A Model for Topoisomerase I-Mediated Insertions and Deletions with Duplex DNA Substrates Containing Branches, Nicks, and Gaps†

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Received November 29, 1994; Revised Manuscript Received January 26, 1995⊗

ABSTRACT: The ability of DNA topoisomerase I to promote insertions and deletions *in vitro* has been studied at nucleotide resolution for structurally diverse DNA substrates that uncouple the cleavage and ligation reactions of the enzyme. Topoisomerase I-mediated ligations afforded DNA duplexes having deletions and insertions with "branched" substrates and deletions up to 18 nucleotides in length with substrates containing nicks or gaps. In addition, a number of the acceptor substrates altered the preferred site of DNA cleavage, thereby increasing the diversity of accessible ligation products. Also demonstrated by the production of two "recombinant" duplexes from a single set of reactants was the potential for amplification of such alterations. These findings illustrate plausible mechanisms by which topoisomerase I-mediated illegitimate recombination may obtain at a molecular level.

Eukaryotic DNA topoisomerase I controls the topological state of DNA through sequential single-strand breakage, strand passage, and rejoining of the phosphodiester backbone of DNA (Gellert, 1981; Wang, 1985). The transient strand breaks involve reversible formation of an intermediate in which the active site tyrosine of topoisomerase I is linked to DNA covalently via a 3′-*O*-phosphorotyrosine bond (Champoux, 1981). Coordinated cleavage and religation restores continuity to the DNA duplex. The uncoupling of the cleavage and ligation reactions can be demonstrated in cell free systems and may well constitute the biochemical basis for the participation of topoisomerase I in illegitimate recombination (Ikeda, 1990; Champoux & Bullock, 1988). Separation of the cleavage and ligation reactions can be achieved by topoisomerase I-mediated site specific cleavage of single-stranded DNA (Been & Champoux, 1981; Halligan et al., 1982), double-stranded DNA containing nicks in the noncleaved strand (McCoubrey & Champoux, 1986), or partially double-stranded substrates (Svejstrup et al., 1991; Christiansen et al., 1993). Cleavage of these "suicide" substrates occurs at high efficiency cleavage sites (Svejstrup et al., 1991; Christiansen et al., 1993) without sequential religation due the instability of the DNA secondary structure downstream from the site of cleavage (*vide infra*).

The topoisomerase I-DNA intermediate trapped using substrates that uncouple the half-reactions can undergo ligation with DNA acceptors, affording new DNA molecules (Been & Champoux, 1981; Halligan et al., 1982; McCoubrey & Champoux, 1986; Svejstrup et al., 1991; Shuman, 1992; Christiansen et al., 1993). In this fashion, it has been possible to demonstrate efficient ligation of the topoisomerase I-DNA intermediate to complementary acceptors (Svejstrup et al., 1991; Shuman, 1992; Christiansen et al., 1993). Where extended regions of complementarity are present, it is also possible to obtain ligation at lower efficiency involving nonhomologous acceptors; hairpins (Shuman, 1992) and partially base-paired duplexes (Christiansen & Westergaard, 1992) have been so obtained. The introduction of deletions and insertions, which would seem to be of greater interest from the perspective of mutagenesis and nonhomologous DNA exchange, has also been studied. Incubation of a single topoisomerase I-DNA intermediate with mixtures of acceptor oligomers gave complex mixtures of products whose size distribution was consistent with the interpretation that insertions and deletions had been introduced (Christiansen & Westergaard, 1992). A similar observation was made using single acceptor oligomers, although two products were clearly formed in the insertion experiment (Shuman, 1992). None of the putative insertion or deletion products in either study was characterized by DNA sequence analysis.

Presently, we describe the use of three different types of DNA substrates whose ligation with single acceptor oligomers affords deletion or insertion products. DNA sequence analysis was used to verify the nature of each product, as well as the surprising finding that the acceptor oligomers could alter the preferred site of DNA cleavage substantially.

EXPERIMENTAL PROCEDURES

Materials. T4 polynucleotide kinase and proteinase K were purchased from United States Biochemicals; trypsin was from Sigma Chemicals. [γ-^{32}P]ATP was obtained from ICN Radiochemical, and Nensorb prep nucleic acid purification cartridges were from DuPont-New England Nuclear.

