DNA Topoisomerase I-Mediated Formation of Structurally Modified DNA Duplexes. Effects of Metal Ions and Topoisomerase I Inhibitors†

Xiangyang Wang, Kristine A. Henningfeld, and Sidney M. Hecht*

Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901

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ABSTRACT: The ability of DNA topoisomerase I to mediate the formation of structurally modified DNA duplexes was studied utilizing suicide substrates containing high-efficiency cleavage sites and acceptor oligonucleotides in which the 5′-terminal nucleotides were varied. When the substrates were nicked duplexes, the divalent cations Mg^{2+} and Ca^{2+} were found to facilitate the topoisomerase I-mediated formation of ligation products containing 3-nucleotide deletions on the scissile strand, but to suppress the formation of 1-nucleotide deletions. The presence of a complementary nucleotide at the 5′-end of the acceptor strand was not required for the ligation reaction to proceed, but duplex formation to produce duplexes containing a mismatch proceeded more slowly than formation of the fully complementary duplex. Topoisomerase I-mediated mismatch formation in the ligation reaction was inhibited more readily by camptothecin than the corresponding ligation reaction to form a fully complementary duplex; the extent of inhibition was comparable for all three mismatches studied. In comparison, the topoisomerase I inhibitors nitidine and coralyne exhibited quite different effects on the same ligation reactions.

Although the exact linear sequence of DNA determines the genetic make-up of an organism, topological relationships within the DNA helix affect virtually every physiological function of the genome (*1, 2*). Intracellularly, the topology of DNA is controlled by enzymes known as topoisomerases (*3, 4*). These enzymes are required for replication and transcription of DNA, condensation/decondensation, and segregation of chromosome structure, as well as recombination (*2, 5*−*7*). As a consequence of their involvement in essential cell processes, topoisomerases are molecular targets for a number of clinically important anticancer drugs (*8*).

Eukaryotic DNA topoisomerases I catalyze topological rearrangements of DNA through sequential single-stranded breakage, strand passage or free rotation (*9*), and rejoining of the phosphodiester backbone of DNA (*1, 10*). The transient strand breaks involve reversible formation of a 3′-*O*-phosphorotyrosine bond between the active site tyrosine of topoisomerase I and the DNA backbone (*11*). Under normal circumstances the cleavage and ligation reactions of topoisomerase I are tightly coupled (*12*); the coordinated cleavage and religation reactions restore continuity to the DNA duplex. However, separation of the cleavage and ligation reactions can be achieved by topoisomerase I-mediated site specific cleavage of partial DNA duplexes (Figure 1) (*13, 14*). Cleavage of such "suicide" substrates occur at high efficiency cleavage sites (*13, 14*) without sequential religation due the instability of the DNA secondary structure downstream from the site of cleavage (vide infra). The topoisomerase I−DNA intermediate thus trapped has been shown to undergo ligation with acceptor strands of varying lengths and sequences, resulting in insertions, deletions, and mismatches in vitro (*15*). This suggested the ability of topoisomerase I to catalyze covalent alterations in DNA connectivity, which has also been demonstrated (*16, 17*).

Presently, we describe several additional facets of topoisomerase I-mediated DNA rearrangement. These include the effects of divalent metal ions on topoisomerase I-mediated deletions in a system that utilized nicked duplexes as substrates. It was found that a 3-nt deletion was formed efficiently, while a 1-nt deletion was suppressed, in the presence of 5 mM Mg^{2+} or Ca^{2+}. Using suicide substrates and acceptor strands differing in the 5′-terminal nucleotide, it was also demonstrated that calf thymus topoisomerase I could facilitate the formation of DNA mismatches. However, enzyme-mediated DNA mismatch formation was slower and more readily inhibited by camptothecin than the corresponding homologous ligation. The efficiency of inhibition of camptothecin (CPT)[1] on topoisomerase I-mediated ligation to form mismatched duplexes was the same regardless of the 5′-terminal nucleotide of the individual acceptor strands. Interestingly, nitidine and coralyne showed completely different effects on topoisomerase I-mediated ligation to form correctly matched and mismatched duplexes.

EXPERIMENTAL PROCEDURES

Materials and General Methods. Coralyne chloride was purchased from Aldrich Chemical Co. Camptothecin and topotecan were obtained from SmithKline Beecham Phar-

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* To whom correspondence should be addressed.

[1] Abbreviations: CPT, camptothecin; TPT, topotecan; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; DMSO, dimethyl sulfoxide.

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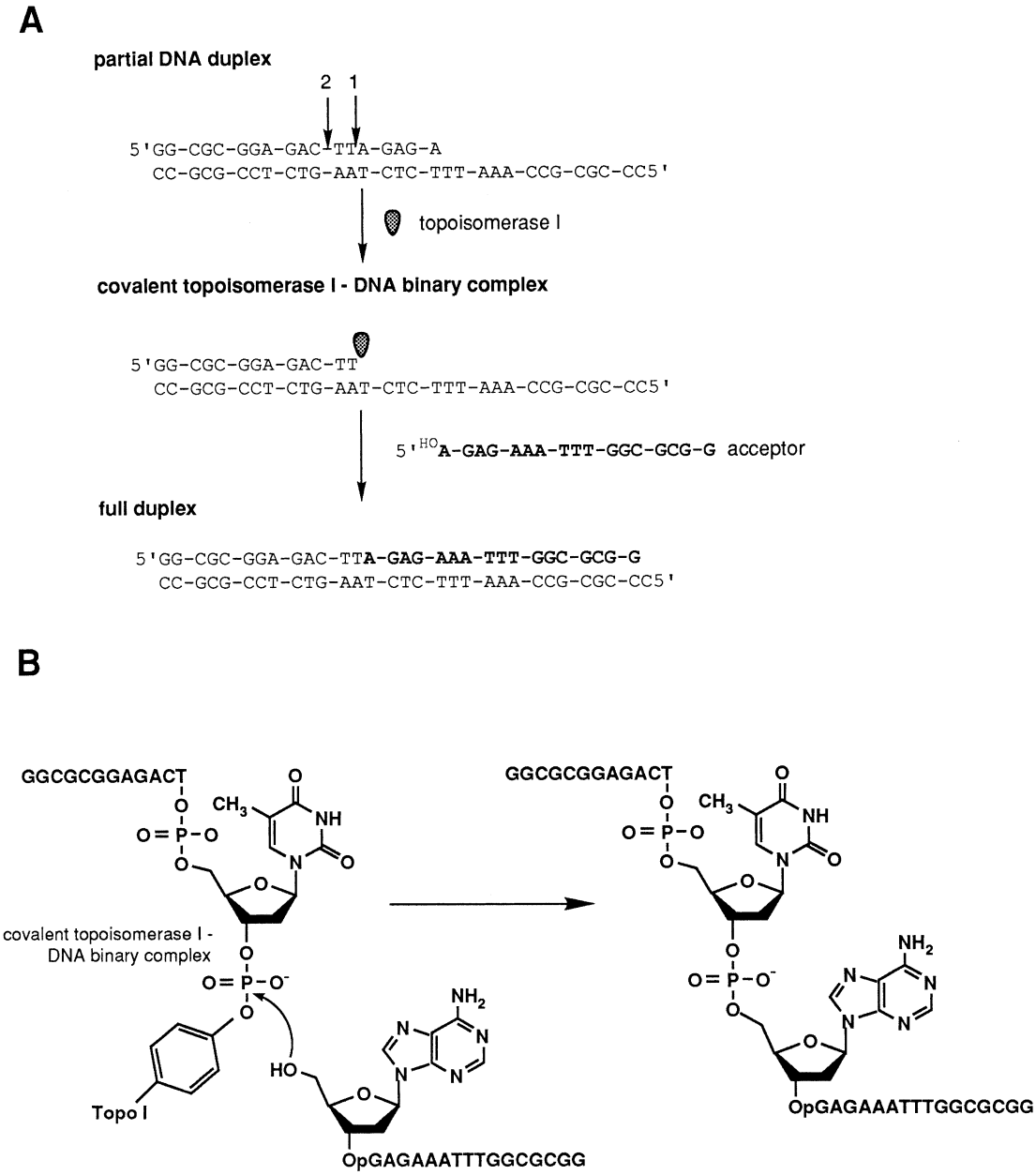


