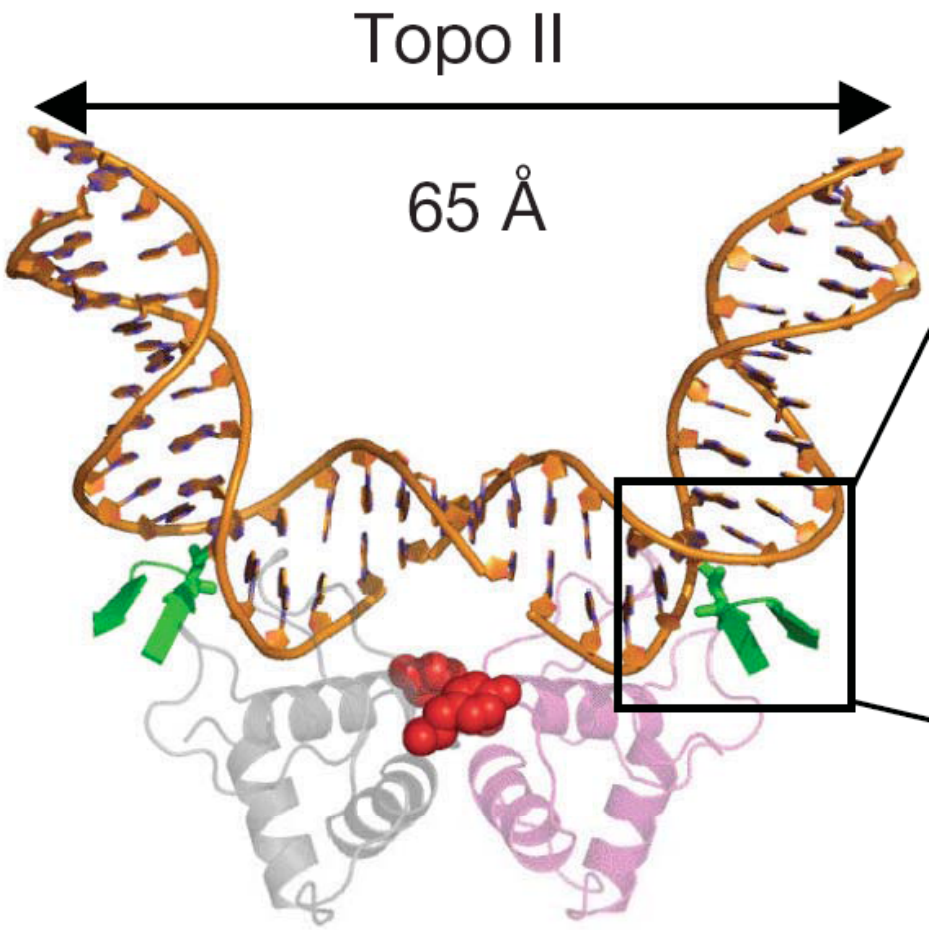

Figure 23: Topoisomerase II severely bends DNA. The non-covalent complex between yeast topoisomerase II and DNA is partially shown above. The double helix colored orange is bent when bound in the active site of topoisomerase II. Figure adapted from ref. (65).

Furthermore, they stimulate the forward rate of scission without inhibiting ligation (64,133,136).

If the hypothesis discussed above is correct, the presence of a nick in the vicinity of the scissile bonds should enhance the flexibility of the double helix and promote DNA cleavage by topoisomerase IIα. However, since DNA strain is greatest at the scissile bonds, placement of a nick precisely at the point of cleavage on one strand should have the greatest effect on cleavage of the opposite strand. Consequently, the relationship between the position of the nick and levels of DNA scission by topoisomerase IIα was determined. As seen in Figure 24, the presence of a nick in the vicinity of the scissile bond generally increased equilibrium levels of cleavage at the opposite scissile bond ~2– to 6–fold. Moreover, the presence of a nick at the scissile bond (-1/+1 position) increased cleavage at the opposite scissile bond ~25–fold. Results from DNA cleavage time courses demonstrated that the increase in DNA cleavage levels, as compared to wild-type, occurs quickly (Figure 25). These findings demonstrate that increased DNA flexibility enhances the ability of topoisomerase IIα to cleave its substrate. The fact that the increase in cleavage was dramatically higher when the nick was placed directly at the scissile bond further supports the proposal that coordination of the two protomer subunits of topoisomerase II results from an enhanced DNA conformational flexibility that follows scission of the first strand.

Nicks as Topoisomerase II Poisons

In addition to stimulating cleavage at the “native” site, the presence of a nick at a position other than that of the scissile bond always generated a novel DNA cleavage band

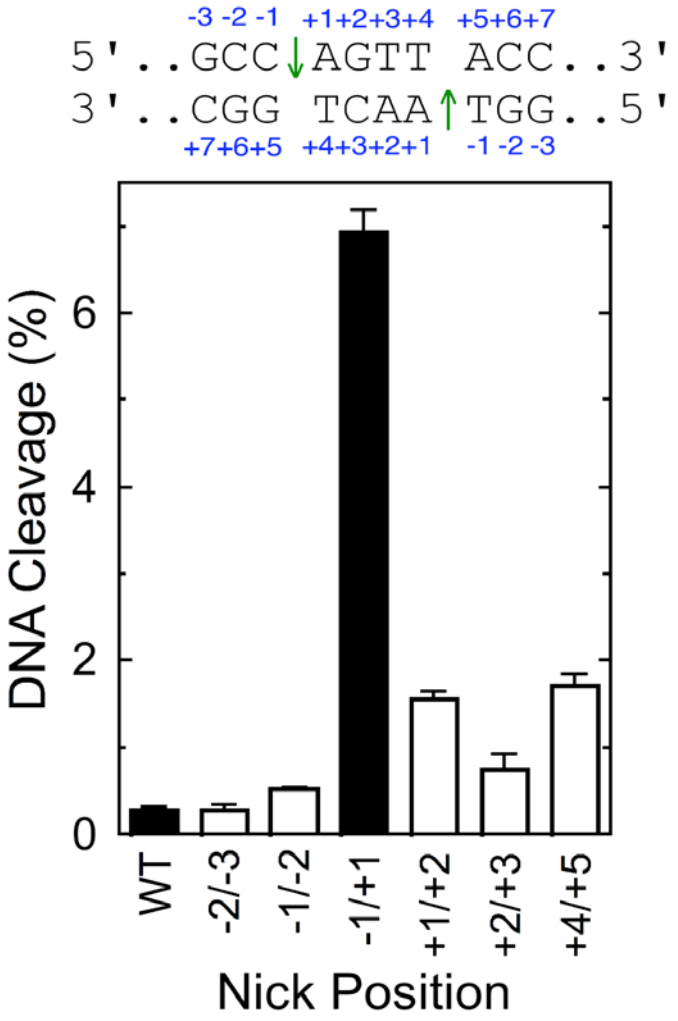

Figure 24: A nick at one scissile bond greatly enhances DNA cleavage at the scissile bond on the opposite strand. The sequence of the central region of the 50-mer oligonucleotide cleavage substrate, along with the positions of the scissile bonds (arrows) is show at top. Levels of topoisomerase IIα-mediated DNA cleavage at the native scissile bond on the bottom strand are shown for the un-nicked oligonucleotide (wild-type, WT) and for substrates containing a nick at the indicated position on the top strand (-2/-3, -1/-2, -1/+1, +1/+2, +2/+3, +4/+5). Cleavage for un-nicked and scissile bond nicked (-1/+1) substrates are depicted in black. Error bars represent the standard deviation of at least three independent experiments.

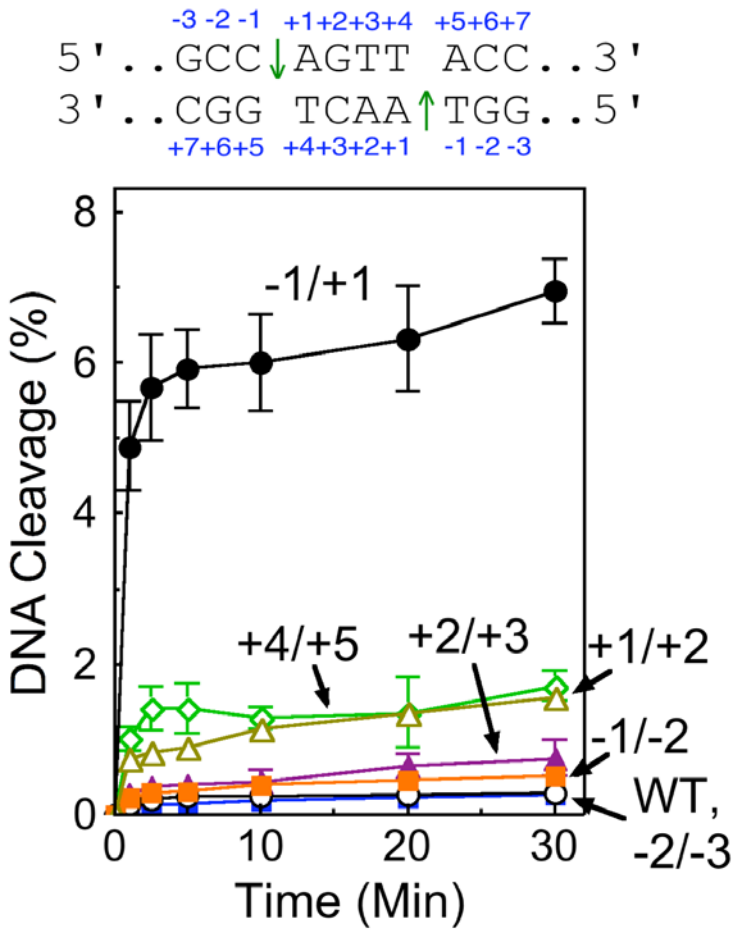

Figure 25: Time courses for topoisomerase IIα-mediated cleavage of nicked substrates. The sequence of the central region of the 50-mer oligonucleotide cleavage substrate, along with the positions of the scissile bonds (arrows) is show at top. Levels of topoisomerase IIα-mediated DNA cleavage on the bottom strand were monitored over 30 min for the un-nicked oligonucleotide (WT, black open circles) and for substrates that contained a nick at the indicated position of the top strand (-2/-3, blue open squares; -1/-2, orange closed squares; -1/+1, black closed circles; +1/+2, gold open triangles; +2/+3, purple closed triangles; and +4/+5, green open diamonds). Error bars represent the standard deviation of at least three independent experiments.

on the opposite strand that was 4 base pairs away from the nick (such that cleavage generated a 4-base 5'-overhang). Cleavage levels at these novel sites were at least equivalent to that seen at the native site, and in some cases were as much as 6–fold higher (Figure 26).

To further investigate the ability of nicks to induce enzyme-mediated cleavage at novel DNA sites, an oligonucleotide substrate that contained no native sites for topoisomerase IIα was utilized. No appreciable level of DNA scission was detected with this substrate in the absence of nicks (Figure 27). Nicks were introduced at single sites on one strand of the double helix and cleavage on the opposite strand was characterized (Figure 27). In all cases, a novel cleavage site located 4 bases away from the nick was generated. These novel sites all had different sequences, with nicks 1, 2, and 3 inducing cleavage between GA, TC, and CG to give products of 25, 21, and 17 bases, respectively. Levels of DNA cleavage were at least comparable to that observed with the native site shown in Figure 24 (0.29%, indicated by the dashed line) and ranged as much as 6–fold higher (Figure 27).

The above findings demonstrate that nicks, like other DNA lesions, are topoisomerase II poisons. However, they differ from the actions of other lesions in a significant fashion. Abasic sites and modified bases are site-specific topoisomerase II poisons that only stimulate cleavage when they are located between the two scissile bonds of a pre-existing site of enzyme action (64,133,136). Furthermore, while they enhance scission at the native site, they do not generate novel sites of cleavage (64,133,136). In contrast, the actions of nicks are not constrained to pre-existing sites of

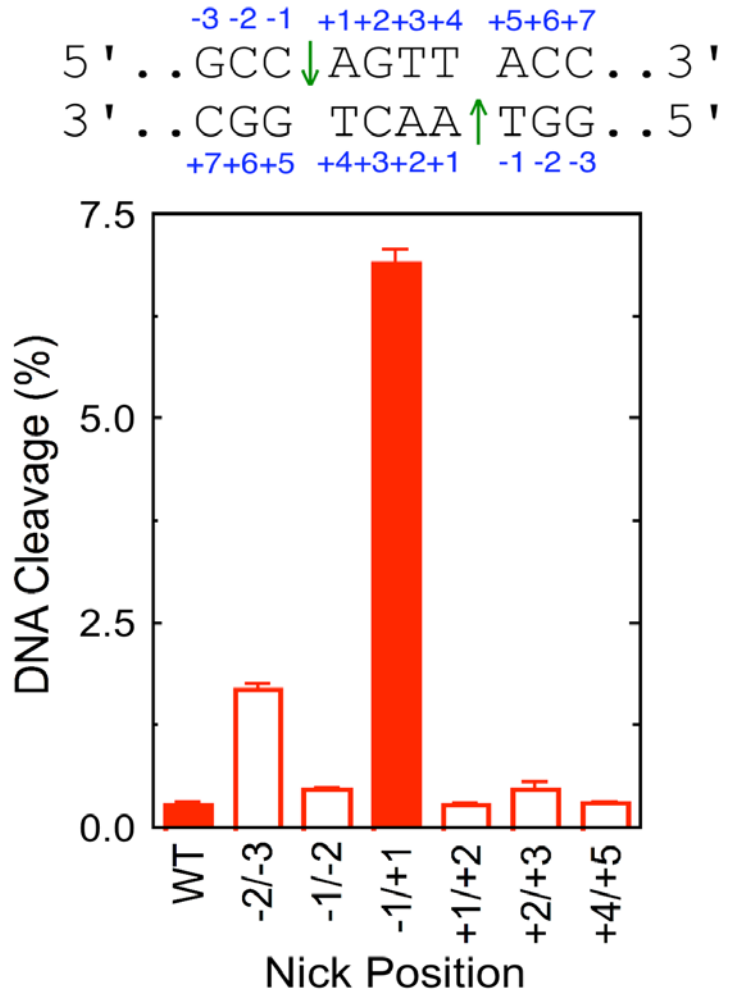

Figure 26: Nicks generate novel DNA cleavage sites for topoisomerase IIα. In addition to stimulating cleavage at the "native" site, the presence of a nick at a position other than that of the scissile bond always generated a novel DNA cleavage band on the opposite strand that was 4 base pairs away from the nick (such that cleavage generated a 4-base 5'-overhang). The position of the native scissile bond is between the -1 and +1 nucleotides. Topoisomerase IIα-mediated DNA cleavage levels on the bottom strand are shown for the un-nicked (WT) oligonucleotide and a substrate that contained a nick (-1/+1) at the native scissile bond on the bottom strand (red bars). Enzyme-mediated DNA cleavage of substrates that contained a nick located at other positions on the top strand (-2/-3, -1/-2, +1/+2, +2/+3, +4/+5) was monitored four base pairs away on the bottom strand (open bars). Error bars represent the standard deviation of at least three independent experiments.