Enzyme Purification. Calf thymus DNA topoisomerase I was isolated and purified by a modification of a published procedure (Wang et al., 1993). The isolated protein exhibited two major bands ($M_r \approx 96\,000$ and 82 000) when analyzed by sodium dodecyl sulfate (SDS[1])−polyacrylamide gel electrophoresis and visualization of the protein by silver staining. The heterogeneity of the isolated topoisomerase I

† This work was supported by Research Grant CA53913 from the National Cancer Institute.

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⊗ Abstract published in *Advance ACS Abstracts,* April 15, 1995.

[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; DEPC, diethyl pyrocarbonate.

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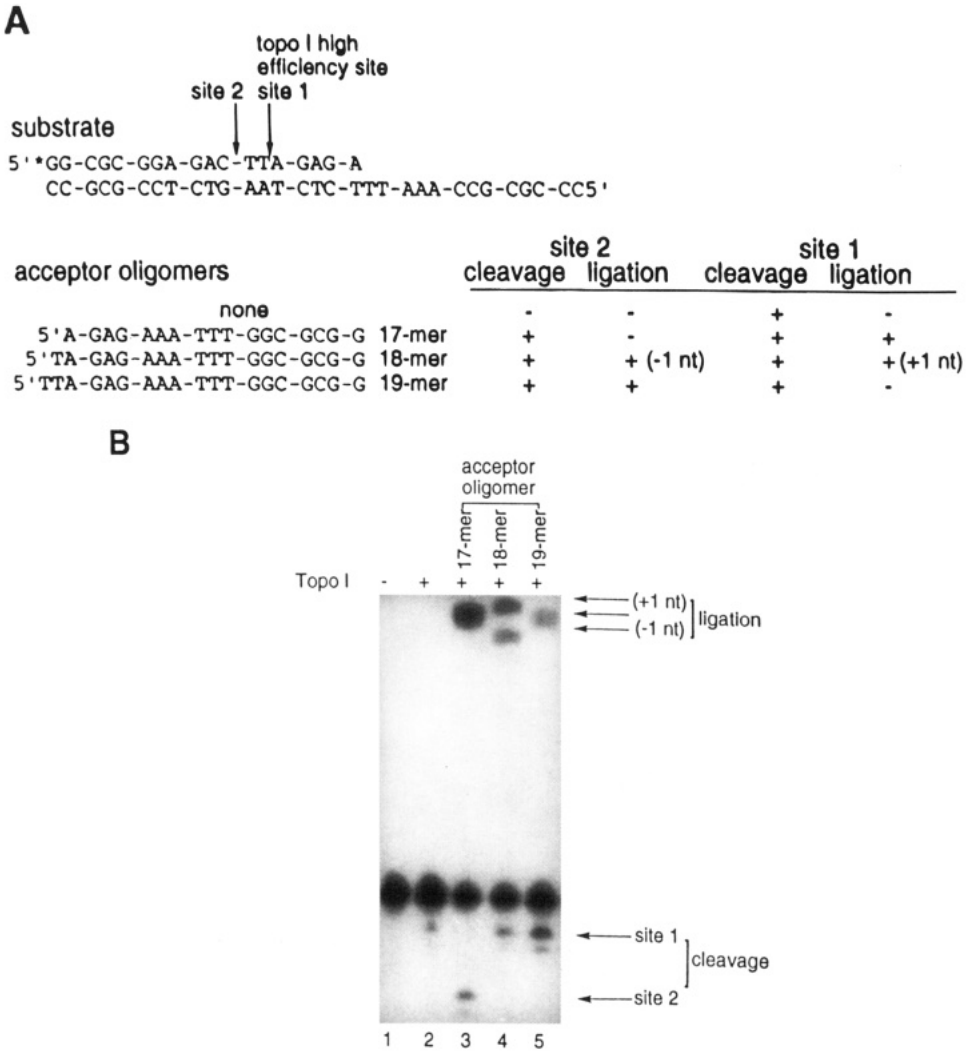


FIGURE 1: Topoisomerase I-mediated cleavage and ligation of *branched substrates*. (A) Sequences of the branched DNA substrates and observed sites of topoisomerase I-mediated cleavage and ligation in the presence of three acceptor oligomers. The position of the radiolabel is indicated by an asterisk. (B) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating topoisomerase I-mediated cleavage and ligation of branched duplex DNAs.

can be attributed to proteolysis during the isolation procedures (Schmitt et al., 1984). The purified protein had a specific activity of 1.4×10^7 units/mg of protein.