FIGURE 1: (A) DNA substrate utilized to uncouple the cleavage and ligation reactions of topoisomerase I, thereby allowing DNA strand exchange. (B) Topoisomerase I-mediated ligation reaction.

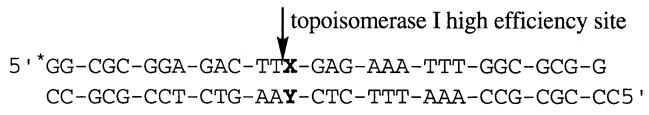
maceuticals with the assistance of Dr. Randall Johnson. Nitidine was obtained from the National Cancer Institute through the courtesy of Dr. Matthew Suffness. Topoisomerase inhibitors were dissolved in DMSO to make 5 mM stock solutions; these were stored at −20 °C. Further dilutions were made in 10% DMSO before use so that the final concentration of DMSO in the incubation mixture was 1%. T4 polynucleotide kinase and proteinase K were purchased from United States Biochemicals; trypsin was obtained from Sigma Chemicals. Uracil DNA glycosylase was from New England Biolabs. [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from ICN Pharmaceuticals. DNA oligonucleotides were purchased from Cruachem, Inc.

Denaturing polyacrylamide gel electrophoresis was carried out on 20% gels (8 M urea; 50 W for 2−3 h). Gels were visualized by autoradiography at −80 °C with Kodak XAR-2 film and quantified utilizing a Molecular Dynamics 400 E phosphorimager using ImageQuant version 3.2 software. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

Enzyme Purification. Calf thymus DNA topoisomerase I was isolated and purified by a slight modification of a published procedure (*18*). The isolated protein exhibited two major bands ($M_r \approx 96\,000$ and 82 000) when analyzed by SDS−polyacrylamide gel electrophoresis and visualization of the protein by silver staining. The heterogeneity of the isolated topoisomerase I can be attributed to proteolysis of the N-terminal domain during the isolation procedures (*19*). This domain contains the nuclear localization signal but does not otherwise affect topoisomerase I function (*19*). The purified protein had a specific activity of 1.4×10^7 units/mg of protein. One unit is the amount of enzyme that relaxes 250 ng of pBR322 supercoiled DNA in 30 min at 37 °C.

Oligonucleotide Substrates. Synthetic oligonucleotides were purchased from Cruachem Inc. All oligonucleotides were purified on preparative 20% denaturing polyacrylamide

Table 1: Topoisomerase I-Mediated Cleavage of DNA Duplexes in the Presence and Absence of CPT[a,b]



substrate		cleavage (%)[c]	
X	Y	−CPT	+CPT
G	C		28.7
U	C	7.3	16.3
abasic	C	13.9	12.1
A	T	4.5	14.9
U	T	6.2	13.1
abasic	T	22.5	17.1

[a] All DNA duplexes were formed and the specific abasic sites were created as described in Experimental Procedures. [b] CPT was employed at 20 μM final concentration. [c] DNA present as cleavage product was calculated as a percentage of all DNA. Radioactivity was quantified using a phosphorimager.

gels. The DNA was visualized by UV shadowing (*20*) or autoradiography, and the bands of interest were excised from the gel. The DNA was eluted (2 M $LiClO_4$) for 12 h at 37 °C and recovered by precipitation with acetone as described (*21*).

Hybridization of Substrates. Oligonucleotides were hybridized in a solution (50-μL total volume) containing 10 mM Tris-HCl, pH 7.6, 40 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. The solution was heated to 80 °C for 5 min and cooled slowly to room temperature under ambient conditions (~3 h). Due to the low DNA strand concentrations, hybridization mixtures contained 65 fmol of the labeled strand and a 100-fold excess of the unlabeled (noncleaved) strand to ensure complete hybridization of the labeled DNA.

Generation of an Abasic Site. The oligonucleotide containing one deoxyuridine 3′ to topoisomerase I high-efficiency site (Table 1, X = U) was obtained from Cruachem. About 700 ng of gel purified DNA was treated with 7.5 units of uracil-DNA glycosylase in a reaction mixture (30-μL total volume) containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT at 37 °C for 10 min. A small aliquot of the reaction mixture was treated with 1 M piperidine at 90 °C for 30 min to confirm the complete removal of uracil. The oligonucleotide containing one abasic site was then 5′-^{32}P end-labeled using T4 polynucleotide kinase, purified by 20% PAGE, and annealed to the complementary strand. Due to the sensitivity of the abasic site to heat, the annealing mixture was heated to 65 °C, instead of 80 °C, for 2 min. Duplex formation was then examined by 16% native PAGE.

DNA Cleavage and Ligation by Topoisomerase I. The 5′-^{32}P end-labeled DNA duplex (6.5 fmol) was treated with 25 ng of calf thymus topoisomerase I in a reaction mixture (20-μL total volume) containing 10 mM Tris-HCl, pH 7.6, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mM DTT. For the branched and nicked substrates, the acceptor oligomers were present in 1000-fold molar excess relative to the radiolabeled oligonucleotide and were present at the time the reactions were initiated. The reaction mixtures were initiated by the addition of topoisomerase I, incubated at 37 °C for the time indicated, and then quenched by treating either with trypsin (1 mg/mL, 37 °C, 30 min) or proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) prior to analysis by 20% denaturing PAGE.