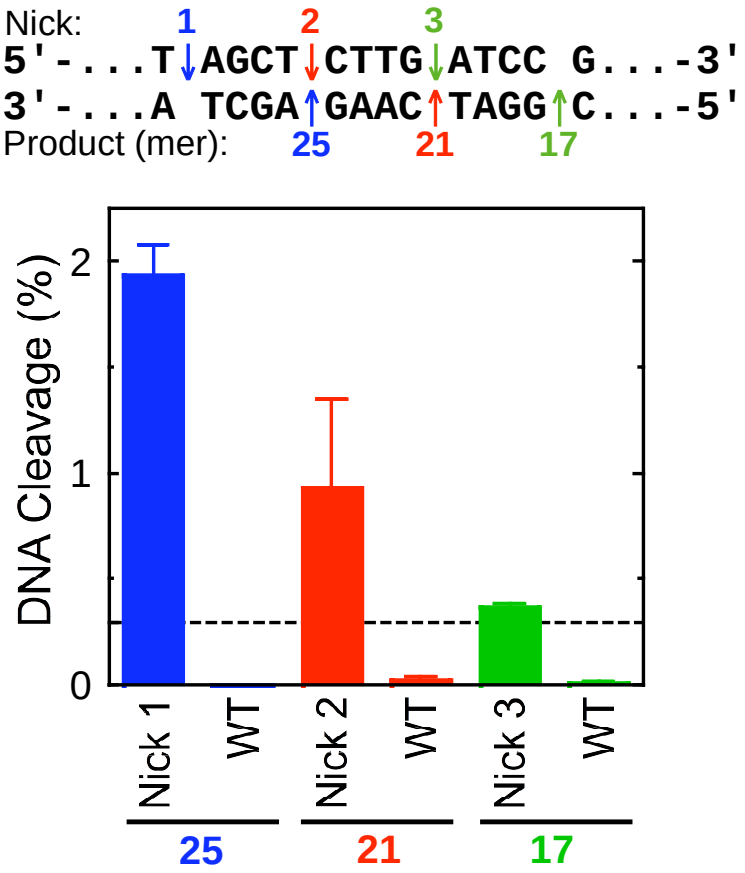

Figure 27: Nicks poison topoisomerase IIα. Nicks were incorporated into the top strand of a 50-mer oligonucleotide (partially shown at top) that did not display any native cleavage sites. Substrates contained a nick at one of three different positions (Nick 1, 2, or 3), and DNA cleavage on the opposite strand was monitored. Cleavage at the corresponding sites in the un-nicked (wild-type, WT) substrate is shown for comparison. The approximate level of DNA cleavage generated at a typical un-nicked native site (0.29%, see Figure 24) is represented by the dashed line. Error bars represent the standard deviation of at least three independent experiments.

topoisomerase II action. The mere presence of a nick in duplex DNA is sufficient to create a novel cleavage site for human topoisomerase IIα.

Conclusions

In summary, nicks have a profound effect on the DNA cleavage activity of topoisomerase IIα. The presence of a nick at one scissile bond dramatically increases the rate of cleavage at the scissile bond on the opposite strand of the double helix. We propose that nicks trigger faster rates of scission by introducing flexibility in the DNA substrate that allows it to attain the acutely bent transition state that is required for efficient cleavage. This enhanced activity at the second strand coordinates the two protomer subunits of topoisomerase II during the DNA cleavage reaction and allows the enzyme to create double-stranded breaks in the genetic material. Finally, nicks can be added to the list of DNA lesions that poison topoisomerase II. This finding may have implications for the actions of type II enzymes during apoptotic events. Moreover, it suggests a mechanism whereby topoisomerase II may impact the physiological effects of endogenous, environmental, or pharmaceutical compounds that damage the genetic material.

CHAPTER V

HUMAN TOPOISOMERASE IIα USES A TWO METAL ION MECHANISM FOR DNA CLEAVAGE

Introduction

Despite the importance of topoisomerase II-mediated DNA cleavage to the regulation of DNA topology and the treatment of human cancers, many aspects of the reaction are poorly understood. To this point, it has long been known that the type II enzyme requires a divalent metal ion in order to cleave DNA (30,61,62,157,158), but the role of the cation is unclear.

Commonly, enzymes that cleave nucleic acids, including exo- and endonucleases, utilize divalent cations in their active sites to stabilize both attacking (*i.e.*, amino acids and/or water) and leaving groups (*i.e.*, DNA backbone oxygen atoms) (68,189-207). However, the number of metal ions per active site and the specific roles of these cations in stabilizing leaving groups (bridging *vs.* non-bridging oxygen) varies. For example, based on structural studies, it has been proposed that several DNA polymerases utilize two divalent cations for both their 3'->5' proofreading exonuclease and 5'->3' polymerization reactions (189,192). Kinetic studies with these enzymes suggest that interactions between one of the active site divalent cations and the bridging oxygen of the DNA backbone play an important role in supporting catalysis (192,195). Although some ribozymes utilize two metal ions to catalyze self-splicing, others require only one (191,198,208-213). Furthermore, interactions with both the bridging and non-bridging oxygens have been described (198,209,212). Finally, structural studies imply that the

restriction endonuclease, *Bgl*II, uses only a single divalent cation and that it coordinates with the non-bridging oxygen of the scissile phosphate (200,214).

Based on mutagenesis and enzymological studies with DNA gyrase, Noble and Maxwell suggested that the prokaryotic type II topoisomerase utilizes a two metal ion mechanism similar to that of DNA polymerases (68). However the specific roles of the divalent cations in DNA gyrase were not characterized.

Recently, a crystal structure of the catalytic core of yeast topoisomerase II complexed with DNA was solved (65). While the structure showed only a single Mg^{2+} atom per active site, the enzyme-DNA complex was non-covalent in nature. Furthermore, it was noted that the active site tyrosine was too far away from the scissile bond to be in a cleavage competent configuration (65). Consequently, the number of divalent metal ions in the active site of eukaryotic type II topoisomerases and their role(s) in the DNA cleavage reaction remain open questions.

To resolve these fundamental issues, the present study utilized a series of divalent metal ions with varying thiophilicities in conjunction with DNA cleavage substrates that replaced the 3'-bridging oxygen of the scissile bond with a sulfur atom (*i.e.*, 3'-bridging phosphorothiolates). Results indicate that human topoisomerase IIα mediates DNA cleavage *via* a two metal ion mechanism and that one of the required divalent cations makes critical interactions with the bridging atom of the scissile bond during this process.

Results and Discussion

DNA Cleavage Mediated by Human Topoisomerase IIα Is Promoted by an Interaction Between a Divalent Metal Ion and the Bridging Atom of the Scissile Phosphate

Although the DNA cleavage reaction of human type II topoisomerases is central to their physiological functions and their roles as targets for anticancer drugs (30,61,62,68,157,158), the mechanism of the reaction has not been fully described. The only cofactor required for DNA scission is a divalent metal ion, which appears to be Mg^{2+} *in vivo* (30,62,68,157,158). However, the contribution of the divalent cation to enzyme function is not understood. Therefore, the role of the metal ion in the DNA cleavage reaction mediated by human topoisomerase IIα was characterized.

Several nucleases that generate a 3'-OH and 5'-phosphate during cleavage require an interaction between a metal ion and the 3'-bridging atom of the scissile phosphate (193,199). Consequently, as a first step in exploring the requirement for a divalent cation in the DNA cleavage reaction of human topoisomerase IIα, the interaction between the divalent cation and the 3'-bridging atom was assessed. Experiments took advantage of the novel phosphorothiolate-containing oligonucleotide substrates described in Chapter III that substituted a 3'-bridging sulfur for the oxygen atom at the scissile phosphate (*i.e.*, S–P scissile bond). Topoisomerase IIα cleaves these oligonucleotides with all of the characteristics of wild-type substrates, with the following exception: since the resulting 3'-terminal –SH moiety is a poor nucleophile at phosphorous, 3'-bridging phosphorothiolates do not support ligation. As a result, these substrates isolate the forward DNA scission event from ligation, and therefore allow high levels of cleavage complexes to accumulate. This is in contrast to wild-type oligonucleotides with 3'-

bridging oxygen atoms (*i.e.*, O–P scissile bond), which establish a rapid DNA cleavage-ligation equilibrium and maintain low levels of cleavage complexes (see Chapter III). Due to the characteristics of DNA scission with these substrates, both rates and levels of cleavage can be determined with S–P oligonucleotides. However, only levels of cleavage can be monitored with O–P oligonucleotides in conventional kinetic experiments.

Interactions between the divalent cation and the 3'-bridging atom of the scissile phosphate were determined by comparing the ability of topoisomerase IIα to cleave DNA substrates containing either a 3'-bridging oxygen or sulfur atom in the presence of metal ions of varying "softness" (*i.e.*, thiophilicity). The ions that were used for this study were Ca^{2+}, Mg^{2+}, Co^{2+}, and Mn^{2+}. Within this series, Co^{2+} and Mn^{2+} are the "softest," or most thiophilic metals, and Mg^{2+} and Ca^{2+} are harder, or less thiophilic (215-217). Softer metal ions often prefer sulfur over oxygen as an inner-sphere ligand, while hard metals usually coordinate more readily with oxygen (192,195,198,208-212,215-217).

The DNA cleavage reaction of human topoisomerase IIα generates a 3'-oxygen leaving group in a divalent metal ion-dependent manner. If there is a direct interaction between the metal ion and the leaving group that facilitates catalysis, relative rates (or levels) of scission with substrates containing a 3'-sulfur in place of the oxygen should increase in the presence of soft (thiophilic) metals (192,195,198,208-212). Conversely, less cleavage should be generated in reactions that contain hard metals (192,195,198,208-212).

The first set of experiments established the capacity of divalent metal ions to support topoisomerase IIα-mediated cleavage of a wild-type O–P substrate (site 2 oligonucleotide). Consistent with previous work with plasmid substrates (158), Mn^{2+},

Ca^{2+}, and Co^{2+} all generated higher levels of DNA cleavage than Mg^{2+} (Figure 28, *left panel*). Levels of cleavage complexes formed in the presence of Mn^{2+} or Ca^{2+} were ~4 times higher than those observed with Mg^{2+}, and those with Co^{2+} were ~2 times higher.

The divalent cation preference for reactions that monitored cleavage of the equivalent S–P oligonucleotide is shown in the *right panel* of Figure 28. Rates and levels of cleavage observed in the presence of the thiophilic metal ions, Mn^{2+} and Co^{2+}, were still higher than those generated with Mg^{2+}. However, results with Ca^{2+}, a hard metal, were markedly different. The initial velocity of DNA cleavage in the presence of Ca^{2+} was 1–2 orders of magnitude slower than that seen with Mg^{2+}. Similar results were found using a different matched pair of O–P and S–P substrates (site 1 oligonucleotide; data not shown).

The precipitous drop in Ca^{2+}-supported cleavage of the S–P, as compared to the O–P substrate, together with the maintenance of high DNA cleavage rates and levels with Mn^{2+} and Co^{2+}, strongly suggest that there is an important interaction between the metal ion and the 3'-bridging atom of the scissile bond in the active site of human topoisomerase IIα.

In order to investigate this interaction in greater detail, rates of DNA cleavage with both the O–P and S–P substrates were compared. Since wild-type substrates attain a cleavage-ligation equilibrium too quickly to ascertain rates in bench top experiments, a rapid chemical quench system was employed. Unfortunately, over the course of these assays (0–3 s), the low levels of cleavage with wild-type O–P oligonucleotides with some cations made the rate constants less reliable. Therefore, a nick was introduced at the scissile bond on the opposite strand from the one being monitored. As discussed in

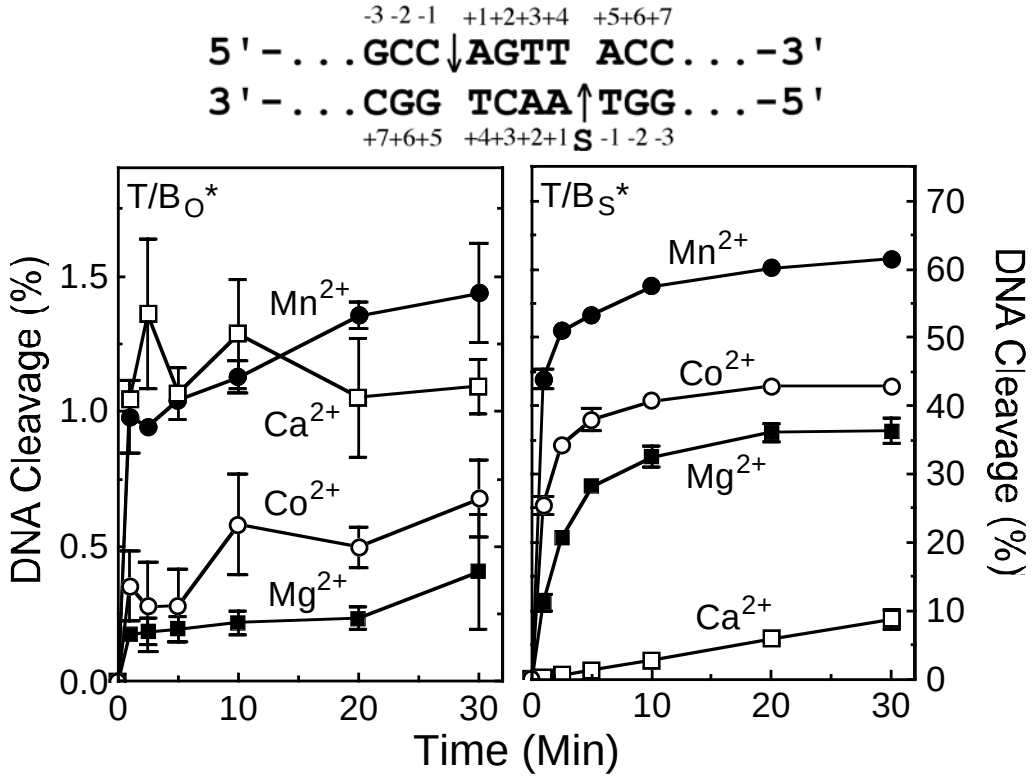

Figure 28: Cleavage of oligonucleotide substrates mediated by human topoisomerase IIα in the presence of different divalent metal ions. Results for wild-type (O–P, T/B_O*, *left*) and phosphorothiolate (S–P, T/B_S*, *right*) substrates are shown. The asterisk denotes the 5'-end-labeled strand. The wild-type duplex containing an O-P scissile bond (subscript O) is denoted by T/B_O* for the labeled bottom strand. The phosphorothiolate substrate containing an S-P scissile bond (subscript S) is denoted by T/B_S* for the labeled phosphorothiolate bottom strand. DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Mn^{2+} (closed circle), Ca^{2+} (open square), or Co^{2+} (open circle). The cleavage sequence (site 2) is depicted above the graphs with the scissile bonds denoted on the top and bottom strands by arrows. "S" on the bottom strand denotes the location of the phosphorothiolate modification. Error bars represent the standard deviation of at least three independent experiments.

Chapter III, these nicked oligonucleotides do not act as "suicide substrates," and rapidly reach a cleavage-ligation equilibrium when opposite an O–P scissile bond. However, they maintain ~10–fold higher levels of cleavage complexes at equilibrium (Figure 10).

As a prelude to rapid quench experiments, bench top assays were carried out with these nicked substrates. Results were similar to those obtained with the corresponding un-nicked oligonucleotides (Figure 29). Accordingly, Mn^{2+}, Ca^{2+}, and Co^{2+} all supported higher levels of DNA cleavage than Mg^{2+} with the O–P substrate (Figure 29, *left panel*). Furthermore, rates and levels of cleavage observed in the presence of Mn^{2+} and Co^{2+} were higher than those generated with Mg^{2+} using the S–P substrate, and the initial velocity of DNA cleavage supported by Ca^{2+} was >10 times slower than that seen with Mg^{2+} (Figure 29, *right panel)*. Once again, these findings support the conclusion that the divalent metal ion interacts with the bridging atom of the scissile bond and promotes the DNA cleavage reaction of topoisomerase IIα.

Rapid quench kinetic data for the cleavage of the O–P and S–P substrates are shown in Figure 30. The earliest time point that could be monitored was 5 ms, and reactions were followed up to 3 s. The data were best fit using a double exponential rate equation. The mechanistic basis for this fit is unclear at the present time (see Chapter III).

Although DNA cleavage levels in the presence of Co^{2+} and Ca^{2+} ultimately were as high or higher than observed with Mg^{2+} and the nicked O–P substrate, reaction velocities at the earliest time points were somewhat slower (*left panel)*. Consequently, rate constants calculated for these divalent metal ions were ~0.77 and 0.050 relative to that derived for Mg^{2+} (Table 1). In contrast, the calculated rate constant for Mn^{2+} was ~1.3 times greater than that for Mg^{2+}.