Oligonucleotide Substrates. Synthetic oligonucleotides were purchased from Midland Certified Reagent Co. or prepared on a Biosearch 8600 series DNA synthesizer using phosphoramidite chemistry. The oligonucleotides synthesized on the Biosearch DNA synthesizer were first purified on Nensorb prep nucleic acid purification cartridges. Synthetic DNA oligonucleotides were 5′-^{32}P-end-labeled with T4 polynucleotide kinase + [γ-^{32}P]ATP. Oligonucleotides were purified on a 20% denaturing (8 M urea) polyacrylamide gel. The DNA was visualized by UV shadowing 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 (Kazakov et al., 1988).

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 strands to ensure complete hybridization of the labeled DNA.

DNA Cleavage and Ligation by Topoisomerase I. The 5′-^{32}P-end-labeled DNA duplex (6.5 fmol) was treated with 8.8 ng of calf thymus topoisomerase I (13:1 enzyme−DNA duplex) 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 substrates, the acceptor oligomer was present in 1000-fold excess relative to the radiolabeled oligonucleotide. The reaction mixtures were incubated at the temperature and for the time indicated, and then the reactions were quenched by increasing the NaCl concentration to 500 mM where indicated and treating with either trypsin (1 mg/mL, 37 °C, 30 min) or proteinase K (1 mg/mL containing 1% SDS, 37 °C, 60 min) prior to mixture analysis by 20% denaturing PAGE.

Isolation and Sequencing of Ligation Products. These reactions were identical to those described above with the exceptions that the DNA and topoisomerase I concentrations were 50−100-fold greater and the reaction volume was 40 μL. The ligation and cleavage products were purified on a

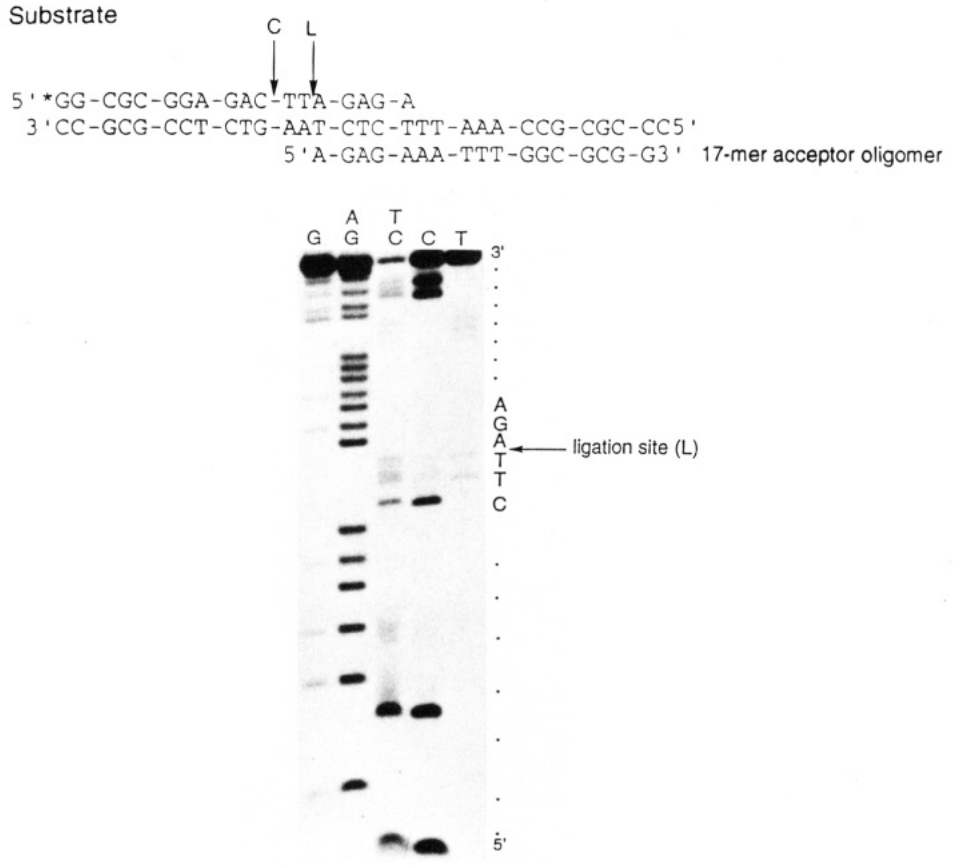


FIGURE 2: Autoradiogram of a 20% denaturing polyacrylamide gel illustrating DNA sequence analysis of the topoisomerase I-mediated ligation product of the branched substrate containing the 17-mer acceptor strand.