Isolation and Sequencing of Ligation Products. To verify the nature of the ligation products, the reactions were scaled up 10-fold and applied to a 20% preparative PAGE. The DNA was isolated from the gel and recovered as described above. Sequencing analysis was performed according to the method of Maxam and Gilbert (*22*).

RESULTS

Effects of Divalent Cations on Topoisomerase I-Mediated Deletions. The effects of the divalent cations Ca^{2+} and Mg^{2+} on topoisomerase I-mediated deletions were determined using a DNA substrate that contained a nick on the scissile strand adjacent to the intended topoisomerase I cleavage site (*15*). The 5′-^{32}P end-labeled nicked duplexes (X = G or T) shown in Figure 2 were incubated with topoisomerase I in the absence of added divalent cation, or in the presence of Mg^{2+} or Ca^{2+} (5 mM) at 37 °C for 60 min. Following proteolysis with trypsin, the reactions were analyzed by 20% denaturing PAGE.

As shown in Figure 2, a somewhat greater site 1 cleavage and subsequent ligation across a 1-nt gap to the 5′-OH group of the acceptor oligomer was observed for the nicked duplex containing 5′ G in the absence of Mg^{2+} and Ca^{2+} (lane 1) compared with the cleavage and ligation in the presence of either one of these divalent cations (lanes 2 and 3). In contrast, while the 3-nt deletion product (X = T) was formed efficiently in the presence of Mg^{2+} or Ca^{2+} (lanes 5 and 6), the ligation did not proceed in the absence of added divalent cation (lane 4). As is evident from the strong cleavage at site 2, the metal dependent step is the ligation reaction rather than DNA strand scission per se. Without an acceptor strand to complete the nicked duplex, little cleavage was observed, and cleavage occurred exclusively at site 2, consistent with earlier reports that cleavage requires the presence of two nucleotides 3′ to the cleavage site (*13*−*15*). This may also contribute to the lack of cleavage at site 1 in Figure 2B.

Topoisomerase I-Mediated Cleavage and Ligation of Correctly Matched and Mismatched Branched Substrates. The sequence requirements for topoisomerase I-mediated strand transfer reaction was investigated by using synthetic DNA substrates containing branches (overlaps) on the scissile strand (Figure 3). The nucleotide at the 5′-terminus of the acceptor strand, as well as the nucleotide opposite the high-efficiency cleavage site (site 1), was varied.

The DNA substrate was 5′-^{32}P end-labeled on the scissile strand, incubated in the presence of topoisomerase I and a 1000-fold excess of one acceptor oligonucleotide at 37 °C for 60 min, and then quenched by the addition of SDS to a final concentration of 1%. Following proteolysis of topoisomerase I covalently bound to DNA with trypsin, the reactions were analyzed by PAGE. The apparent lack of cleavage at site 1 is an artifact, resulting from adventious comigration of intact substrate with cleavage product at site 1 containing a trypsin-resistant peptide.

Ligation with all modified acceptors was observed when the initial substrate (scissile and noncleaved strands) did not contain a mismatch, i.e., when X = T (Figure 3, lanes 7, 11, 15, and 19). That the full-length product actually resulted from the ligation of the 17-mer acceptor strand to the

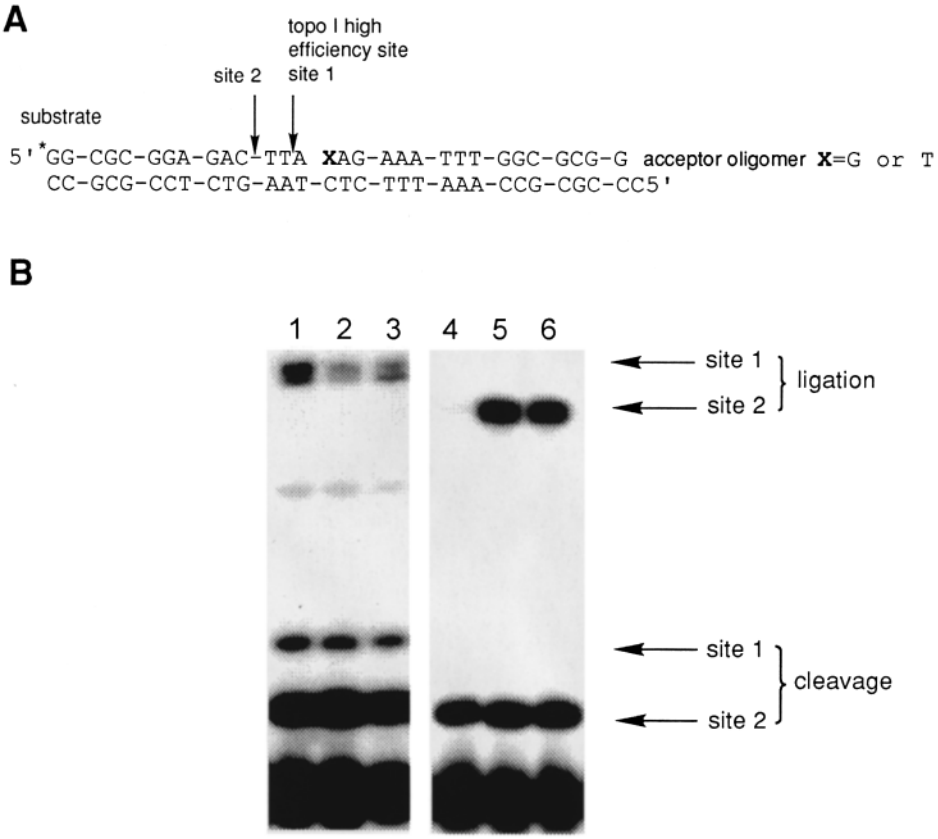


FIGURE 2: The effects of divalent cations on the topoisomerase I-mediated ligation reactions involving 1-nt and 3-nt deletions. (A) Sequences of the nicked DNA substrates. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end-labeled on the scissile strand; reactions were carried out as described in the Experimental Procedures. Lanes 1−3, nicked duplex containing the 5′-G acceptor strand + topoisomerase I; lanes 4−6, nicked duplex containing the 5′-T acceptor strand + topoisomerase I. Lanes 1 and 4, no added divalent cations; lanes 2 and 5, 5 mM Mg^{2+}; lanes 3 and 6, 5 mM Ca^{2+}. The lesser mobility of the cleavage product reflects the presence of a trypsin-resistant peptide (*13*).

DNA covalently bound to enzyme at site 1 was verified by DNA sequence analysis as described in Experimental Procedures. Shown in Figure 1 of the Supporting Information as an example is the sequence analysis of the ligation product derived from the branched substrate (Y = A, X = T).