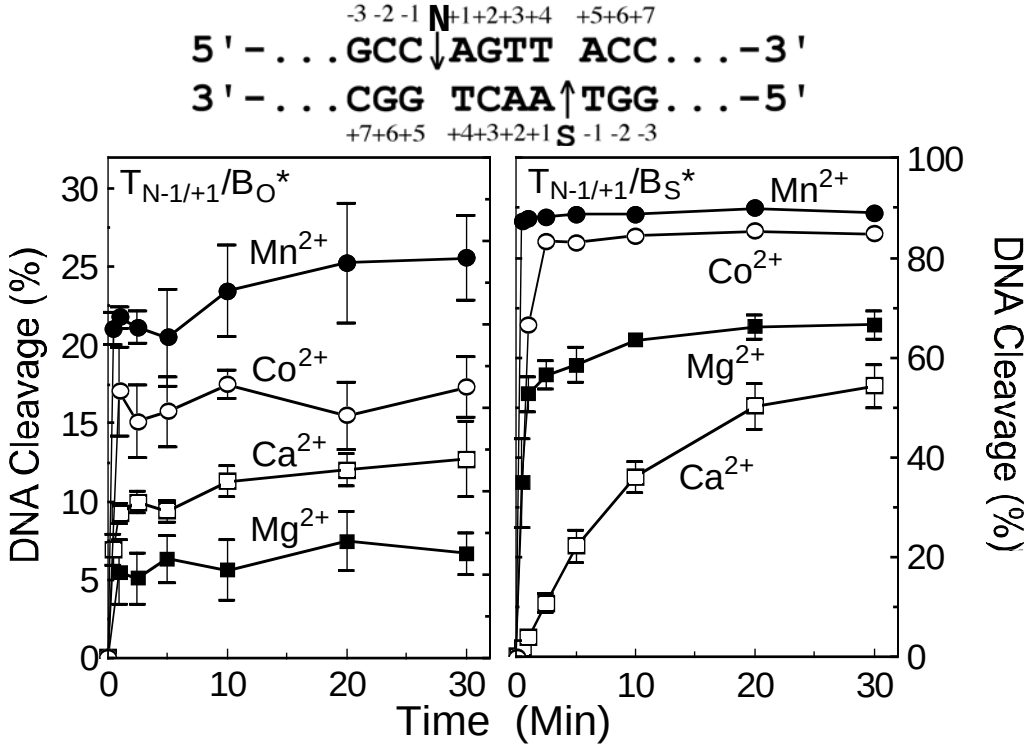

Figure 29: Cleavage of nicked oligonucleotide substrates by topoisomerase IIα in the presence of different divalent metal ions. The nick is located at the scissile bond on the unlabeled strand (-1/+1 position). Results for the O–P ($T_{N-1/+1}/B_O$*, *left*) and S–P ($T_{N-1/+1}/B_S$*, *right*) nicked substrates are shown. DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Mn^{2+} (closed circle), Ca^{2+} (open square), or Co^{2+} (open circle). The cleavage sequence (site 2) is depicted above the graphs with the scissile bonds denoted on the top and bottom strands by arrows. "N" on the top strand denotes the location of the single-stranded nick, and "S" on the bottom strand denotes the location of the phosphorothiolate modification. Error bars represent the standard deviation of at least three independent experiments.

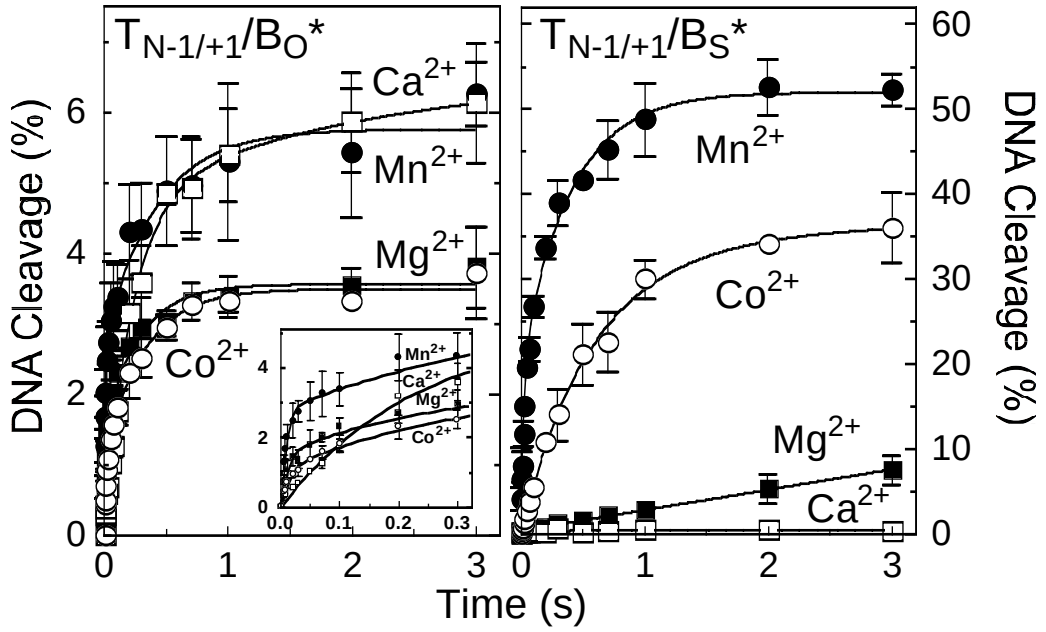

Figure 30: Pre-equilibrium cleavage of nicked oligonucleotide substrates by topoisomerase IIα in the presence of different divalent metal ions. Reaction times varied from 5 ms to 3 s. Results for the O–P ($T_{N-1/+1}/B_O$*, *left*) and S–P ($T_{N-1/+1}/B_S$*, *right*) nicked substrates are shown. The nick was located at the scissile bond on the unlabeled strand (-1/+1 position, see Figure 29). DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Mn^{2+} (closed circle), Ca^{2+} (open square), or Co^{2+} (open circle). A double exponential curve is plotted for each reaction. The *inset* in the *left panel* shows reaction times up to 300 ms. Error bars represent the standard deviation of at least three independent experiments.

Table 1. Observed double exponential rate constants for metal ion-promoted DNA cleavage mediated by human topoisomerase IIα

Cation	Oligonucleotide[a]		
	O-P	S-P	R_P/S_P
	k_{obs}, M^{-1} s^{-1} ($k_{obs}/k_{obs}Mg^{2+}$)[b]		
Mg^{2+}	92 ± 20 (1.0)	$5.1x10^{-7}$ ± 0.15 (1.0)	29 ± 7.0 (1.0)
Mn^{2+}	120 ± 26 (1.3)	35 ± 6.2 ($6.8x10^{7}$)	19 ± 5.6 (0.66)
Co^{2+}	71 ± 20 (0.77)	1.8 ± 0.99 ($3.5x10^{6}$)	70 ± 26 (2.4)
Ca^{2+}	4.6 ± 1.0 (0.050)	N.D.	7.3 ± 3.8 (0.25)

N.D., not determined

[a]Wild type, O-P; 3'-bridging phosphorothiolate, S-P; and racemic non-bridging phosphorothioate, R_P/S_P.

[b]Included in parenthesis is the relative observed rate constant compared to the value for Mg^{2+} (k_{obs}/k_{obs} Mg^{2+}).

Results with the nicked S–P oligonucleotide (Figure 30, *right panel)* paralleled those seen in bench top experiments. Relative to the rate constant derived for Mg^{2+}, those for the thiophilic divalent cations, Mn^{2+} and Co^{2+}, were ~$6.8\mathbf{x}10^{7}$ and $3.5\mathbf{x}10^{6}$ times greater, respectively (Table 1). Conversely, rates for Ca^{2+}-supported reactions were so low that a rate constant could not be calculated. As above, these data indicate that the metal ion interacts with the bridging atom of the scissile bond and that this interaction is important for the DNA cleavage event mediated by topoisomerase IIα.

Divalent Metal Ion Interactions with the Non-bridging Atom of the Scissile Phosphate

Interactions between a metal ion and the non-bridging oxygen atom of the scissile phosphate have been observed or postulated for a number of enzymes that cleave or join nucleic acids (68,189,192,200,214). In order to determine whether such an interaction takes place in the active site of human topoisomerase IIα, a racemic phosphorothioate substrate (site 2 oligonucleotide) that substituted a sulfur atom for either the R_P or S_P non-bridging oxygen atom of the scissile phosphate was employed. The first experiment was carried out on the bench top and monitored DNA cleavage with an un-nicked oligonucleotide (Figure 31). Results were similar to those observed in Figure 28 for the wild-type substrate that contained no sulfur atoms. It is notable that non-bridging phosphorothiolates also have little effect on levels of DNA cleavage mediated by *E. coli* gyrase (218).

The second experiment utilized the rapid quench kinetic system in conjunction with a nicked phosphorothioate substrate to generate rate constants for DNA cleavage supported by the four divalent cations (Figure 32). Once again, results were similar to

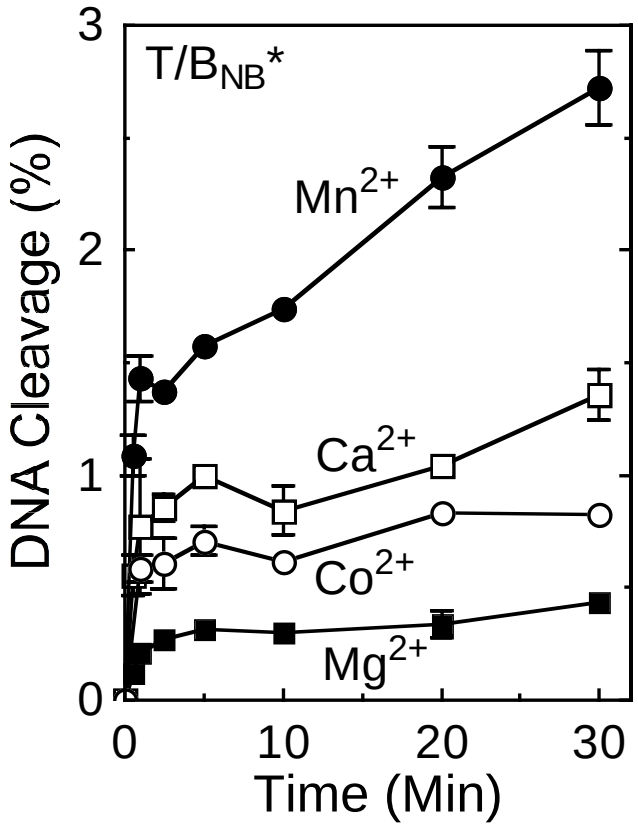

Figure 31: Cleavage of an oligonucleotide substrate containing a non-bridging phosphorothioate by topoisomerase IIα in the presence of different divalent metal ions. Reactions included a racemic mixture of phosphorothioate substrates in which the non-bridging oxygen atoms at the scissile phosphate on the labeled strand were replaced by sulfur atoms. DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Mn^{2+} (closed circle), Ca^{2+} (open square), or Co^{2+} (open circle). Error bars represent the standard deviation of at least three independent experiments.

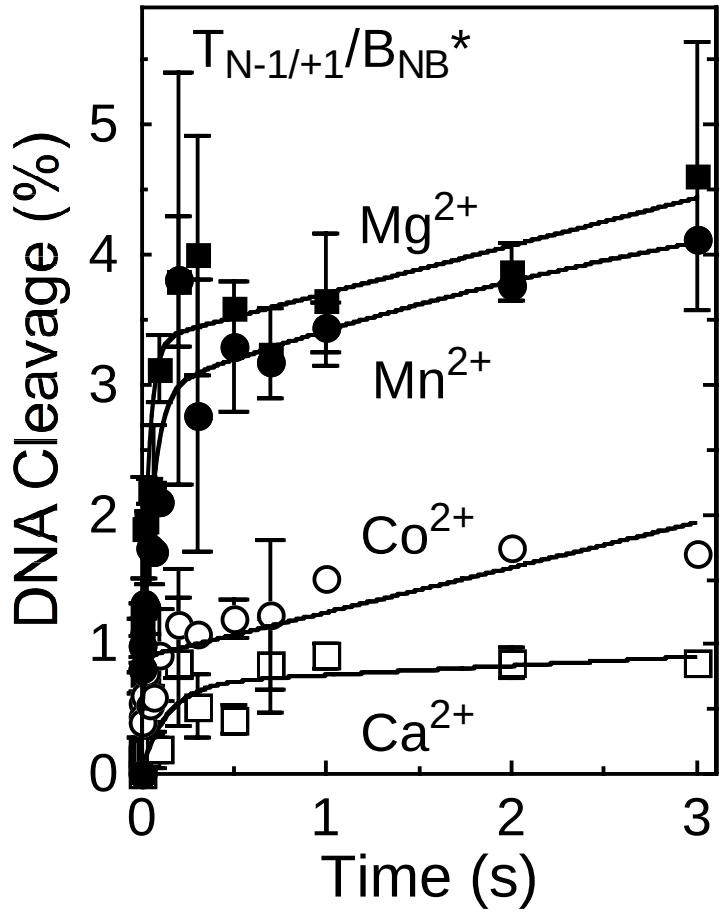

Figure 32: Pre-equilibrium cleavage of a nicked oligonucleotide substrate containing a non-bridging phosphorothioate by topoisomerase IIα in the presence of different divalent metal ions. Reaction times varied from 5 ms to 3 s. The substrate is the same as used in Figure 31 except that there is a nick at the scissile bond of the unlabeled strand (-1/+1 position). DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Mn^{2+} (closed circle), Ca^{2+} (open square), or Co^{2+} (open circle). A double exponential curve is plotted for each reaction. Error bars represent the standard deviation of at least three independent experiments.

those calculated with the nicked O–P oligonucleotide (Table 1). Relative to Mg^{2+}, calculated rate constants for Mn^{2+}, Co^{2+}, and Ca^{2+} were ~0.66, 2.4, and 0.25, respectively.

The above data with the racemic phosphorothioate substrate cannot rule out an interaction between a divalent cation and one of the non-bridging oxygen atoms of the scissile phosphate. However, if such an interaction exists in the active site of topoisomerase IIα, its effect on the DNA cleavage reaction of the enzyme appears to be equivocal.

Human Topoisomerase IIα Uses Two Divalent Metal Ions for DNA Cleavage

A number of nucleic acid enzymes, including polymerases, nucleases, ribozymes, and DNA gyrase have been postulated to use two divalent metal ions in their active sites (190,191,193,194,196-198,200-207). In the case of most protein-based enzymes, evidence comes primarily from crystallographic studies and the kinetic analysis of mutant enzymes in which amino acids that interact with active site metal ions are altered (68,189,190,192,196,197,200,201). This latter approach was used with DNA gyrase (68). In the case of ribozymes and the 3'–>5' exonuclease activity of *E. coli* DNA polymerase I, strong evidence for the use of two metal ions comes from kinetic analysis of RNA sequences that replaced oxygen atoms of the scissile phosphate with sulfur atoms (191,198,208-213). We employed this latter strategy to determine whether human topoisomerase IIα utilizes one or two divalent metal ions during its DNA cleavage reaction.