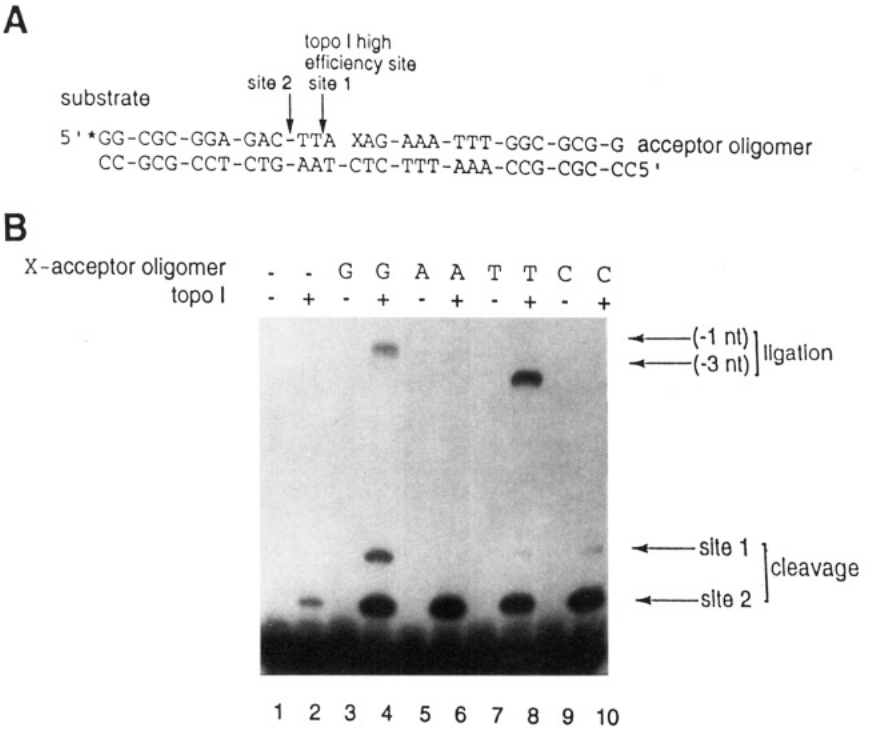
20% denaturing PAGE and recovered as described above. Sequencing analysis was performed according to the method of Maxam and Gilbert (1980).

Conformational Mapping. The gapped substrates, 5′-^{32}P-end-labeled on the noncleaved strand, were treated with 10 mM Tris-HCl, pH 7.6, containing 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 4 μg of unlabeled calf thymus DNA, 1×10^6 dpm of labeled duplex, and either 1 μL of DEPC (20 μL total volume) or 0.4 mM $KMnO_4$ (40 μL total volume). The reactions were maintained at 37 °C for 10 min and then quenched by ethanol precipitation ($KMnO_4$ reactions were first treated with 15 μL of allyl alcohol) followed by strand scission of the modified bases with 1 M piperidine at 90 °C for 30 min.

RESULTS

Topoisomerase I-Mediated Cleavage and Ligation of Branched Substrates. Topoisomerase I-mediated intramolecular ligation of synthetic DNA substrates containing branches on the scissile strand was investigated using substrates differing in the length of the acceptor strand (Figure 1A). The acceptor strands (17-, 18-, and 19-mers) all have a free 5′-OH group and are fully complementary to the noncleaved strand up to the high-efficiency cleavage site. They differ in that the 18- and 19-mer acceptor strands are extended 1 and 2 nt, respectively, past the topoisomerase I high-efficiency cleavage site. The DNA substrate was 5′-^{32}P-end-labeled on the scissile strand and then incubated in the presence of topoisomerase I and an excess of one acceptor oligomer at 37 °C for 30 min and the reaction quenched by the addition of NaCl. Under conditions of high ionic strength, topoisomerase I catalyzes the joining of induced nicks; therefore, only sites of irreversible cleavage arerevealed (Champoux, 1976). Following proteolysis of topoisomerase covalently bound to DNA with proteinase K, the reactions were analyzed by PAGE.