Efficient ligation with substrates containing a mismatch was also observed when the acceptor strand contained a 5′-T (Figure 3, lanes 13 and 16). DNA sequence analysis (data not shown) indicated that in these cases the ligation products were formed by topoisomerase I-mediated strand transfer across a 3-nt gap to the enzyme−DNA complex at site 2. The absence of ligation at site 2 when X = A and Y = T (lane 14) no doubt reflects the Watson−Crick complementarity of these bases. The apparent multiplicity of ligation bands in Figure 3B was a consequence of incomplete duplex denaturation in some experiments, as established by suitable control experiments.

Inhibition of Topoisomerase I-Mediated DNA Mismatch Formation by CPT. Further characterization of differences in the ligation for DNA mismatch formation involved determination of the time course of the ligation reaction and the influence of the topoisomerase I inhibitor CPT (Figure 4) (*23, 24*) on the ligation kinetics (Figures 5 and 6). The acceptor strands all had free 5′-OH groups and were fully complementary to the noncleaved strand with the exception of the nucleotide at the 5′-end. The 5′-^{32}P end-labeled partial duplex was incubated with an excess of the individual acceptor oligonucleotides in the presence or absence of 20 μM CPT for various times, and then the reactions were quenched by the addition of SDS, treated with proteinase K, and analyzed by 20% denaturing PAGE. The apparent heterogeneity at some of the cleavage bands in Figure 5 reflects incomplete proteolytic digestion of the covalently bound enzyme by proteinase K. The gels were quantified by phosphorimager analysis.

As shown in Figure 5B, topoisomerase I-mediated DNA mismatch formation (X = G) proceeded somewhat more slowly in the absence of CPT, and the reaction was inhibited to a greater extent by CPT than that observed for the homologous ligation reaction (X = A). It has been reported previously that most of the CPT-induced topoisomerase I sites have T at position −1 and the most intense sites have G at position +1 (*25*). The observed G preference has led to a suggested model in which CPT stacks against the base of the acceptor strand with G providing a particularly favorable interaction (*26*). A modification of the partial duplex shown in Figure 5 converted the branched substrate to one having G at position +1 on the scissile strand (Figure 6A). As shown in Figure 6B, the topoisomerase I-mediated ligation to produce DNA mismatches (X = A, T, or C) was slower than that observed for the homologous ligation (X = G). CPT was least effective at inhibiting the homologous ligation (X = G) which must form through a covalent binary complex reported (*25*) to be a preferred site for CPT binding. The efficiency of inhibition observed for the mismatched acceptors (X = A, T, and C) at site 1 throughout the time

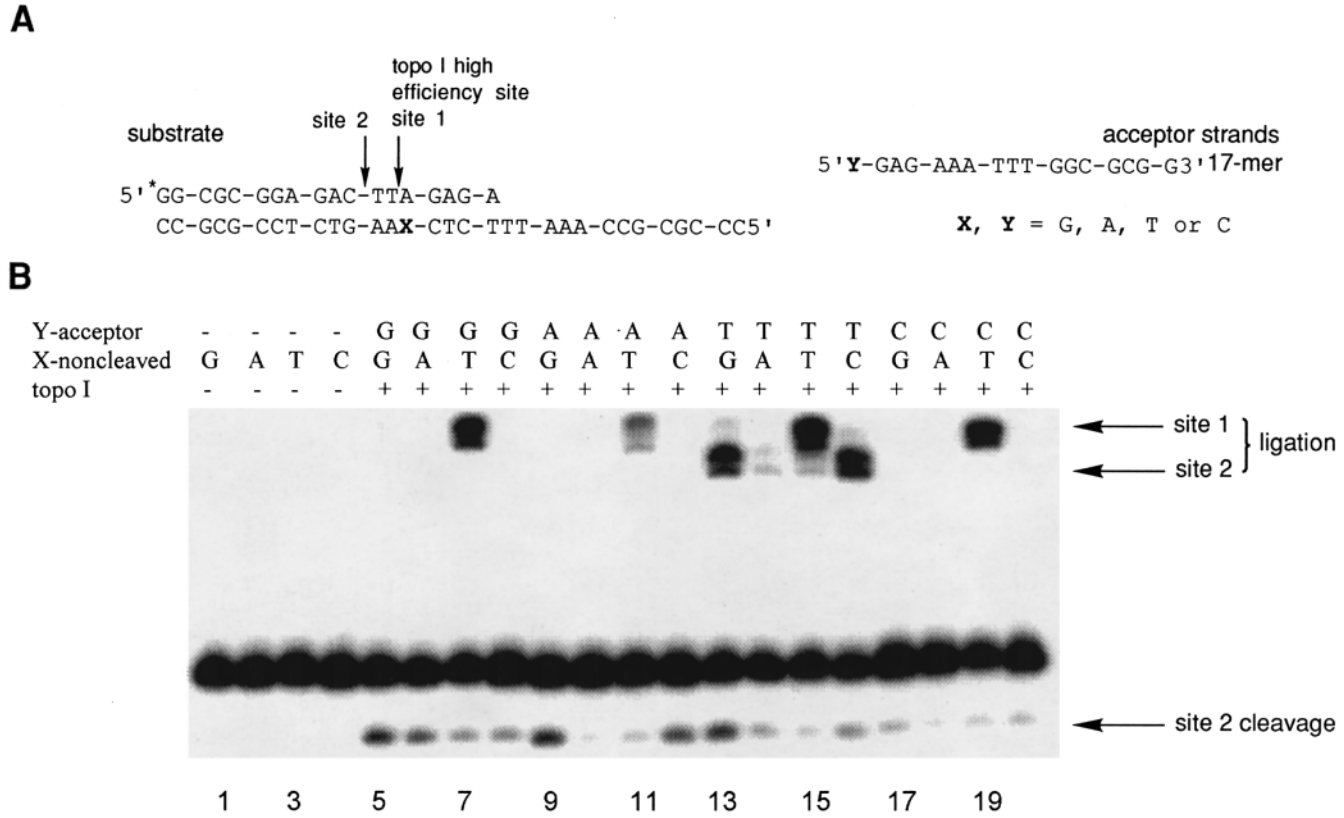


FIGURE 3: Effects of sequence variation in acceptor and noncleaved strands on the topoisomerase I-mediated ligation reaction. (A) Sequences of the DNA oligonucleotide substrates. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The substrate was 5′-^{32}P end-labeled on the scissile strand; reactions were carried out as described in the Experimental Procedures.

course of ligation was comparable (70−80%) regardless of the 5′-terminal nucleotide on the individual acceptor strands.

Topoisomerase I-Mediated Cleavage of Full DNA Duplexes Containing Mismatches and Abasic Sites. Topoisomerase I-mediated formation of structurally modified DNA duplexes was also investigated by using homologous full duplexes, as well as duplexes containing a mismatch or an abasic site at position +1 on the scissile strand (Table 1). Topoisomerase I-mediated cleavage reactions were carried out in the presence or absence of 20 μM CPT as described in the Experimental Procedures. The DNA present as cleavage product was calculated as a percentage of total DNA, as quantified using a phosphorimager.