As a first approach, a cleavage substrate (shown in Figure 33) was used that contained a 3'-bridging phosphorothiolate located one base 3' (+1/+2 position) to the

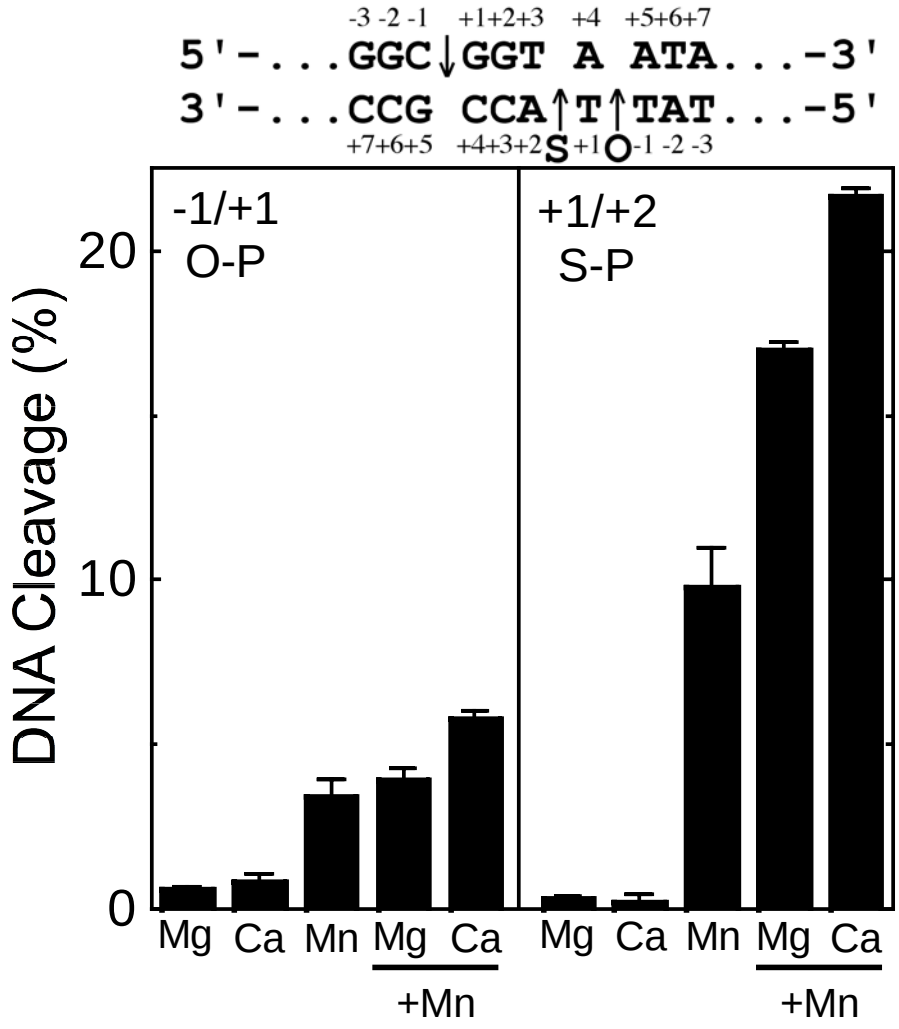

Figure 33: Cleavage of a phosphorothiolate linkage that is located one base 3' to the intrinsic scissile bond. DNA cleavage levels were quantified at both the original O–P scissile bond (-1/+1, O-P, *left*) and the S–P bond (+1/+2, S-P, *right*). The sequence (site 1b) is shown above the graphs, and the location of the wild-type scissile bond ("O") and the phosphorothiolate bond ("S") are denoted. Reactions included Mg^{2+}, Ca^{2+}, or Mn^{2+} alone, as well as mixtures of Mg^{2+} and Mn^{2+}, or Ca^{2+} and Mn^{2+}. All reactions had a total divalent cation concentration of 5 mM (2.5 mM of each divalent cation for reactions with two different divalent cations). Results shown are for a 30 min time point. Error bars represent the standard deviation of at least three independent experiments.

normal scissile bond (-1/+1 position). Site 1b, as opposed to site 2, was employed for this study because it contained a thymine residue at the +1 position. Due to the chemistry involved in generating the 3'-bridging phosphorothioamidite, only modified thymines were available.

DNA cleavage mediated by topoisomerase IIα was monitored simultaneously at the normal (-1/+1) O–P scissile bond as well as the off-site (+1/+2) S–P bond. Since these bonds are adjacent to one another, the enzyme is forced to choose between the two sites.

Mg^{2+}, Ca^{2+}, and Mn^{2+} were used for these experiments. Time courses for cleavage were followed up to 30 min. Data for the 30 min time point are displayed in Figure 33. Results for cleavage at the normal O–P scissile bond are shown in the *left panel*. Consistent with the data from Figure 28, 5 mM Mn^{2+} supported higher levels of DNA scission than either Mg^{2+} or Ca^{2+}. A mixture (2.5 mM + 2.5 mM) of Mg^{2+} and Mn^{2+} produced levels of DNA cleavage that were similar to that of 5 mM Mn^{2+} alone. However, the corresponding mixture of Ca^{2+} and Mn^{2+} supported cleavage that was ~1.7 times greater than that seen with Mn^{2+} alone. This result suggests that topoisomerase IIα may utilize two divalent metal ions in its cleavage reaction.

More dramatic results were seen at the off-site bond (Figure 33, *right panel*). Of the three divalent cations employed, only 5 mM Mn^{2+} was able to support efficient cleavage at the +1/+2 S–P site. This probably is due to the fact that Mn^{2+} is considerably softer then either Mg^{2+} or Ca^{2+} (192,195,198,208-212), and is therefore able to alter the cleavage specificity of topoisomerase IIα in the presence of a 3'-bridging sulfur. Mixtures (2.5 mM + 2.5 mM) of Mg^{2+} + Mn^{2+} or Ca^{2+} + Mn^{2+} resulted in levels of cleavage that were ~1.7– or 2.2–fold higher than observed with 5 mM Mn^{2+} alone. As above, this

finding argues for the use of two divalent metal ions in the active site of topoisomerase IIα.

In light of the above results, two additional approaches were employed to further explore whether two metal ions are involved in catalysis. In the first, DNA cleavage was monitored over a range of divalent cation concentrations. These experiments utilized the site 2 substrate that contained a nick at the scissile bond (–1/+1 position) on the opposite strand. As discussed earlier, this substrate generates higher levels of DNA cleavage than observed with the fully wild-type oligonucleotide. Mn^{2+} (a soft cation) and Ca^{2+} (a hard cation) were used for these studies. As seen in Figure 34, the concentration of Mn^{2+} that was required to promote DNA scission mediated by topoisomerase IIα was considerably lower than that seen with Ca^{2+}.

When cleavage of the O–P oligonucleotide was monitored (Figure 34, *left panel*), both divalent cations displayed a biphasic concentration dependence. In addition, the initial phase that was seen with Ca^{2+} was more pronounced than observed with Mn^{2+}. When cleavage of the S–P oligonucleotide was monitored (Figure 34, *middle panel*), the opposite was seen. While the initial phase observed with Mn^{2+} became more prominent, that with Ca^{2+} essentially disappeared. The biphasic metal ion concentration dependence implies that there are two divalent cation sites in the DNA cleavage-ligation site of topoisomerase IIα and that both need to be filled in order to support scission. The finding that the initial (*i.e.*, high affinity) phase with the S–P substrate became more prominent with the softer metal ion (Mn^{2+}) and was lost with the harder metal ion (Ca^{2+}), suggests that the first site that is filled by the divalent cation is the one that interacts with the

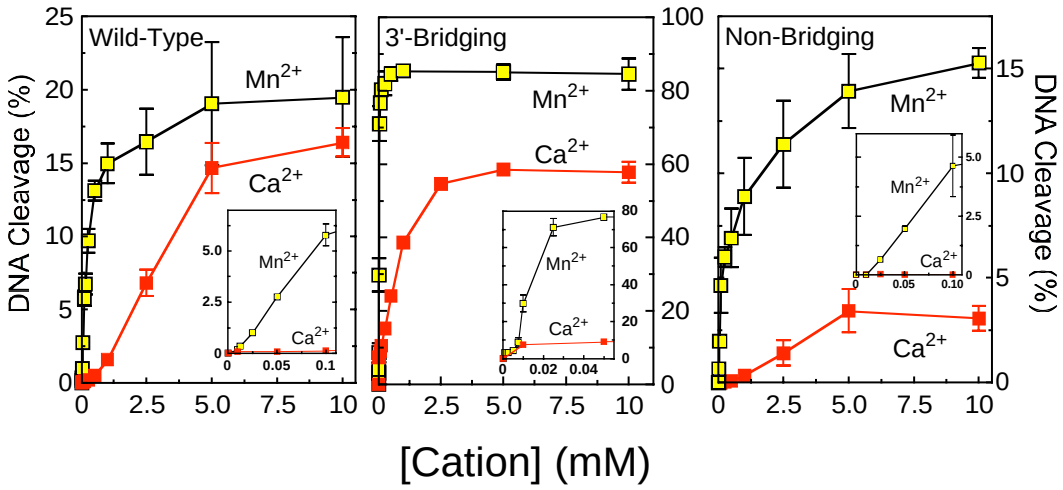

Figure 34: Cation concentration dependence for DNA cleavage of wild-type, phosphorothiolate, and phosphorothioate substrates by topoisomerase IIα. Results for the nicked (-1/+1 position) O–P (Wild-Type, *left*), S–P (3'-Bridging, *middle*), and phosphorothioate substrate (Non-Bridging, *right*) are shown. *Left panel*, Mn^{2+} (yellow squares) or Ca^{2+} (red squares) were titrated from 10 μM to 10 mM. Reactions were incubated for 30 min prior to stopping the reaction. The *inset* shows DNA cleavage at concentrations up to 100 μM. *Middle panel*, Mn^{2+} (yellow squares) or Ca^{2+} (red squares) were titrated from 1 μM (for Mn^{2+}) or 10 μM (for Ca^{2+}) to 10 mM. Reactions were incubated for 30 min prior to stopping the reaction. The *inset* shows concentrations up to 50 μM. *Right panel*, Mn^{2+} (yellow squares) or Ca^{2+} (red squares) were titrated from 10 μM to 10 mM. Reactions were incubated for 30 min prior to stopping the reaction. The *inset* shows DNA cleavage at concentrations up to 100 μM. Error bars represent the standard error of the mean of two independent experiments or the standard deviation of at least three independent experiments.

bridging atom of the scissile bond. Results from the non-bridging phosphorothioate (Figure 34, *right panel*) substrate were similar to that of the wild-type substrate.

A second approach was employed to confirm that two metal ions are required to support DNA cleavage mediated by human topoisomerase IIα. These studies took advantage of the enhanced affinity of Mn^{2+} and the decreased affinity of Ca^{2+} for substrates with 3'-bridging sulfur atoms. Experiments compared levels and rates of DNA scission monitored in the presence of Ca^{2+}, Mn^{2+}, or a combination of the two divalent cations. Near saturating concentrations of Ca^{2+} (5 mM and 2.5 mM with the O–P and S–P oligonucleotides, respectively) were paired with concentrations of Mn^{2+} that bound the scissile bond site without appreciably filling the second site (25 μM and 5 μM with the O–P and S–P oligonucleotides, respectively). These limiting concentrations of Mn^{2+} supported levels of enzyme-mediated DNA cleavage that were ~10–fold lower than generated at the concentrations of Ca^{2+} that were used (see Figure 35).

Combining metal ions in reactions that utilized a wild-type or a non-bridging phosphorothioate substrate had little effect (Figure 35, *left* and *right panels*). Levels of DNA cleavage with the wild-type were only slightly higher than predicted by adding the amount of cleavage generated in the presence of the two individual divalent cations. This result was not surprising, since cleavage of the O–P bond of the site 1b oligonucleotide (Figure 33) was stimulated only moderately by the presence of the two metal ions. Similarly, levels of cleavage mediated by wild-type DNA gyrase were not enhanced in the presence of two different metal ions as compared to the individual cations (68). Stimulation of DNA cleavage by two divalent cations was observed only when residues

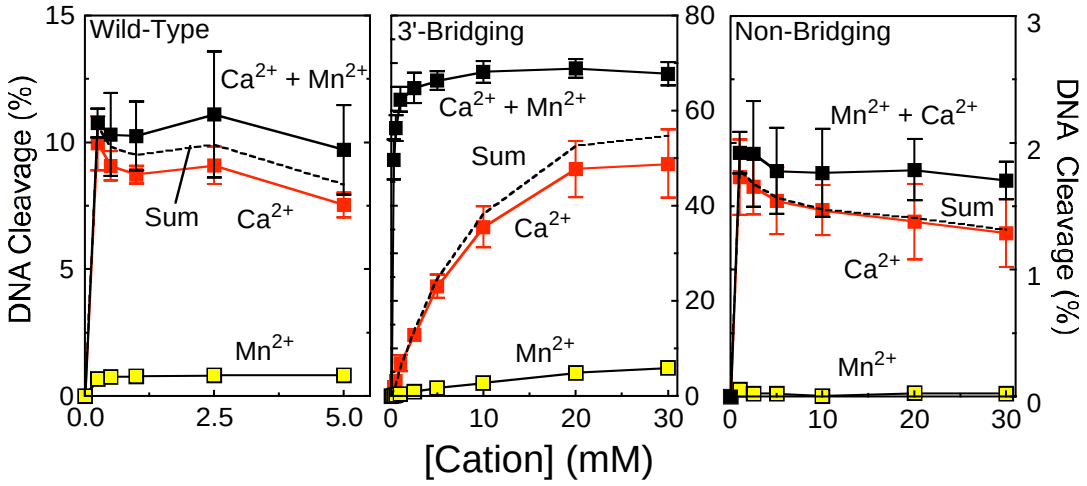

Figure 35: Cleavage of oligonucleotide substrates by topoisomerase IIα in the simultaneous presence of two different divalent metal ions. Results for the nicked (-1/+1 position) O–P substrate (Wild-Type, *left*), S–P (3'-Bridging, *middle*), and phosphorothioate substrate (Non-Bridging, *right*) are shown. *Left panel*, time courses (up to 5 min) were carried out in the presence of 5 mM Ca^{2+} alone (red squares), 25 μM Mn^{2+} alone (yellow squares), or a mixture of 5 mM Ca^{2+} and 25 μM Mn^{2+} (closed squares). The calculated sum of the data from reactions containing Ca^{2+} or Mn^{2+} alone is also plotted (dashed line, "Sum"). *Middle panel*, time courses (up to 30 min) were carried out in the presence of 2.5 mM Ca^{2+} alone (red squares), 5 μM Mn^{2+} alone (yellow squares), or a mixture of 2.5 mM Ca^{2+} and 5 μM Mn^{2+} (closed squares). Once again, the calculated sum of the data from reactions containing Ca^{2+} or Mn^{2+} alone is also plotted (dashed line, "Sum"). *Right panel*, time courses (up to 30 min) were carried out in the presence of 2.5 mM Ca^{2+} alone (red squares), 10 μM Mn^{2+} alone (yellow squares), or a mixture of 2.5 mM Ca^{2+} and 10 μM Mn^{2+} (closed squares). The calculated sum of the data from reactions containing Ca^{2+} or Mn^{2+} alone is also plotted (dashed line, "Sum"). Error bars represent the standard deviation of three independent experiments or in some cases the standard error of the mean of two independent experiments.

that interacted with metal ions in the active site of the prokaryotic type II enzyme were altered (68).

In contrast to results with O–P DNA, a dramatic difference was seen in reactions that utilized the S–P substrate (Figure 35, *middle panel*). The initial velocity of DNA scission in reactions that contained both 2.5 mM Ca^{2+} and 5 μM Mn^{2+} (~3.5% · s^{-1}) was nearly 40 times faster than the calculated rate derived from the sum of cleavage observed in the presence of the individual metal ions (~0.09% · s^{-1}).

In order to more fully investigate any evidence for two metal ions, a series of reactions with short time points and a range of Mn^{2+} concentrations were performed. As seen in Figure 36, results with the wild-type substrate demonstrate a somewhat more-than-additive effect in the presence of both divalent cations. This effect is more noticeable at 25 and 50 μM Mn^{2+}. Moreover, when the phosphorothiolate substrate was used in similar reactions, a much larger effect was observed (Figure 37). The significant enhancement in the rate and level of DNA scission (compared to calculated sums) was observed when 2.5 mM Ca^{2+} was combined with a range of Mn^{2+} concentrations (1–10 μM) that partially filled or saturated the high affinity scissile bond site (Figure 37). It is notable that once the scissile bond site was saturated with Mn^{2+} (7.5 μM), the inclusion of higher levels of Mn^{2+} did not stimulate DNA cleavage further. Like the wild-type, results with the non-bridging phosphorothioate (Figure 38) also displayed evidence that the two metal ions together were more efficient at inducing cleavage than either of them separately. Taken together, these results provide strong evidence that human topoisomerase IIα utilizes two metal ions in its cleavage-ligation active site and that both must be present in order for the enzyme to cleave the DNA backbone.