As shown in Figure 1B (lane 2) and reported previously for related systems (Svejstrup et al., 1991; Christiansen & Westergaard, 1992; Christiansen et al., 1993), treatment of the DNA substrate with topoisomerase I in the absence of any acceptor resulted in site specific cleavage, i.e., in the uncoupling of cleavage and religation reactions. The heterogeneity in the cleavage bands resulted from incomplete proteolytic digestion of the bound enzyme by proteinase K. That cleavage actually occurred at site 1 was verified by DNA sequence analysis of the products formed following ligation of the resulting topoisomerase I−DNA binary complex to two different acceptor oligonucleotides (*vide infra*); the pentanucleotide fragment released concomitant with topoisomerase I cleavage was also 3′-end labeled and shown to comigrate on a 20% denaturing PAGE with an authentic synthetic standard. Consistent with earlier reports of a double-strand recognition site (Svejstrup et al., 1991; Christiansen & Westergaard, 1992; Christiansen et al., 1993), the addition of acceptor oligomers complementary to the single-strand region downstream from the intended cleavage site facilitated substrate cleavage by topoisomerase I; unexpectedly, the acceptor oligonucleotide also promoted cleavage at a second site two nucleotides upstream from the intended cleavage site.



C

acceptor oligomer	site 2 cleavage	site 2 ligation	site 1 cleavage	site 1 ligation
none	+	-	-	-
X = G	+	-	+	+ (-1 nt)
X = A	+	-	-	-
X = T	+	+ (-3 nt)	-	-
X = C	+	-	-	-

FIGURE 3: Topoisomerase I-mediated cleavage and ligation of *nicked substrates*. (A) Sequences of the nicked DNA substrates. (B) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating topoisomerase I-mediated cleavage and ligation of nicked DNA substrates. (C) Sites of observed cleavage and ligation for individual substrates.

For all three acceptor oligonucleotides, cleavage of the DNA substrate occurred at sites 1 and 2. In the presence of the 17-nt oligomer, a single ligated product was observed (lane 3); DNA sequence analysis (Figure 2) indicated that this was the full duplex resulting from ligation of the 17-mer at cleavage site 1. Accordingly, the band corresponding to cleavage at site 1 was absent. In the presence of the 17-mer acceptor, the topoisomerase I−DNA intermediate formed by cleavage at site 2 did not afford a ligation product. Conversely, in the presence of the 19-nt acceptor, cleavage and ligation occurred only at site 2 (lane 5); DNA sequence analysis (supplementary material, Figure 1) indicated that this species was a full duplex, i.e., having the same sequence as the product in lane 3. The intermediate formed by cleavage at site 1 failed to undergo ligation with the 19-nt acceptor.

In the presence of the 18-mer acceptor, which positions the acceptor 5′-OH group in between cleavage sites 1 and 2, two ligation products were observed; densitometric analysis indicated that these were of approximately equal abundance. One of these was shown by sequence analysis (supplementary material, Figure 2) to be a 1-nt insertion product resulting from ligation of the intermediate formed at site 1; the other was a 1-nt deletion product formed by ligation to the intermediate formed at site 2 (supplementary material, Figure 2).

Topoisomerase I-Mediated Cleavage and Ligation of Nicked Substrates. Further evaluation of the ability of topoisomerase I to produce deletions via intramolecular cleavage and ligation was made using a DNA substrate that contained a nick on the scissile strand adjacent to the intended topoisomerase I cleavage site. The 5′-^{32}P-end-labeled substrates were treated with topoisomerase I for 30 min at 45 °C and treated with trypsin prior to PAGE analysis (Figure 3A). Absent the acceptor strand that completes the nicked duplex, little cleavage was seen upon addition of topoisomerase I, and cleavage occurred exclusively at a site two nucleotides upstream from the intended site (Figure 3B, lane 2) because two nucleotides are required 3′ to the cleavage site (Svejstrup et al., 1991; Shuman, 1992; Christiansen et al., 1993). The lessened mobility of the cleavage product relative to the substrate is due to the presence of a trypsin-resistant peptide which retards the cleavage fragment the equivalent of five nucleotides (Svejstrup et al., 1991). In the presence of the acceptor strand (X = G), more substantial cleavage occurred at the two sites and a ligation product was formed. DNA sequence analysis (Figure 4) indicated that the ligation product resulted from topoisomerase I cleavage