As shown in Table 1, substrate DNA having A at position +1 on the scissile strand (X = A, Y = T) was cleaved to the extent of 4.5%, and the cleavage was increased to about 15% in the presence of CPT. In comparison, the fully base paired substrate having G at position +1 was not cleaved at all in the absence of CPT; however, admixture of CPT resulted in almost 29% cleavage at site 1. A one-base mismatch (X = U, Y = C or T) at position +1 diminished the facility of religation so that more cleavage products were observed even in the absence of CPT. This was enhanced by CPT, presumably due to its further inhibition of the ligation reaction. The abasic site (X = abasic, Y = C or T) at position +1 appeared to have disfavored the ligation reactions to about the same extent as CPT. The strongest cleavage was observed for both substrates containing an abasic site in the absence of CPT. Admixture of CPT had no further effect on DNA cleavage and may actually have shifted the equilibrium toward ligated duplex.

Effects of Topotecan, Nitidine, and Coralyne on Topoisomerase I-Mediated Ligation Reactions of Correctly Matched and Mismatched Substrates. The effects of the topoisomerase I inhibitors topotecan, nitidine, and coralyne on topoisomerase I-mediated ligation reactions of correctly matched and mismatched substrates were investigated and compared with that observed for CPT. The branched substrate having G at position +1 on the scissile strand was utilized, in the presence of four 17-nt acceptors, each having a different nucleotide at the 5′-end. Topoisomerase I inhibitors were employed at 20 μM final concentration. Topoisomerase I-mediated ligations were carried out at 37 °C for 5 min; the reaction mixtures were then treated with SDS−proteinase K. After 20% denaturing PAGE and phosphorimager analysis, the calculated extents of ligation in the presence of topoisomerase I inhibitors were normalized to the ligation obtained with the same acceptor oligomer in the absence of these inhibitors.

As shown in Table 2, topotecan (TPT) inhibited topoisomerase I-mediated ligation of mismatched acceptors (X = A, C, or T) to a greater extent than the ligation of the fully complementary acceptor (X = G). The inhibition efficiency of TPT was very close to that observed for CPT itself. Under the same experimental conditions, nitidine showed a similar extent of inhibition of ligation of the fully complementary acceptor oligomer as CPT, but the effects on ligation of mismatched acceptors differed dramatically according to the identity of the 5′-nucleotide (X). Further determination of the time course of the ligation reactions (Figure 7) showed that mismatch formation in the presence of 20 μM nitidine was actually slightly enhanced when X =

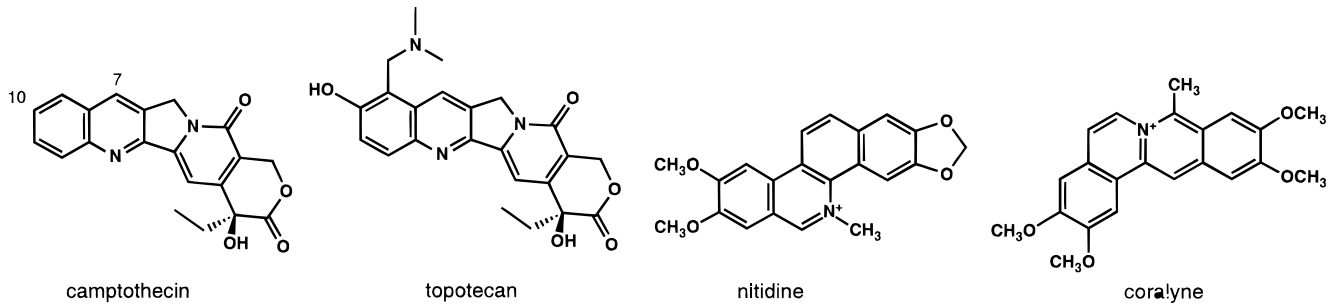


FIGURE 4: Structures of the topoisomerase I inhibitors camptothecin, topotecan, nitidine, and coralyne.

Table 2: Effects of CPT, TPT, Nitidine, and Coralyne on Topoisomerase I-Mediated Ligation Reactions[a,b]

site 1 ↓ X-GAG-AAA-TTT-GGC-GCG-G acceptor oligonucleotide

5'*GG-CGC-GGA-GAC-TTG-GAG

CC-GCG-CCT-CTG-AAC-CTC-TTT-AAA-CCG-CGC-CC5'

acceptor (X)	extent of ligation (%)[c]			
	CPT	TPT	nitidine	coralyne
G	61	54	62	94
A	20	20	110	98
C	31	26	24	64
T	16	12	98	77

[a] Topoisomerase I inhibitors were employed at 20 μM final concentration. [b] Topoisomerase I-mediated ligation reactions were carried out at 37 °C for 5 min and then quenched by treatment with SDS-proteinase K as described in the Experimental Procedures. [c] The calculated extents of ligation in the presence of topoisomerase I inhibitors were normalized to the values obtained for ligation with the same acceptor oligomer in the absence of these inhibitors.

A, strongly inhibited when X = C, and unaffected when X = T. Another topoisomerase I inhibitor, coralyne (*27*), had no effect on the ligation reaction to produce a duplex when X was G or A but inhibited duplex formation (to produce mismatched duplexes) when X was C or T (Table 2).

DISCUSSION

DNA molecules containing branches in the scissile strand are known to be good substrates for separating the partial reactions of topoisomerase I in vitro (*14*, *28*), thus allowing nonhomologous DNA strand exchange. Using a DNA substrate containing a nick in the scissile strand, it has been shown that topoisomerase I-mediated ligations afford DNA products with deletions of as many as three nucleotides (*15*). The known role of divalent cations in enhancing topoisomerase I activity (*29*) and maintaining DNA polymerase fidelity (*30*) prompted us to determine their influence on topoisomerase I-mediated deletions.

As shown in Figure 2, the formation of the 3-nt deletion products was dependent on the presence of 5 mM divalent Mg^{2+} or Ca^{2+}. This requirement most likely reflects a need for stabilization of substrate secondary structure, which controls the position of the acceptor oligonucleotide relative to the topoisomerase I−DNA binary complex. The increased efficiency of the 3-nt deletion observed in the presence of Ca^{2+} or Mg^{2+} may be also facilitated by the ability of the acceptor oligonucleotide to form a base pair at the site of ligation. Westergaard and co-workers also studied the effect of divalent cations on the cleavage and ligation reactions separately, using suicide DNA substrates analogous to those employed in our assay (*13*). The acceptor, however, was fully complementary to the noncleaved strand, forming a homologous duplex upon ligation. These workers reported an enhancement of the cleavage reaction by Mg^{2+} or Ca^{2+} but no effect on the ligation reaction. However, earlier studies investigating the effects of Mg^{2+} on the recircularization of linear DNA to which topoisomerase was covalently linked demonstrated a requirement for at least 10 mM $MgCl_2$ for the ligation reaction (*31*). This latter substrate very likely requires the stabilization of a secondary structure.