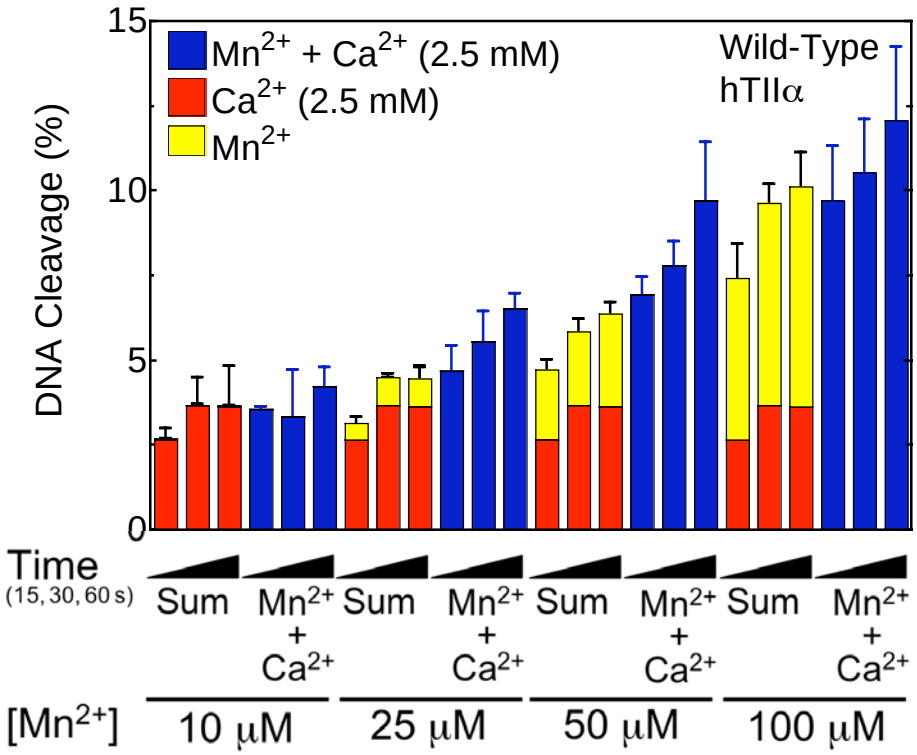

Figure 36: Cleavage of nicked wild-type oligonucleotide substrates by topoisomerase IIα in the presence of divalent metal ion combinations. DNA cleavage reactions with topoisomerase IIα were carried out for 15, 30 or 60 s in the presence of 2.5 mM Ca^{2+} alone (red bars), 10–100 μM Mn^{2+} alone (yellow bars), or a mixture of 2.5 mM Ca^{2+} and 10–100 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity.

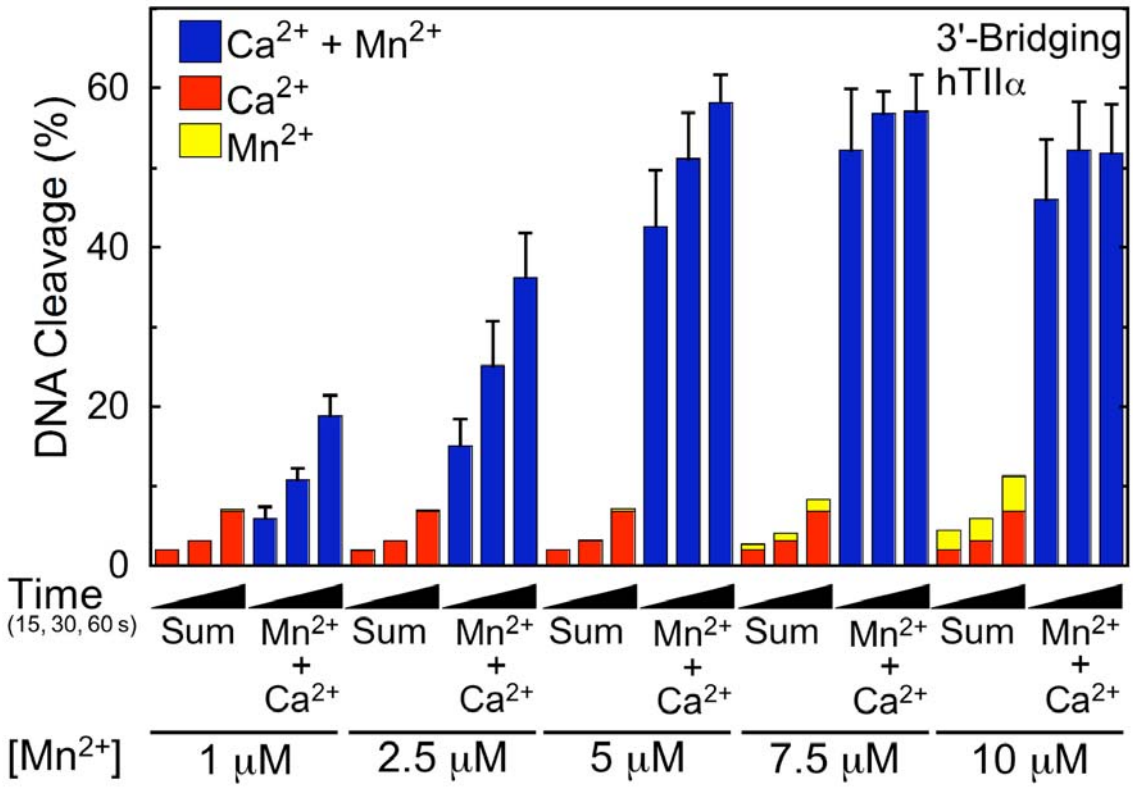

Figure 37: Cleavage of nicked 3'-bridging phosphorothiolate oligonucleotide substrates by topoisomerase IIα in the presence of divalent metal ion combinations. DNA cleavage reactions were carried out for 15, 30 or 60 s in the presence of 2.5 mM Ca^{2+} alone (red bars), 1–10 μM Mn^{2+} alone (yellow bars), or a mixture of 2.5 mM Ca^{2+} and 1–10 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity. However, the standard deviation for these latter reactions never exceeded 1.7%.

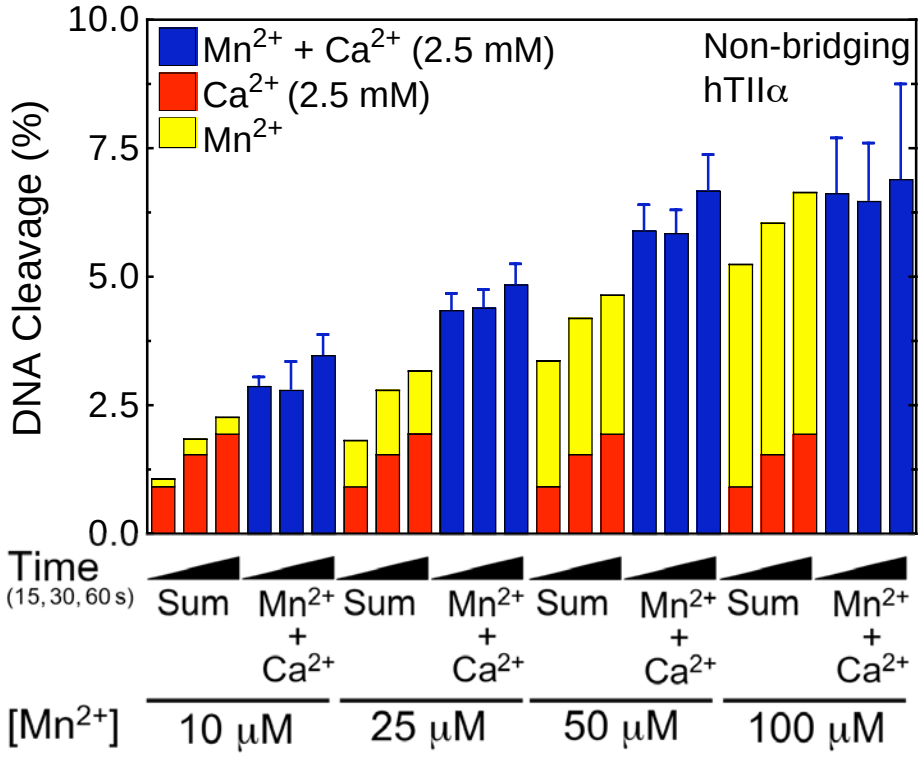

Figure 38: Cleavage of nicked non-bridging phosphorothioate oligonucleotide substrates by topoisomerase IIα in the presence of divalent metal ion combinations. DNA cleavage reactions with topoisomerase IIα were carried out for 15, 30 or 60 s in the presence of 2.5 mM Ca^{2+} alone (red bars), 10–100 μM Mn^{2+} alone (yellow bars), or a mixture of 2.5 mM Ca^{2+} and 10–100 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity.

Amino Acid Residues Involved in Divalent Metal Coordination in Topoisomerase IIα

Amino acids that are postulated to interact with the metal ions in the active site of topoisomerase IIα are D541, D543, D545, and E461. These assignments are based on previous enzymological studies of altered *E. coli* gyrase proteins in which homologous residues were mutated (68), as well as structural studies of prokaryotic topoisomerase III, primase (dnaG), and other DNA enzymes that contain the Toprim domain (66,219,220). Moreover, these assignments are consistent with a recent crystal structure of the yeast topoisomerase II catalytic core in a complex with nicked DNA (65). In this structure, residues homologous to D541, D543, and E461 of the human enzyme were in close proximity to the observed Mg^{2+} atom. However, as noted earlier, the yeast topoisomerase II-DNA complex was non-covalent in nature and the active site tyrosine was too far away from the scissile bond to promote cleavage.

To address the validity of these assignments in the human enzyme, all three Asp residues (D541, D543, and D545) and the Glu residue (E461) were individually mutated to either Ala or Cys. Each of these mutant enzymes was purified and characterization has begun. As seen in figure 39, compared to the wild-type human topoisomerase IIα, levels of cleavage in the presence of a wild-type substrate are dramatically reduced for all of the mutant enzymes. Mutations at D545 and E461 result in enzymes that display almost no cleavage activity with the wild-type substrate. Mutations at D543 and D541 still display some activity but tend to cleave more efficiently in the presence of Mg^{2+} or Ca^{2+} than Mn^{2+}. Further, the ability to cut the phosphorothiolate is significantly reduced in all cases, except D543C, which retains considerable activity (Figure 40). It is interesting to note again that both E461 mutations display very little activity again, while D545C and

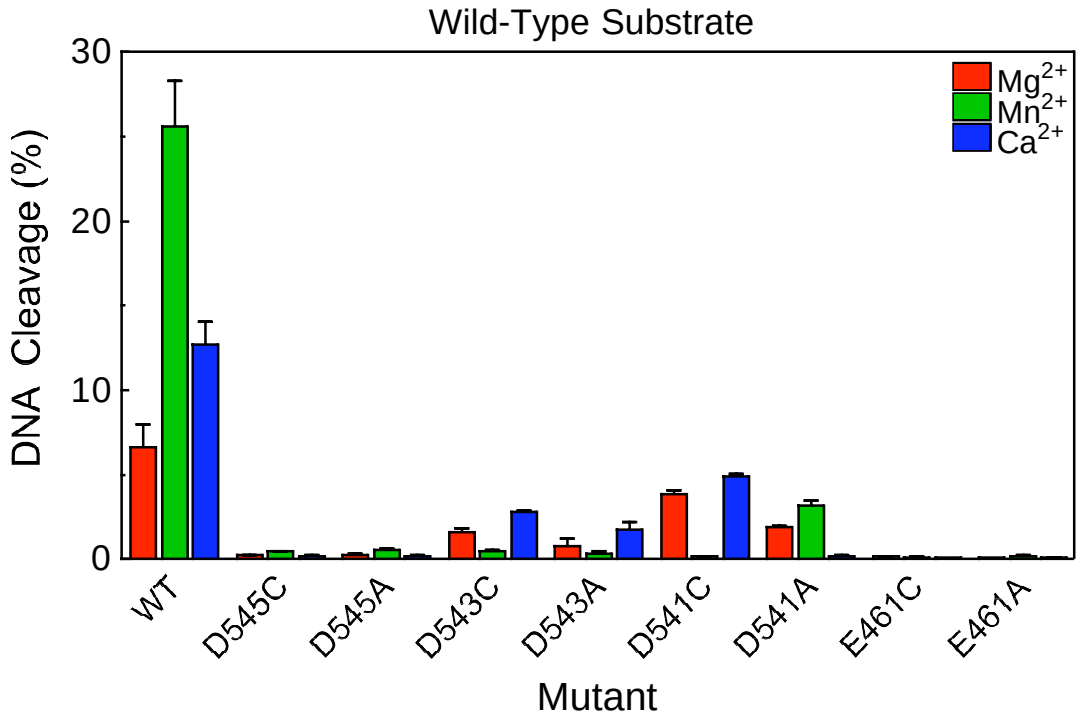

Figure 39: Cleavage of a wild-type substrate mediated by human topoisomerase IIα point mutants. The nicked wild-type site 2 substrate was cleaved in the presence of 5 mM Mg^{2+} (red bars), Mn^{2+} (green bars), or Ca^{2+} (blue bars) with wild-type (WT), D545C, D545A, D543C, D543A, D541C, D541A, E461C, or E461A human topoisomerase IIα. Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.

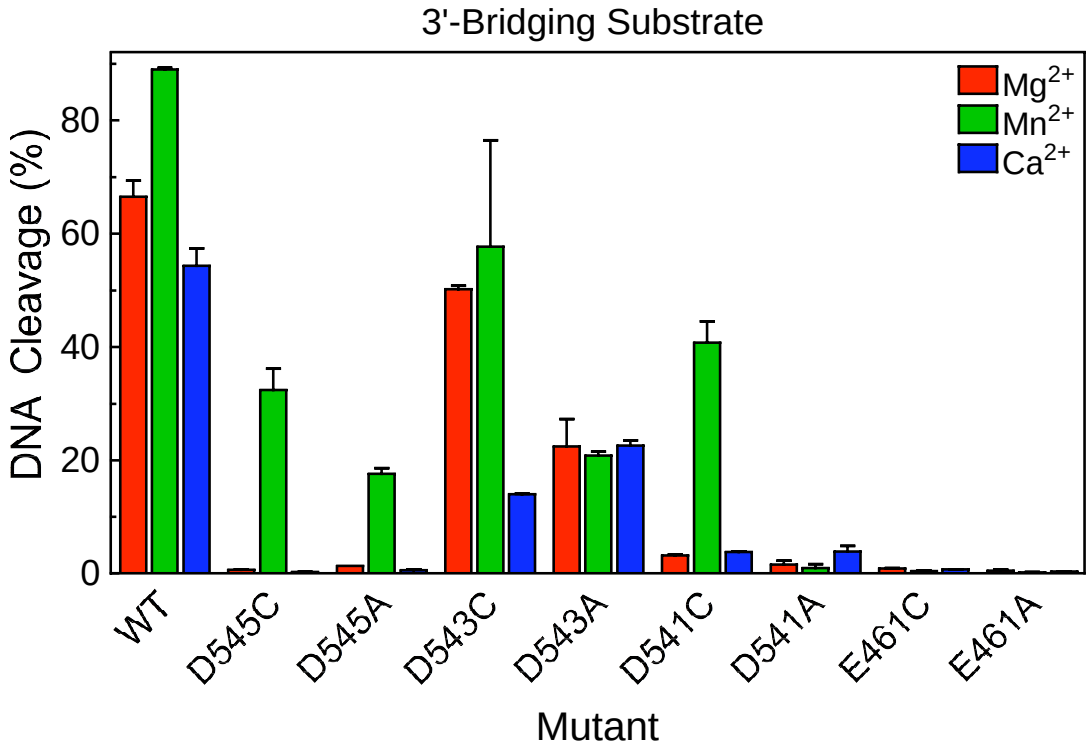

Figure 40: Cleavage of a 3'-bridging phosphorothiolate substrate mediated by human topoisomerase IIα point mutants. The nicked 3'-bridging phosphorothiolate site 2 substrate was cleaved in the presence of 5 mM Mg^{2+} (red bars), Mn^{2+} (green bars), or Ca^{2+} (blue bars) with wild-type (WT), D545C, D545A, D543C, D543A, D541C, D541A, E461C, or E461A human topoisomerase IIα. Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.

D545A cleave the phosphorothiolate efficiently only in the presence Mn^{2+}. In fact, both D543C and D541C show a significant "rescue effect" when Mn^{2+} is used as the divalent cation in the reaction. When D543A and D541A are compared with the Cys mutations at those positions, cleavage levels are generally lower for the Ala mutations, especially in the presence of Mn^{2+} (except for D543A in the presence of Ca^{2+}). Results with the non-bridging phosphorothioate substrate (Figure 41) were similar to those seen with the wild-type substrate. In all cases, phosphorothioate cleavage was reduced compared to the wild-type enzyme, and the mutations at D543 and D541 maintained the highest levels of DNA cleavage.

Conclusions

A model for the role of the divalent metal ion in the DNA cleavage reaction of human topoisomerase IIα is proposed in Figure 42. Our model for topoisomerase IIα has two salient features. The first is a critical interaction between one of the divalent metal ions and the 3'-bridging atom of the scissile phosphate. This interaction greatly accelerates rates of enzyme-mediated DNA cleavage and most likely is needed to stabilize the leaving 3'-oxygen. While kinetic studies cannot rule out an interaction between a metal ion and the non-bridging atoms of the scissile phosphate, such an interaction (if it exists) does not appear to have a major effect on rates of DNA scission.