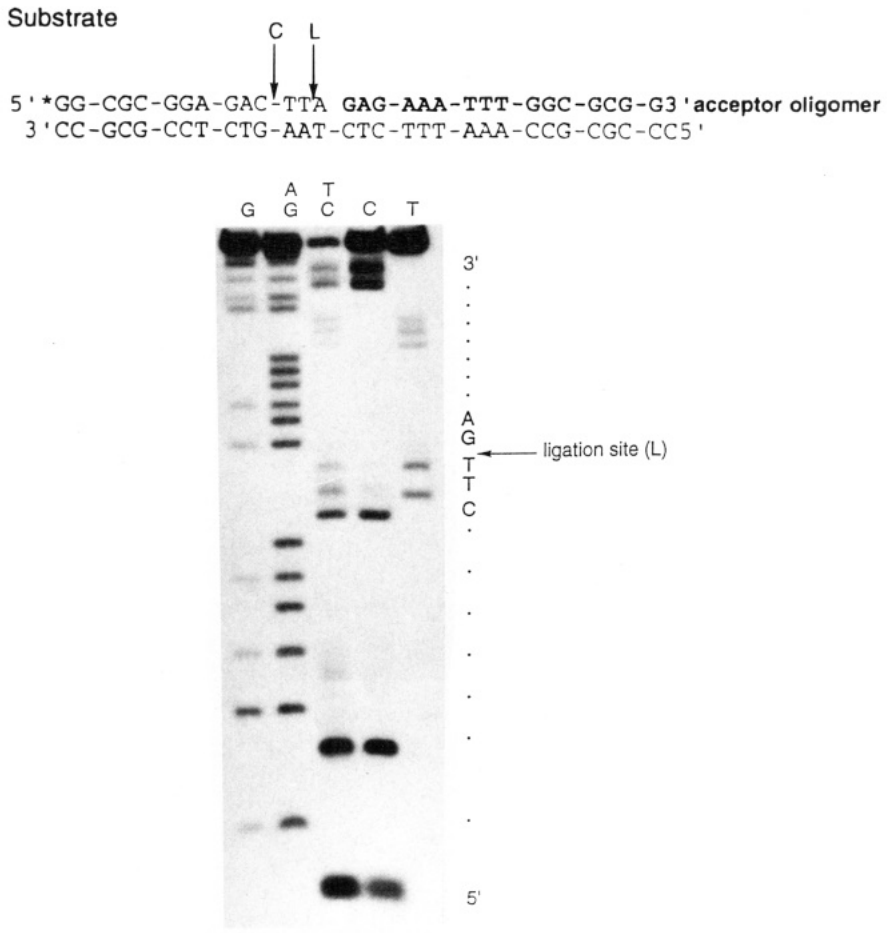


FIGURE 4: Autoradiogram of a 20% denaturing polyacrylamide gel illustrating DNA sequence analysis of the topoisomerase I-mediated ligation product of the nicked substrate containing the 5′-G acceptor strand.

at site 1 followed by ligation across a 1-nt gap to the 5′-OH group of the acceptor oligomer, thus affording a product with a 1-nt deletion (Figure 3C).

To assess the possible sequence specificity for heterologous strand transfer across a gap, the 5′-terminal nucleotide of the acceptor strand was varied. Although DNA substrate cleavage was observed in each case (Figure 3B), particularly at site 2, only weak ligation (at site 1) was observed for the acceptor having a 5′-A (lane 6); none was apparent when the 5′-terminal nucleotide was C (lane 10). As is evident in lane 8, however, the acceptor strand containing a 5′-T underwent ligation more efficiently than any of the others. DNA sequence analysis (supplementary material, Figure 3) of the ligation product in lane 8 indicated that it had formed by topoisomerase I-mediated strand transfer across a 3-nt gap to the enzyme−DNA complex formed at site 2. Thus, the availability of a nucleotide on the noncleaved strand complementary to the 5′-terminal nucleotide on the acceptor facilitated ligation.