It is less obvious why the presence of 5 mM Mg^{2+} or Ca^{2+} should suppress the 1-nt deletion reaction (Figure 2, lanes 1−3). There are a few potential mechanisms whereby Mg^{2+} could affect topoisomerase I activity. For example, Mg^{2+} is known to compensate the negative charge of the phosphate backbone of duplex DNA, thus diminishing interstrand electrostatic repulsion and allowing the two strands to bind more cohesively (*32*). Alternatively, Mg^{2+} could bind to topoisomerase I, causing conformational changes. Additionally, the magnitude of the stimulatory effect of Mg^{2+} on recombinant human topoisomerase I relaxation activity has been reported to be inversely proportional to monovalent salt concentration (*33*). There has been no previous report of the effects of divalent cations on topoisomerase I-mediated in vitro illegitimate recombination. However, it is conceivable, as shown here, that the concentrations and types of metal ions present may have an effect on enzyme-mediated reorganization of DNA structure through insertion and deletion reactions.

The relationship between the complementarity within the substrate at position +1 and the facility of topoisomerase I-mediated ligation was then studied utilizing branched substrates containing each of the four possible nucleotides at the 5′-end (Figure 3). Although the efficiency of ligation was variable, ligation was observed with all acceptors when the initial substrate did not contain a mismatch at position +1 (X = T). This suggested that topoisomerase I does not require complementarity of the acceptor strand at the site of ligation as long as the cleavage reaction to produce the enzyme−DNA covalent binary complex is not inhibited. The only acceptor strand that underwent ligation in the presence of a mismatch at the high-affinity cleavage site was the species having thymidine at the 5′-end (Y = T). This may be the result of diminished facility for topoisomerase I binding and cleavage at site 1 as a result of the mismatch. Consequently, cleavage may occur at site 2; the adenosine on the noncleaved strand opposite the site of cleavage would then be expected to facilitate ligation of the acceptor oligonucleotide having thymidine at the 5′-end. Control experiments demonstrated that 2-nt complementarity at the 5′-end of the acceptor oligonucleotide was insufficient to

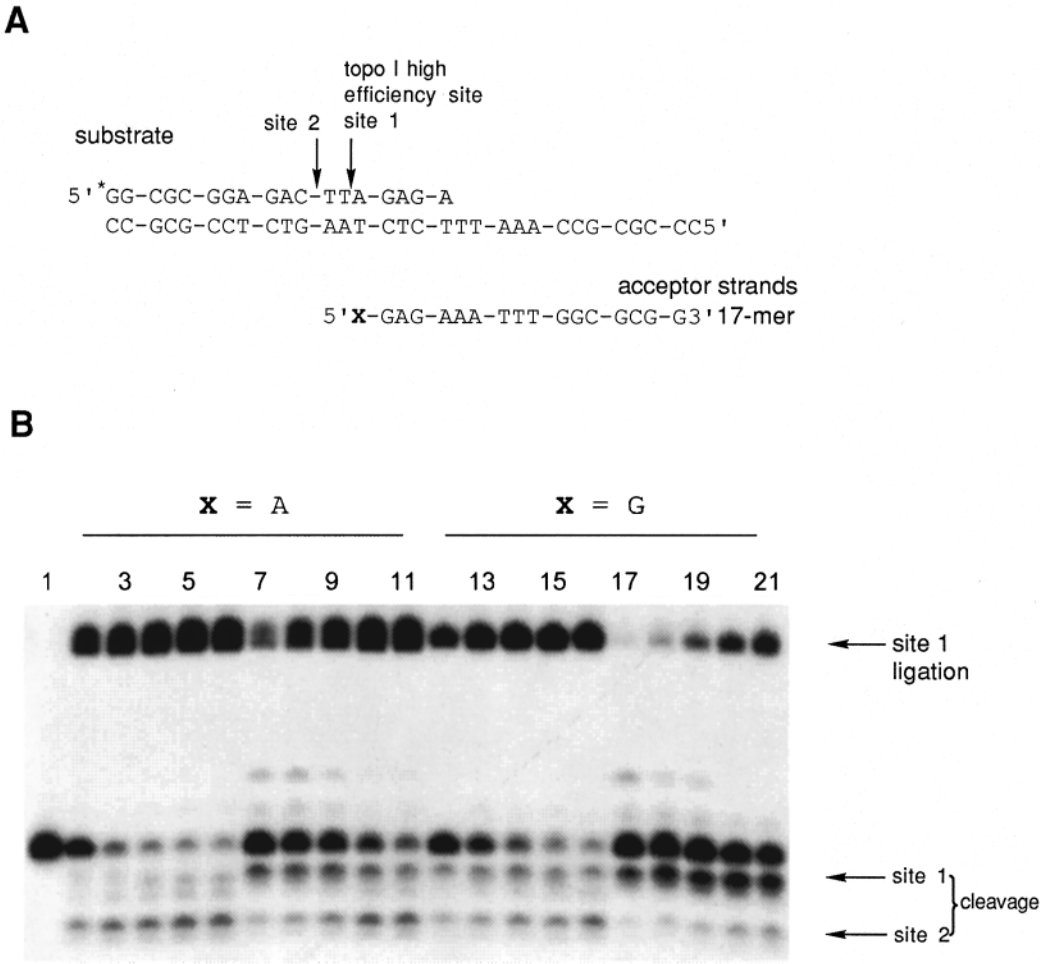


FIGURE 5: Time course of topoisomerase I-mediated ligation in the presence and absence of CPT utilizing branched substrates having A in position +1 on the scissile strand and A or G at the 5′-end of the acceptor strand. (A) Sequences of the DNA oligonucleotide substrates. (B) Autoradiogram of a 20% denaturing polyacrylamide gel. The partial duplex was 5′-^{32}P end-labeled on the scissile strand; reactions were carried out as described in the Experimental Procedures. Lanes 2−6, 12−16, no CPT; lanes 7−11, 17−21, 20 μM CPT. Lane 1, branched substrate containing the 5′-A acceptor strand. Lanes 2−6, 7−11, 12−16, and 17−21, 1, 3, 5, 15, and 30 min, respectively.

promote efficient ligation if the acceptor could not hybridize to the substrate (Supporting Information, Figure 2).