The other important feature is a requirement for a second divalent metal ion in the DNA cleavage-ligation active site of the human type II enzyme. Although our data provide strong evidence for the presence of this second metal ion, they offer no direct insight into the specific role that it plays during DNA scission. However, based on a

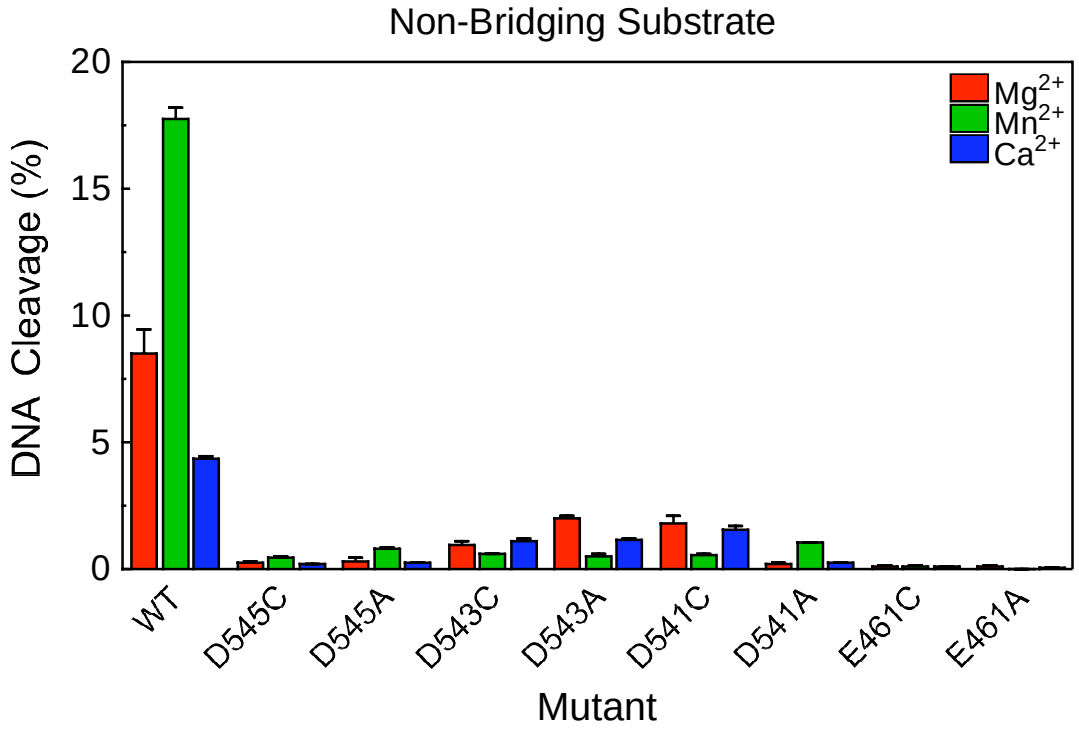

Figure 41: Cleavage of a non-bridging phosphorothioate substrate mediated by human topoisomerase IIα point mutants. The nicked nonbridging phosphorothioate site 2 substrate was cleaved in the presence of 5 mM Mg^{2+} (red bars), Mn^{2+} (green bars), or Ca^{2+} (blue bars) with wild-type (WT), D545C, D545A, D543C, D543A, D541C, D541A, E461C, or E461A human topoisomerase IIα. Error bars represent the standard error of the mean of two independent experiments or the standard deviation of three independent experiments.

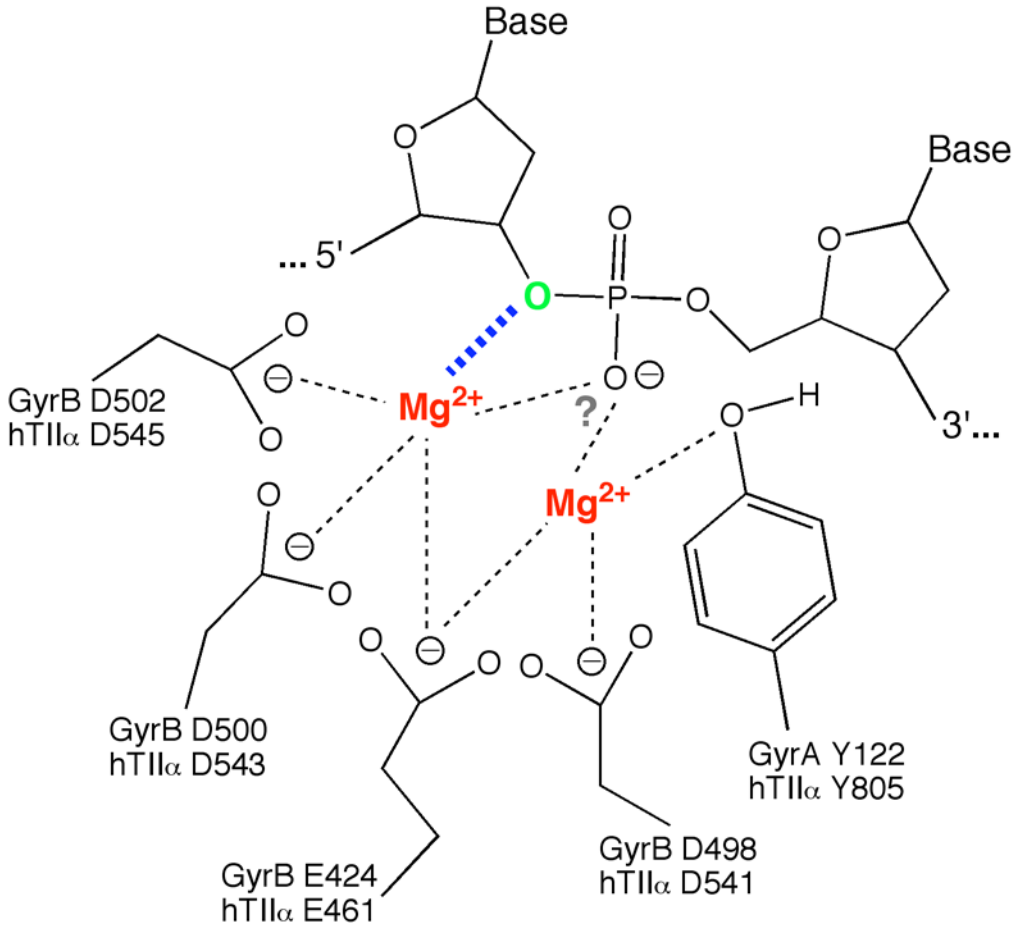

Figure 42: A two-metal ion model for DNA cleavage by human topoisomerase IIα. Details are described in the text. Amino acids that are postulated to interact with the metal ions in the active site of topoisomerase IIα are D541, D543, D545, and E461. Corresponding amino acids in the A subunit of *E. coli* DNA gyrase are shown for comparison. The model postulates that one of the metal ions (left, shown in red) makes a critical interaction with the 3'-bridging atom of the scissile phosphate (bond shown in blue), which most likely is needed to stabilize the leaving 3'-oxygen (shown in green). While a second metal ion (right, shown in red) is required for DNA scission, our data provide no direct insight into its specific role during cleavage. However, based on a model proposed for *E. coli* DNA gyrase (68), this divalent cation may stabilize the DNA transition state and/or help deprotonate the active site tyrosine (Y805). Finally, if an interaction between one of the metal ions (or both) and the non-bridging atoms of the scissile phosphate exists, it does not appear to have a major effect on rates of DNA scission. The figure was adapted from Ref. (68).

model proposed for *E. coli* DNA gyrase (68), we postulate that this divalent cation may stabilize the DNA transition state and/or help deprotonate the active site tyrosine (Y805).

The mutant enzyme evidence with the phosphorothiolate substrate supports the model shown in Figure 42 in several ways. First, "rescue" of cleavage activity in the specifically presence of Mn^{2+} suggests that D545 interacts with a metal ion that coordinates with the 3'-bridging position during scission. A similar conclusion can be reached for D543 when comparing the activity of the Cys mutation with that of the Ala mutation, which is much lower in the presence of Mg^{2+} or Mn^{2+}. Second, the lack of activity in the presence of the E461 mutations supports the model that this position may contact both of the divalent cations, making it essential to scission. Third, mutations at D541 also dramatically reduce activity but do not eliminate it, which might be expected if this position is not the only residue coordinating to the metal ion.

CHAPTER VI

USE OF DIVALENT METAL IONS IN THE DNA CLEAVAGE REACTION OF HUMAN TOPOISOMERASE IIβ

Introduction

Humans encode two isoforms of the type II topoisomerase: topoisomerase IIα and IIβ (5,8,22,42). As discussed in Chapter I, these isoforms differ in their protomer molecular masses, are encoded by separate genes, and display distinct physiology (8,10,22,42). Despite the distinct expression patterns and cellular roles of the two isoforms, topoisomerase IIα and IIβ display a high degree (~70%) of amino acid sequence identity and often show similar enzymological characteristics (10,22,42). However, numerous studies of enzyme-mediated DNA cleavage and ligation suggest that there are significant (but subtle) differences in the active sites of topoisomerase IIα and IIβ.

First, the DNA cleavage specificities of the two isoforms are similar, but not identical (63,115,221). Second, some anticancer drugs that enhance topoisomerase II-mediated DNA cleavage display preferential effects on one isoform or the other (101,115,222). Third, covalent topoisomerase IIα-cleaved DNA complexes (*i.e.*, cleavage complexes) that are formed during scission generally persist longer than equivalent complexes with topoisomerase IIβ (222,223). Fourth, topoisomerase IIβ is more sensitive than topoisomerase IIα to alterations in the scissile bond of DNA. For example, topoisomerase IIβ is less able to cleave a DNA substrate that contains a 3'-bridging sulfur atom (*i.e.*, phosphorothiolate) in place of the normal oxygen atom (Figure 11). It also

ligates a nick that has been activated by the addition of a 5'-*p*nitrophenyl group more slowly (169).

All type II topoisomerases require a divalent metal ion in order to cleave and ligate DNA. In the previous chapter, it was shown that human topoisomerase IIα utilized a two-metal-ion mechanism similar to that used by primases, some DNA polymerases, and bacterial DNA gyrase (68,189,192,224). Given the differences in the active sites of topoisomerase IIα and IIβ, we wanted to determine whether the two isoforms used divalent metal ions in a similar manner. Results indicate that topoisomerase IIβ also utilizes a two-metal-ion mechanism for DNA cleavage. Furthermore, they provide the first kinetic evidence for an important interaction between a divalent metal ion and the non-bridging atom of the scissile phosphate in the DNA cleavage reaction of type II topoisomerases. Please note: Amber M. Burch, a Vanderbilt Summer Science Academy Student, started this project under my direct supervision. Consequently, Ms. Burch generated some of the data presented in this Chapter.

Results and Discussion

Interactions Between Divalent Metal Ions and Scissile Phosphate Atoms During DNA Cleavage Mediated by Topoisomerase IIβ

As a first step towards defining the requirement for a divalent metal ion in the DNA cleavage reaction of human topoisomerase IIβ, interactions between the cation and the scissile phosphate atoms were assessed. All DNA cleavage substrates utilized in the present study had the same sequence. However, experiments took advantage of three alterations in the scissile bonds of these oligonucleotides. First, all substrates contained a

nick at the scissile bond on the strand that was not being monitored for cleavage. As described for topoisomerase IIα in Chapter V, the presence of this nick on the opposite strand greatly enhances the sensitivity of the DNA cleavage reaction, stimulating both rates and levels of scission ~10–fold.

Second, a phosphorothiolate substrate was employed that substituted a sulfur for the 3'-bridging oxygen atom at the scissile phosphate (*i.e.*, S–P scissile bond). Topoisomerase IIβ cleaved these oligonucleotides with all of the characteristics of wild-type substrates, with the following exception: since the resulting 3'-terminal –SH moiety is a poor nucleophile at phosphorous (153,171), the 3'-bridging phosphorothiolate did not support ligation (data not shown). Thus, as described in Chapter III for topoisomerase IIα, S–P substrates isolate the forward DNA scission event from ligation, allowing high levels of cleavage complexes to accumulate.

Third, a substrate was utilized that substituted a sulfur atom for the non-bridging oxygen at the scissile phosphate. This phosphorothioate substrate was a racemic mixture that replaced either the R_P or S_P non-bridging oxygen atom with a sulfur atom. In contrast to the 3'-bridging phosphorothiolate oligonucleotide, the non-bridging phosphorothioate substrate maintained a DNA cleavage-ligation equilibrium and cleavage complexes did not accumulate over time.

Interactions between divalent cations and the scissile phosphate atoms were determined by comparing the ability of topoisomerase IIβ to cleave DNA substrates containing an oxygen atom or a sulfur atom at the 3'-bridging or non-bridging position in the presence of metal ions of varying "softness" (*i.e.*, thiophilicity). The ions used for

this study were Ca^{2+}, Mg^{2+}, and Mn^{2+}. As discussed in Chapter V, Mn^{2+} is the "softest," or most thiophilic metal, and Mg^{2+} and Ca^{2+} are harder, or less thiophilic (215-217).

The first set of experiments established the ability of divalent metal ions to support cleavage of a wild-type O–P substrate by topoisomerase IIβ. Consistent with previous work with topoisomerase IIα, Mn^{2+} and Ca^{2+} generated higher levels of DNA cleavage than Mg^{2+} (Figure 43, *top left panel*). Levels of cleavage complexes formed in the presence of Mn^{2+} or Ca^{2+} were ~5 or 2 times higher, respectively than those observed with Mg^{2+}. Despite the low equilibrium levels of cleavage seen with Mg^{2+}, initial rates of scission (determined by rapid chemical quench over the first seconds of the cleavage reaction) with the divalent cation were comparable to those generated in the presence of Mn^{2+} (*top right panel*).

The previous chapter demonstrated that there is an important interaction between the metal ion and the 3'-bridging atom of the scissile bond in the active site of human topoisomerase IIα that significantly enhances the ability of the enzyme to cleave DNA. Therefore, a second set of experiments examined the divalent cation preference for cleavage of the 3'-bridging S–P (phosphorothiolate) oligonucleotide by topoisomerase IIβ (Figure 43, *middle panels*). Levels and rates of cleavage observed in the presence of the thiophilic metal ion, Mn^{2+}, were considerably higher than those generated with the hard metal, Ca^{2+}. Both the levels and rate of cleavage for reactions that contained Ca^{2+} fell significantly below those with Mg^{2+}. The drop in Ca^{2+}-supported scission of the S–P, as compared to the O–P DNA substrate, together with the relative rise in cleavage levels and rates with Mn^{2+}, strongly suggest that the divalent cation contacts the 3'-bridging atom of the scissile bond in the active site of human topoisomerase IIβ. Furthermore, like

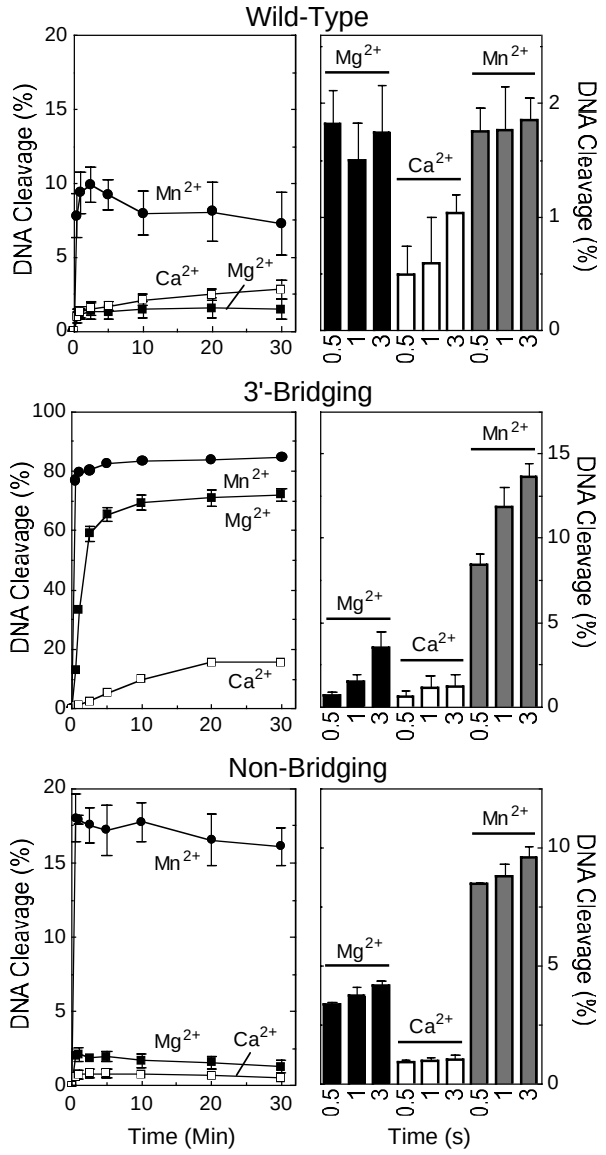

Figure 43: Cleavage of nicked oligonucleotide substrates by topoisomerase IIβ in the presence of different divalent metal ions. The nick is located at the scissile bond on the unlabeled strand. Thirty-minute time courses (*left panels*) and rapid quench time points (*right panels*) for the wild-type (*top*), 3'-bridging phosphorothiolate (*middle*), and non-bridging phosphorothioate (*bottom*) are shown. DNA cleavage was carried out in the presence of 5 mM Mg^{2+} (closed square), Ca^{2+} (open square), and Mn^{2+} (closed circle). Rapid quench results for 0.5, 1, and 3 seconds are shown in the presence of Mg^{2+} (black bars), Ca^{2+} (open bars), and Mn^{2+} (gray bars). Error bars represent the standard deviation of at least three independent experiments.

topoisomerase IIα, this metal ion-DNA interaction mediated by topoisomerase IIβ stimulates rates of enzyme-mediated scission.