Topoisomerase I-Mediated Cleavage and Ligation of Gapped Substrates. To determine whether topoisomerase I could promote the deletion of large stretches of DNA, another series of substrates was investigated (Figure 5A). Topoisomerase I cleavage at the high-efficiency site would result in the formation of a large gap between the covalently bound enzyme and the acceptor oligomer. The ability of various oligomers to serve as acceptors was studied. The acceptors included an oligonucleotide 21 nt in length that was fully complementary to the noncleaved strand; the gap between the covalently bound enzyme and this acceptor substrate was 18 nt. Also studied was a 21-nt acceptor containing a 2-base mismatch and a 19-nt acceptor containing a 1-base mismatch. The 5′-^{32}P-end-labeled substrates were incubated with topoisomerase I for 60 min at 37 °C and digested with proteinase K prior to PAGE analysis. As shown in Figure 5B, topoisomerase I-mediated ligation was observed with the 21-nt acceptor oligomer containing a 2-base mismatch; this product was shown by DNA sequence analysis (supplementary material, Figure 4) to have resulted from ligation across the formed 18-nt gap (Figures 5 and 6). Densitometric analysis using a phosphorimager indicated the yield of ligated product to be 8%. The DNA substrates constituted with the two other potential acceptors did not promote ligation, although substrate cleavage was noted in both cases.

The noncleaved strand of the gapped substrates contains a sequence derived from the ColE1 cruciform (underlined sequences, Figure 5A), which has previously been shown to form a thermodynamically stable hairpin under conditions similar to those utilized here (Blatt et al., 1993). We propose that ligation of the 21-nt acceptor is facilitated by folding of the noncleaved strand into a hairpin, which brings the 5′-end of the acceptor oligomer into close spatial proximity to the covalently bound enzyme (Figure 6). Consistent with this analysis, it may be noted that the fully complementary 21-nt acceptor would likely hinder hairpin formation and thereby fail to undergo ligation as noted, while the 19-nt acceptor would be predicted (Welch et al., 1993) to be too short to bridge the formed hairpin.

Conformational Mapping of "Intact" or "Cleaved" Gapped DNA. Direct support for the proposed hairpin intermediate