Using branched substrates and acceptor oligonucleotides differing in the identity of the 5′-terminal nucleotide, the effect of camptothecin on topoisomerase I-mediated DNA mismatch formation was investigated (Figures 5 and 6). Camptothecin stabilizes the covalent topoisomerase I−DNA cleavable complex by preventing DNA religation (*23*, *24*). Previous binding studies with radiolabeled CPT indicated that camptothecin forms a ternary complex in which it is bound noncovalently to the covalent enzyme−DNA binary complex (*34*). While the exact structure of the ternary complex is not known, the available evidence suggests that CPT must bind at the interface formed between protein and DNA in the binary complex since one type of electrophilic CPT analogue (having a reactive functionality at position 10) alkylated the enzyme (*34*), while a second electrophilic CPT analogue (reactive functionality at position 7) alkylated the G at position +1 on the scissile strand (*35*, *36*). On the basis of the observed sequence preference for guanosine at position +1 for efficient topoisomerase I cleavage, Leteurtre et al. (*26*) have proposed that the planar, heterocyclic ring system of camptothecin interacts, possibly through hydrogen bonding or stacking interactions, with the nucleobase at position +1 on the scissile strand. The characterization of the effect of CPT on topoisomerase I-mediated homologous ligation and mismatch formation (Figures 5 and 6) demonstrated that camptothecin inhibited mismatch formation to a greater extent than homologous ligation. The efficiency of inhibition of mismatch formation after 5 min was about the same (70∼80% inhibition) regardless of the 5′-terminal nucleotide present on the acceptor oligomer. This observation of lack of base specificity may simply reflect the fact that all of the nonhomologous ligation reactions (Figures 5 and 6) were intrinsically less efficient than the homologous system and thus presumably more easily disrupted. However, this observation might also be thought to argue against a single unique orientation of CPT within all covalent binary complexes and especially against a model in which good inhibition requires stacking of the CPT with the nucleobase at the 5′-end of the acceptor oligonucleotide. Likewise, the fact that nitidine and coralyne exhibited rather different differential inhibitions of homologous and nonhomologous ligation reactions (Figure 7, Table 2) argues strongly that different types of inhibitors may employ somewhat different molecular strategies to disfavor the topoisomerase I-mediated ligation reaction. While the results obtained in the present study for CPT and its structural analogue TPT indicated rather similar effects on topoisomerase I-mediated ligation (Table 2), it is not inconceivable that other CPT analogues

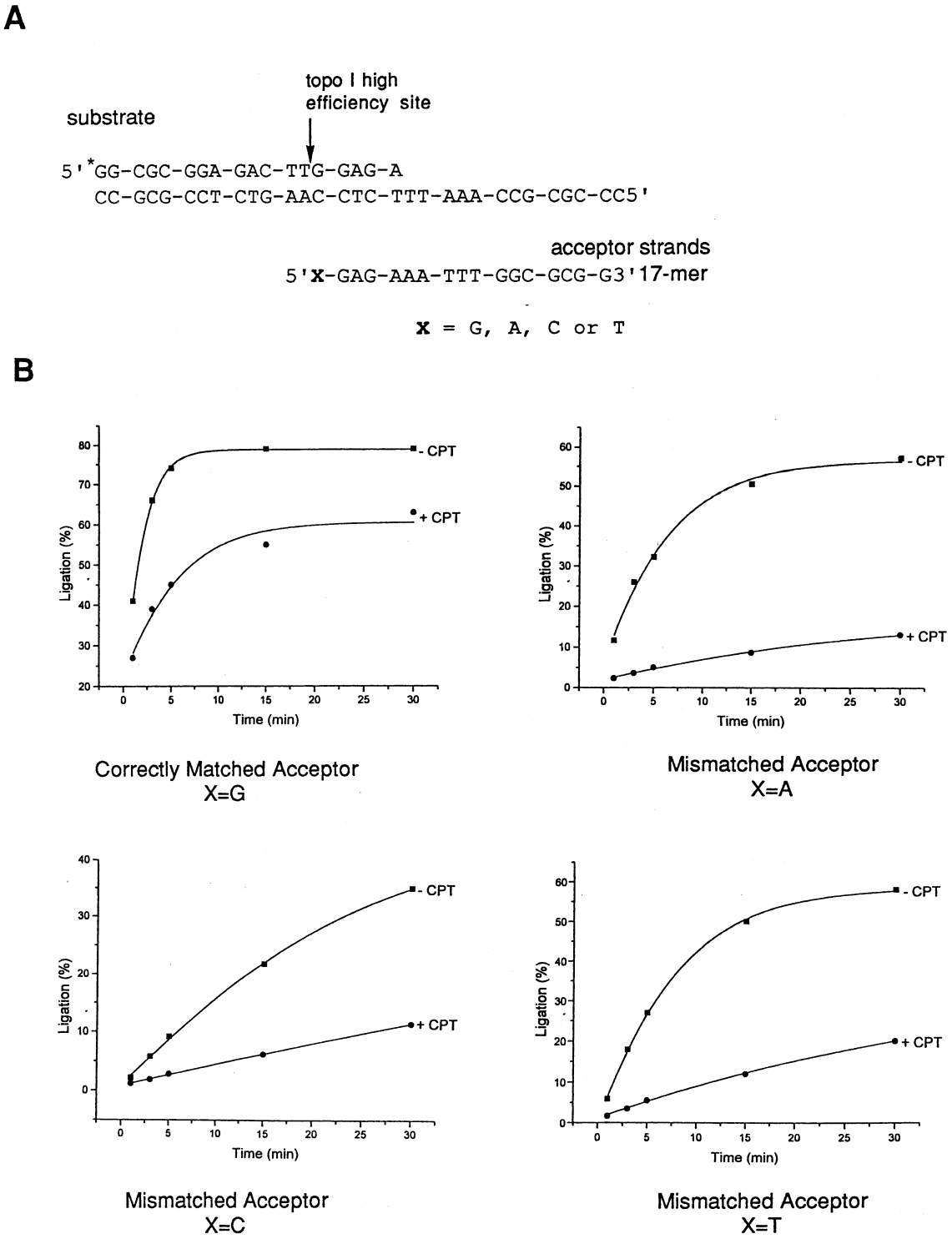


FIGURE 6: Time course of topoisomerase I-mediated ligation in the presence and absence of 20 μM CPT utilizing branched substrates having G in position +1 on the scissile strand and G, A, C, or T at the 5′-end of the acceptor strands. The substrate was 5′-^{32}P end-labeled on the scissile strand; reactions were carried out as described in the Experimental Procedures. (A) Sequences of the DNA oligonucleotide substrates. (B) Phosphorimager quantification of the time course of ligation (filled rectangles, −CPT; filled circles, +CPT).

or other assay systems might reveal differences even between inhibitors that are closely related structurally.