Models proposed for the active site geometry of *E. coli* DNA gyrase (68) and human topoisomerase IIα (Figure 42) both postulate interactions between divalent metal ions and a non-bridging oxygen atom of the scissile phosphate. However, no direct evidence supporting this interaction has been reported. To this point, replacement of non-bridging oxygen atoms with sulfur had little effect on levels of DNA scission generated by either enzyme (218) [Figure 31]. Thus, it was concluded that if these postulated interactions between metal ions and non-bridging oxygen atoms exist, their effects on DNA cleavage mediated by these enzymes were equivocal.

Based on mutagenesis studies, it has been proposed that topoisomerase IIβ utilizes divalent cations in a manner similar to that of DNA gyrase (225,226). Nonetheless, a third set of experiments was carried out to determine whether interactions could be observed between the metal ion and a non-bridging atom of the scissile phosphate in the active site of human topoisomerase IIβ.

In marked contrast to the findings with DNA gyrase (218) and topoisomerase IIα (Chapter V), dramatic differences were seen with a substrate that contained a sulfur atom in the non-bridging position (Figure 43, *bottom panels*). Levels of cleavage generated by topoisomerase IIβ in the presence of Mn^{2+} were ~8–fold higher than those seen with Mg^{2+}, and rates of scission were 2 to 3 times faster. Furthermore, levels and rates of cleavage for reactions that contained Ca^{2+} dropped below those observed with Mg^{2+}. Finally, as compared to results with the wild-type substrate (*top panel*), substitution of sulfur at the non-bridging position increased cleavage ~2–fold in the presence of Mn^{2+}

and decreased it ~3–fold in the presence of Ca^{2+}. These results provide the first evidence for an interaction between the divalent metal ion and a non-bridging atom of the scissile phosphate for any type II topoisomerase and suggest that this interaction enhances the ability of topoisomerase IIβ to cleave DNA.

A Two-Metal-Ion Mechanism for DNA Cleavage Mediated by Human Topoisomerase IIβ

On the basis of metal ion mixing experiments, DNA gyrase and topoisomerase IIα were demonstrated to use a two-metal-ion mechanism for DNA cleavage (68) [Figure 37]. Two approaches were utilized to determine whether human topoisomerase IIβ uses a similar mechanism to mediate DNA scission. In the first, DNA cleavage was monitored over a range of divalent cation concentrations. These experiments utilized Mn^{2+} (a soft cation) and Ca^{2+} (a hard cation). As seen in Figure 44, the concentration of Mn^{2+} that was required to promote DNA scission mediated by topoisomerase IIβ was considerably lower than that seen with Ca^{2+}.

When cleavage of the wild-type O–P oligonucleotide was monitored (Figure 44, *top panels*), both divalent cations displayed a mild biphasic concentration dependence. This finding suggests that topoisomerase IIβ utilizes more than one divalent metal ion to mediate DNA cleavage.

Introduction of a 3'-bridging phosphorothiolate had a significant effect on cation titrations, and resulted in a dramatically more pronounced initial phase with Mn^{2+} (Figure 44, *middle panels*). This biphasic metal ion concentration dependence suggests that there are two divalent cation sites in the DNA cleavage-ligation domain of topoisomerase IIβ and that both need to be filled in order to support scission. The finding that the initial

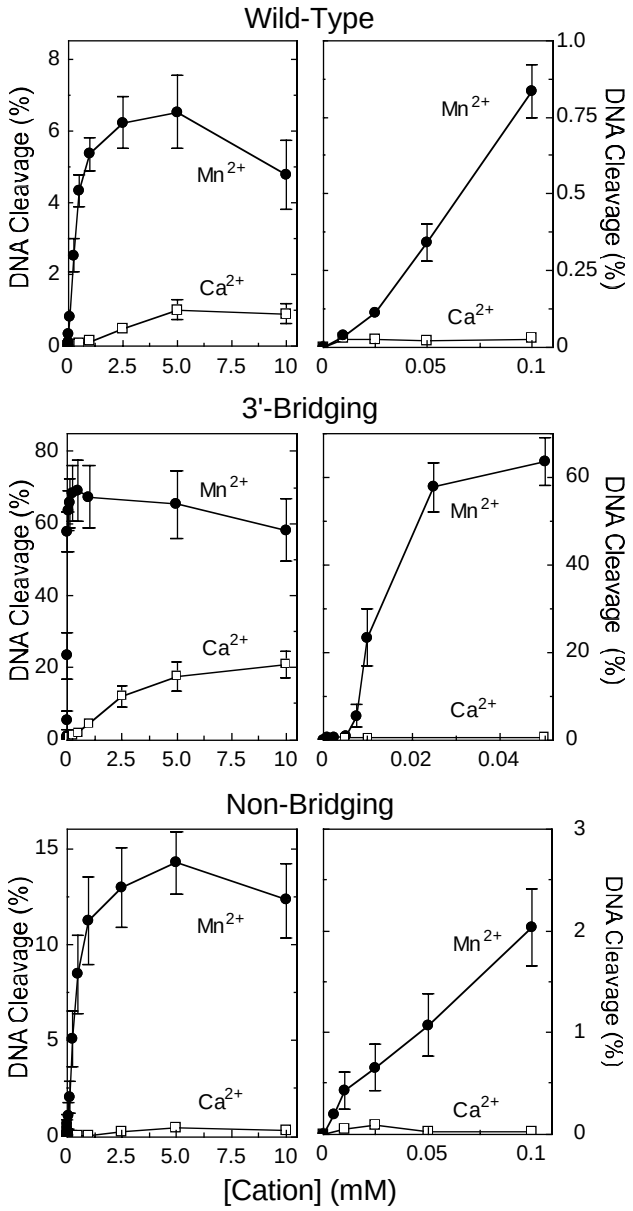

Figure 44: Metal ion concentration dependence for DNA cleavage of nicked wild-type, phosphorothiolate, and phosphorothioate substrates by topoisomerase IIβ. Results for the wild-type (*top*), 3'-bridging phosphorothiolate (*middle*), and non-bridging phosphorothioate (*bottom*) are shown. *Left panels*, Mn^{2+} (closed circles) or Ca^{2+} (open squares) were titrated from 10 μM to 10 mM for wild-type, 1 μM to 10 mM for 3'-bridging substrates, or 5 μM to 10 mM for non-bridging. *Right panels*, expanded DNA cleavage at concentrations up to 100 μM for wild-type and non-bridging substrates or 50 μM for the 3'-bridging substrate are shown. All reactions were incubated for 30 min prior to stopping the reaction. Error bars represent the standard error of the mean of two independent experiments or the standard deviation of at least three independent experiments.

(*i.e.*, high affinity) phase with the 3'-bridging phosphorothiolate substrate became more prominent with the soft metal ion (Mn^{2+}) implies that the first site that is filled by the divalent cation is the one that interacts with the bridging atom of the scissile bond.

Introduction of a sulfur at the non-bridging position resulted in an ~2–fold increase in levels of DNA cleavage in the presence of Mn^{2+} and an ~2–fold decrease in the presence of Ca^{2+} (Figure 44, *bottom panels*) as compared to the wild-type substrate. Although this finding provides further evidence for an important interaction between a metal ion and the non-bridging atom of the scissile phosphate, no prominent initial phase was observed.

Therefore, a second approach was utilized to confirm that two metal ions are required for DNA cleavage mediated by human topoisomerase IIβ. These studies took advantage of the enhanced scission observed for substrates that contained a sulfur atom in either the 3'-bridging or the non-bridging positions in the presence of Mn^{2+}. Experiments compared levels of DNA cleavage monitored in the presence of Ca^{2+}, Mn^{2+}, or a combination of the two divalent cations. Near saturating concentrations of Ca^{2+} (5 mM) were paired with sub-saturating concentrations of Mn^{2+} (up to 100 μM depending on the cleavage substrate). These limiting concentrations of Mn^{2+} supported levels of enzyme-mediated DNA scission that were <15% of the observed maxima.

Figures 45-47 display results for topoisomerase IIβ-mediated DNA cleavage of the wild-type, 3'-bridging phosphorothiolate, or non-bridging phosphorothioate substrate, respectively.

Combining metal ions in reactions that utilized topoisomerase IIβ and a wild-type substrate had a small but discernable effect (Figure 45). Levels of DNA cleavage in

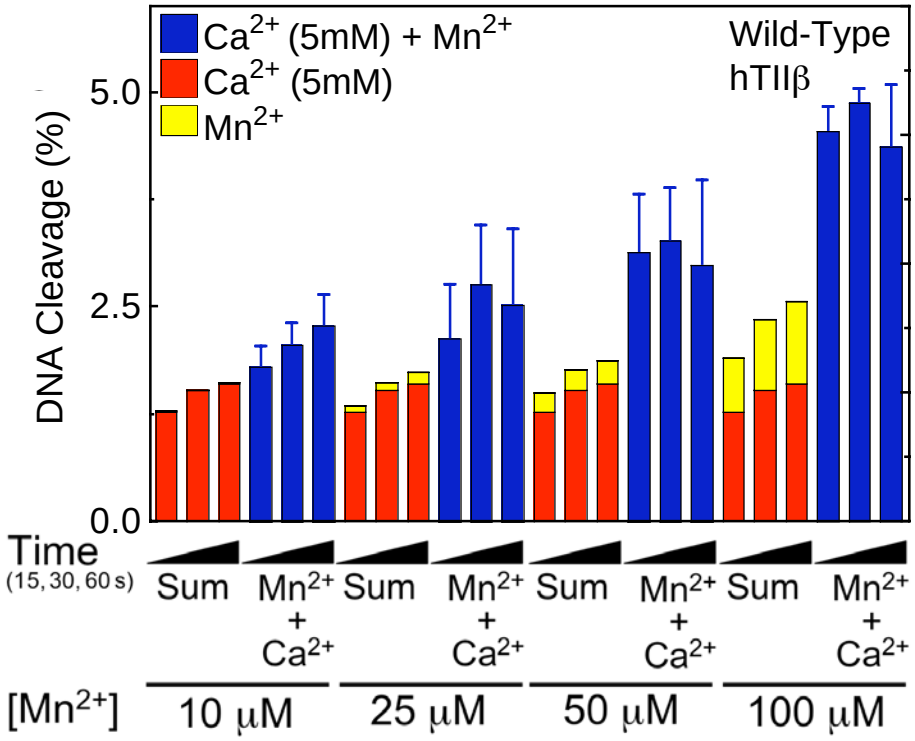

Figure 45: Cleavage of nicked wild-type oligonucleotide substrates by topoisomerase IIβ in the presence of divalent metal ion combinations. DNA cleavage reactions with topoisomerase IIβ were carried out for 15, 30 or 60 s in the presence of 5 mM Ca^{2+} alone (red bars), 10–100 μM Mn^{2+} alone (yellow bars), or a mixture of 5 mM and 10–100 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity.

reactions that contained both divalent cations were slightly higher (~1.5– to 2–fold) than predicted by summing the amount of cleavage generated in reactions that contained either Mn^{2+} or Ca^{2+}. This result supports a two-metal-ion mechanism for DNA cleavage mediated by the β isoform and indicates that minor differences between the two cation sites can be discerned even in the presence of an unmodified substrate.

In contrast to the above results, a dramatic difference was seen in experiments that utilized the 3'-bridging S–P substrate (Figure 46). Levels of DNA scission in reactions that contained both Ca^{2+} and Mn^{2+} were ≥10 times greater than the calculated levels derived from the sum of cleavage observed in the presence of the individual metal ions. Furthermore, a large enhancement in the rate of DNA scission (compared to calculated sums) was observed when Ca^{2+} and Mn^{2+} were combined. It should be noted that these experiments used Mn^{2+} concentrations (1–10 μM) that partially filled or saturated the high affinity scissile bond site (see Figure 44, *middle panel*) without appreciably filling the second site. These data confirm the two-metal-ion mechanism as well as the importance of the metal ion interaction with the 3'-bridging atom of the scissile phosphate.

As seen in Figure 47, the inclusion of a non-bridging sulfur at the scissile phosphate also stimulated DNA cleavage in reactions that contained a mixture of Ca^{2+} and Mn^{2+}. Levels of cleavage in reactions that contained both metal ions were ~5 times higher than predicted by the calculated sums. Once again, these data confirm the two-metal-ion mechanism for DNA cleavage mediated by topoisomerase IIβ, and they also validate the significance of the metal ion interaction with the non-bridging atom of the scissile

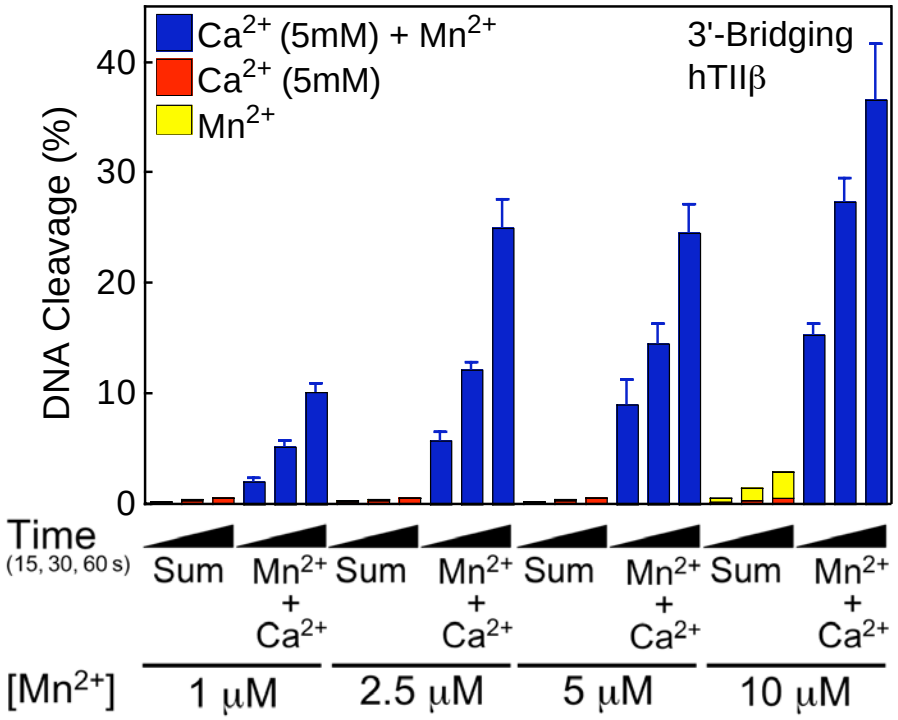

Figure 46: Cleavage of nicked 3'-bridging phosphorothiolate oligonucleotide substrates by topoisomerase IIβ in the presence of divalent metal ion combinations. DNA cleavage reactions with topoisomerase IIβ were carried out for 15, 30 or 60 s in the presence of 5 mM Ca^{2+} alone (red bars), 1–10 μM Mn^{2+} alone (yellow bars), or a mixture of 5 mM Ca^{2+} and 1–10 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity.