Abasic (apurinic/apyrimidinic) sites are among the most commonly formed endogenous lesions in DNA (~10 000/mammalian cell/day) (*37*−*39*). It has been reported that an abasic site immediately adjacent to the topoisomerase I cleavage site irreversibly trapped topoisomerase I cleavable complexes and increased topoisomerase I cleavage in the presence or absence of camptothecin (*40*). Also noted previously has been the nicking of DNA at the sites of mismatches (*41*). Consistent with the report of Pourquier et al. (*40*), abasic sites enhanced the topoisomerase I-mediated cleavage reaction more than DNA mismatches in our model system (Table 1). Quantification of the cleavage products using phosphorimager analysis further indicated that abasic sites most likely hindered the religation reaction to the same extent as CPT. Unlike the case for mismatches, there was no further enhancement by CPT of topoisomerase

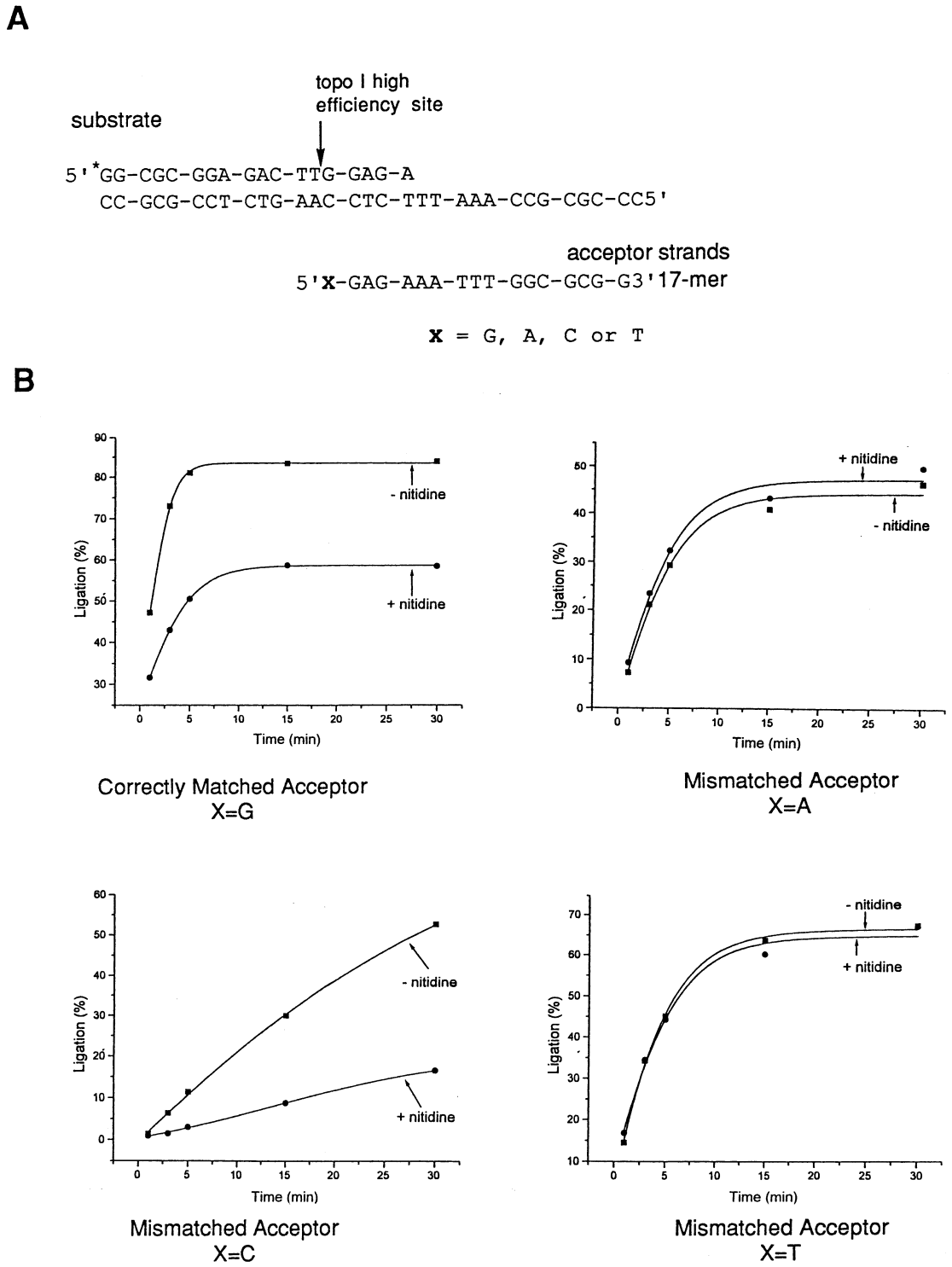


FIGURE 7: Effect of 20 μM nitidine on topoisomerase I-mediated ligation utilizing branched substrates with G in position +1 on the scissile strand and 5′-G, -A, -C, or -T acceptor strands. The substrate was 5′-^{32}P end-labeled on the scissile strand; reactions were carried out as described in the Experimental Procedures. (A) Sequences of the substrates. (B) Phosphorimager quantification of the time course of ligation (filled rectangles, −nitidine; filled circles, +nitidine).

I-mediated cleavage of full duplexes containing an abasic site adjacent to the cleavage site.

The demonstration that topoisomerase I is the molecular target for camptothecin (*23*) has stimulated intense research and afforded a variety of CPT derivatives such as topotecan (*42*). A number of other types of inhibitors with diverse chemical structures have also been identified (reviewed in refs *43* and *44*). Among these, coralyne derivatives and nitidine derivatives were found to be cytotoxic to two CPT-resistant human tumor cell lines (*45*). The cytotoxicity of these agents toward CPT-resistant lines could reflect the different strategies that these species employ for binding to the topoisomerase I−DNA covalent binary complex, as is readily apparent in Figure 7 and Table 2, but could also result from other biochemical and pharmacological effects of these inhibitors (*18*, *46*). Benzophenanthridine alkaloids related to nitidine have also been reported to have other biochemical effects, including inhibiting protein kinase C (*47*) and taxol-

promoted tubulin polymerization (*48*). Clearly, it would be of great interest to determine the extent to which nitidine and coralyne facilitate topoisomerase I-dependent DNA cleavage and inhibit ligation in CPT-resistant tumor cell lines.

Also of considerable interest is the development of an understanding of the importance of specific effects of individual topoisomerase inhibitors on the expression of antitumor activity. The ability of such agents to inhibit topoisomerase I-mediated alteration of DNA structure, in addition to inhibition of DNA ligation, constitutes one obvious parameter of inhibitor function that seems worthy of study.

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SUPPORTING INFORMATION AVAILABLE

Autoradiogram of 20% denaturing polyacrylamide gels illustrating DNA sequence analysis of the ligation product derived from the branched substrate containing a homologous 17-nt acceptor strand (Figure 1) and the requirement for acceptor hybridization for topoisomerase I-mediated ligation (Figure 2) (3 pages). Ordering information is given on any current masthead page.

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