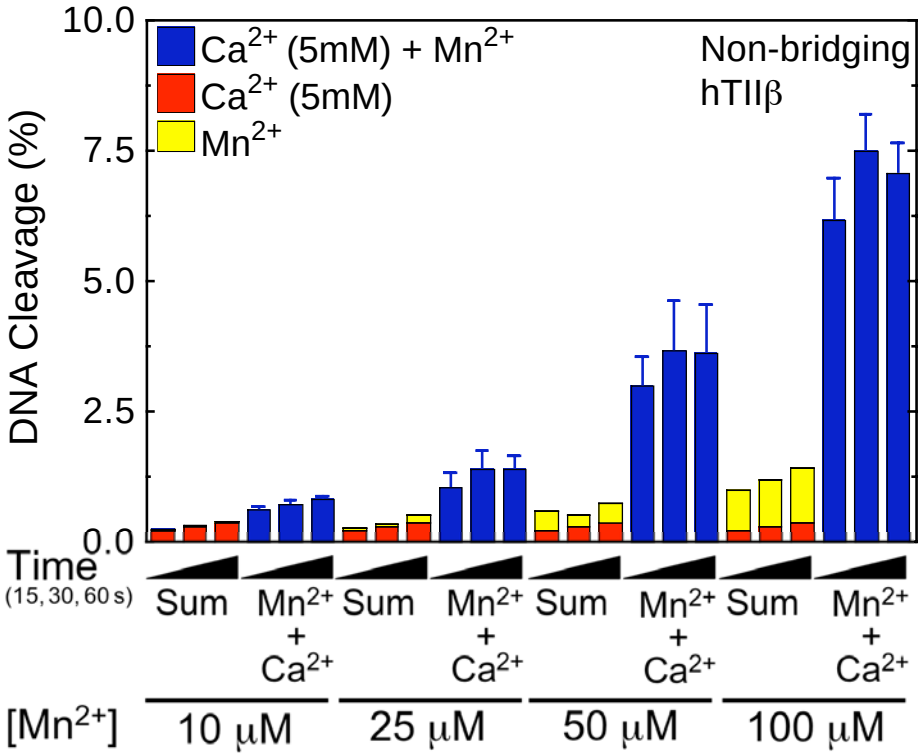

Figure 47: Cleavage of nicked non-bridging phosphorothioate oligonucleotide substrates by topoisomerase IIβ in the presence of divalent metal ion combinations. DNA cleavage reactions with topoisomerase IIβ were carried out for 15, 30 or 60 s in the presence of 5 mM Ca^{2+} alone (red bars), 10–100 μM Mn^{2+} alone (yellow bars), or a mixture of 5 mM Ca^{2+} and 10–100 μM Mn^{2+} (Ca^{2+} + Mn^{2+}, blue bars). The calculated sum of the enzyme-mediated DNA cleavage from reactions containing either Ca^{2+} or Mn^{2+} alone is also shown (Sum, stacked open and stippled bars, respectively). All data represent the average of at least three independent experiments. Error bars for reactions carried out in the presence of Ca^{2+} + Mn^{2+} are shown. Error bars for reactions carried out in the presence of Ca^{2+} or Mn^{2+} alone are not shown for simplicity.

phosphate. It is notable that results with the non-bridging substrate were markedly different with topoisomerase IIα (Figure 38).

Scission levels generated by topoisomerase IIα in the presence of Ca^{2+} and Mn^{2+} were marginally higher compared to those predicted by the calculated sums (Figure 47) or by those observed in the presence of both divalent cations with the wild-type oligonucleotide (compare with Figure 45). These findings indicate that human topoisomerase IIα and IIβ utilize divalent metal ions differently for some aspects of their DNA cleavage reactions. The interaction between a metal ion and the non-bridging atom of the scissile phosphate is important for the DNA cleavage reaction of topoisomerase IIβ and substantially raises levels of scission. However, if the parallel interaction in topoisomerase IIα exists, it has little effect on DNA scission.

Conclusions

A model for the use of divalent metal ions in the DNA cleavage reaction of human type II topoisomerases is proposed in Figure 48. Amino acids that are postulated to interact with the metal ions in the active site of topoisomerase IIα and IIβ are assigned based on previous enzymological studies of mutated *E. coli* DNA gyrase and human topoisomerase IIβ proteins (68,225,226), as well as structural studies of bacterial topoisomerase III, yeast topoisomerase II, and other DNA enzymes that contain the Toprim domain (65,66,219,220).

Our model has several features. First, topoisomerase IIα and IIβ both employ a two-metal-ion mechanism to support DNA cleavage. Second, both enzyme isoforms utilize an interaction between a divalent metal ion (metal ion 1) and the 3'-bridging atom of the

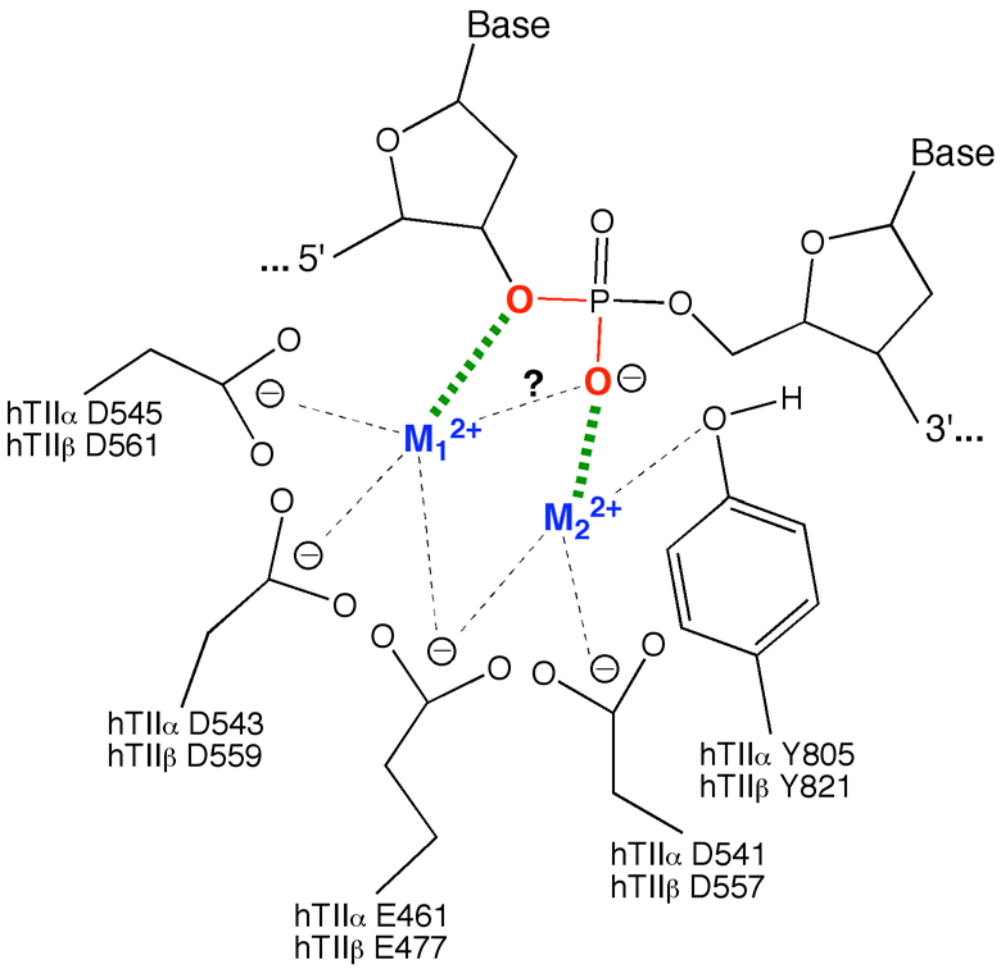

Figure 48: A two-metal-ion model for DNA cleavage by human topoisomerase IIα and IIβ. Details are described in the text. Amino acids that are postulated to interact with the metal ions in the active site of both isoforms are shown. The model postulates that the metal ions bind to topoisomerase II in an ordered fashion, in which the binding of metal ion 1 (M_1^{2+}, shown in blue) is a prerequisite for the binding of metal ion 2 (M_2^{2+}, shown in blue). M_1^{2+} makes a critical interaction with the 3'-bridging atom (left, shown in red) of the scissile phosphate (left, bond shown in green), which most likely is needed to stabilize the leaving 3'-oxygen (left, shown in red). M_2^{2+} is required for DNA scission and likely interacts with the non-bridging oxygen (right, shown in red) during scission. This divalent cation may stabilize the DNA transition state and/or help deprotonate the active site tyrosine. While interactions between M_2^{2+} and the non-bridging atom of the scissile phosphate significantly enhance DNA cleavage mediated by topoisomerase IIβ, effects on DNA scission mediated by topoisomerase IIα are equivocal. The model was adapted from Ref. (68).

scissile phosphate to accelerate rates of enzyme-mediated DNA cleavage, most likely by stabilizing the leaving 3'-oxygen. At the present time, it is not known whether metal ion 1 also has interactions with the non-bridging oxygen. Third, a second metal ion (metal ion 2) appears to contact a non-bridging atom of the scissile phosphate in the active site of topoisomerase IIβ. This interaction plays a significant role in DNA cleavage mediated by the β isoform and greatly stimulates scission. As proposed previously, this metal ion is believed to stabilize the DNA transition state and/or help deprotonate the active site tyrosine (68,226). Although topoisomerase IIα has an absolute requirement for metal ion 2, the role of this divalent cation in its DNA cleavage reaction is unclear. Results of the present study cannot rule out an interaction between the metal ion and the non-bridging oxygen in the active site of topoisomerase IIα. However, if the interaction exists, it does not affect rates of DNA cleavage.

CHAPTER VII

CONCLUSIONS

Type II topoisomerases are ubiquitous enzymes that regulate the topological state of DNA. These enzymes are involved in a number of critical cellular processes, such as replication, transcription, and recombination, and are effective and commonly used targets for anticancer drugs. Despite their essential physiological functions, the basic mechanism of type II topoisomerases is not completely understood. Important questions concerning the mechanism of cleavage, the coordination of the two protomer subunits, and the role of the required divalent cations have been difficult to address. Previously, there were no systems established to directly tackle these challenging concepts. The work described in this dissertation uses several oligonucleotide-based approaches to help unravel the complexities of the human isoforms of topoisomerase II.

Isolation of the Forward DNA Cleavage Reaction of Topoisomerase II

The low level of DNA cleavage at equilibrium and the tight coupling of the cleavage and ligation reactions have made it difficult to characterize the mechanism by which topoisomerase II cuts the double helix. Therefore, Chapter III of this dissertation describes the development of a system that isolates topoisomerase II-mediated DNA scission from ligation. This goal was accomplished using oligonucleotide substrates that contained a 3'-bridging phosphorothiolate at the scissile bond. Results demonstrate that the characteristics of topoisomerase IIα-mediated cleavage of phosphorothiolate

oligonucleotides were identical to those seen with wild-type substrates, except that no ligation was observed.

This unidirectional accumulation of cleavage complexes provided an important advantage over traditional "suicide substrates" that require a time-dependent conversion from a reversible to an irreversible, covalent complex. As seen in Chapter III, a +2/+3 nicked suicide substrate may leave the impression that etoposide enhances the forward rate of DNA cleavage. However, experiments with phosphorothiolate substrates demonstrate that there is no enhancement in the forward rate of scission in the presence of etoposide. This result is consistent with the fact that etoposide is known to strongly inhibit topoisomerase II-mediated DNA ligation. Results also indicate that the two enzyme subunits are partially coordinated and that cleavage at one scissile bond increases cleavage at the other. Further, abasic sites that increase levels of cleavage complexes without affecting ligation stimulate the forward rate of scission.

Not only do phosphorothiolate substrates provide significant advantages over traditional "suicide substrates", but they will also be valuable for future studies on DNA scission and the topoisomerase II-DNA cleavage complex. For example, these substrates currently are being used to crystallize covalent topoisomerase II-DNA complexes for structural studies in collaboration with Dr. James Berger, University of California at Berkeley.

Coordination of the Two Protomer Subunits of Topoisomerase II

Since topoisomerase II modulates DNA topology by generating double-stranded breaks in DNA, the two protomer subunits must be relatively well-coordinated. As seen

in Chapter III, cleavage at one strand appears to increase the rate of cleavage of the opposite strand. These studies were extended in Chapter IV by using wild-type substrates in the presence or absence of scissile bond nicks. Results confirm that the presence of a nick at one scissile bond dramatically increases the rate of cleavage by human topoisomerase IIα at the scissile bond on the opposite strand. This enhanced rate of cleavage of the second strand likely coordinates the two protomer subunits of topoisomerase II and allows the enzyme to create double-stranded breaks. As determined by crystallography of yeast topoisomerase II, there is a severe bend in DNA bound in the active site of the enzyme. We propose that the increase in rate at the second strand is caused by the increased flexibility imposed by the introduction of a nick in the first strand. Such flexibility would allow the substrate to bend into position in the active site more readily.

It was also found that the presence of a nick on one strand induces cleavage four bases away on the opposite strand, even in the absence of a native cleavage site. Thus, nicks are topoisomerase II poisons that generate novel sites of DNA cleavage. This finding may have implications for the actions of type II enzymes during apoptotic DNA fragmentation. Moreover, it suggests a mechanism whereby topoisomerase II may impact the physiological effects of endogenous, environmental, or pharmaceutical compounds that damage the genetic material. For example, events or compounds that result in the generation of nicks in DNA could lead to an increase in topoisomerase II cleavage complexes and threaten cell survival.

Metal Ion Usage by Type II Topoisomerases

Chapters V and VI discuss the role of divalent metal ions in the DNA cleavage reaction of human topoisomerase IIα and IIβ. First, Chapter V demonstrated that the "thio effect" could be used to examine metal ion usage by topoisomerase IIα. This work utilized a series of divalent metal ions with varying thiophilicities in conjunction with DNA cleavage substrates that substituted a sulfur atom for the 3'-bridging oxygen or the non-bridging oxygens of the scissile phosphate. Based on the results, we propose that topoisomerase IIα mediates DNA cleavage *via* a two metal ion mechanism. In this model, one of the metal ions makes a critical interaction with the 3'-bridging atom of the scissile phosphate. This interaction greatly accelerates rates of enzyme-mediated DNA cleavage, and most likely is needed to stabilize the leaving 3'-oxygen.

The model was tested further by mutagenesis of topoisomerase IIα. By changing three conserved Asp residues (D545, D543, and D541) and one Glu residue (E461) to either Cys or Ala, the results in Chapter V demonstrate that each of these positions impacts the level of DNA cleavage. These highly conserved residues are found in several different nucleic acid-acting enzymes that utilize metal ions in their reaction chemistry and are believed to function in coordinating metal ion(s) around the active site. Results from the mutant enzymes support the model proposed for amino acid involvement in topoisomerase IIα. It is notable that E461 is the only residue proposed in the model to coordinate to both metal ions and a mutation at this position to either Cys or Ala had the greatest effect on DNA cleavage of any of the mutations, essentially abolishing activity. Further work with these mutants will be needed to more fully elucidate the role of these interactions.

As seen in Chapter VI, our work on metal ions was extended by examining the cleavage reaction of human topoisomerase IIβ. The data demonstrate that similar to topoisomerase IIα, topoisomerase IIβ employs a two-metal-ion mechanism to support DNA cleavage. Again, one metal ion makes an interaction with the 3'-bridging atom of the scissile phosphate. However, unlike the α isoform, there is an important interaction between a divalent second metal ion and a non-bridging atom of the scissile phosphate that stimulates DNA cleavage mediated by topoisomerase IIβ. If this interaction exists in topoisomerase IIα, its effects on DNA cleavage are equivocal. This result is the first evidence for an interaction between a metal ion and the non-bridging position of the DNA during scission by a type II topoisomerase.

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