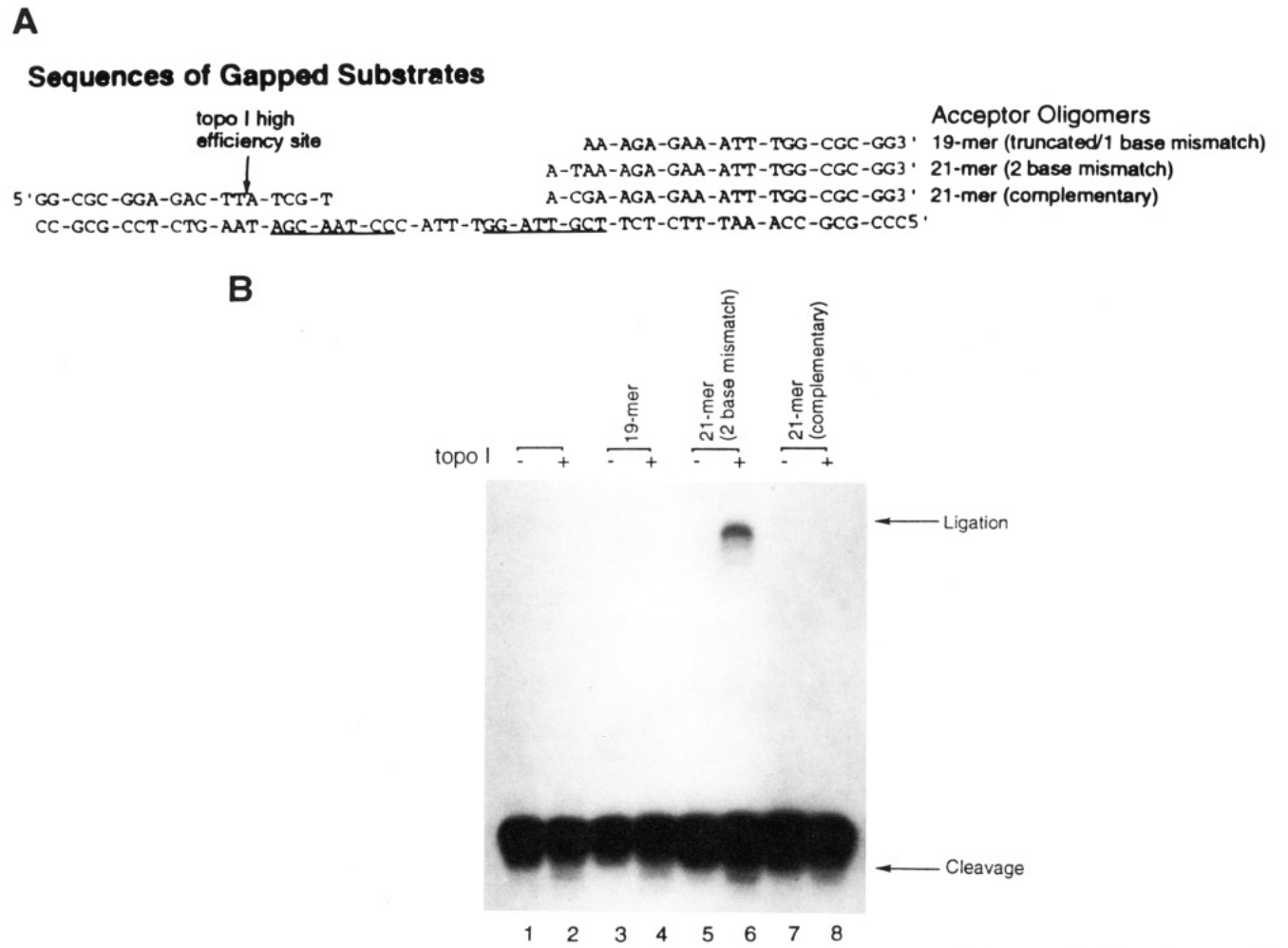


FIGURE 5: Topoisomerase I-mediated cleavage and ligation of *gapped substrates*. (A) Sequences of the gapped DNA substrates. (B) Autoradiogram of a 20% denaturing polyacrylamide gel illustrating topoisomerase I-mediated cleavage and ligation of gapped substrates.

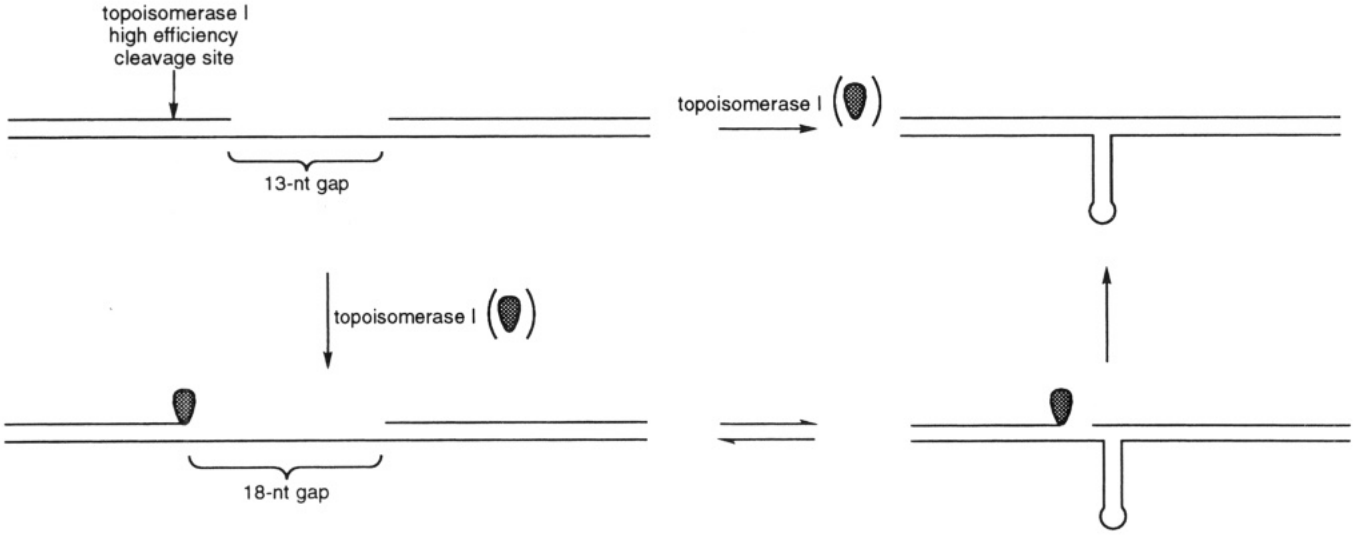
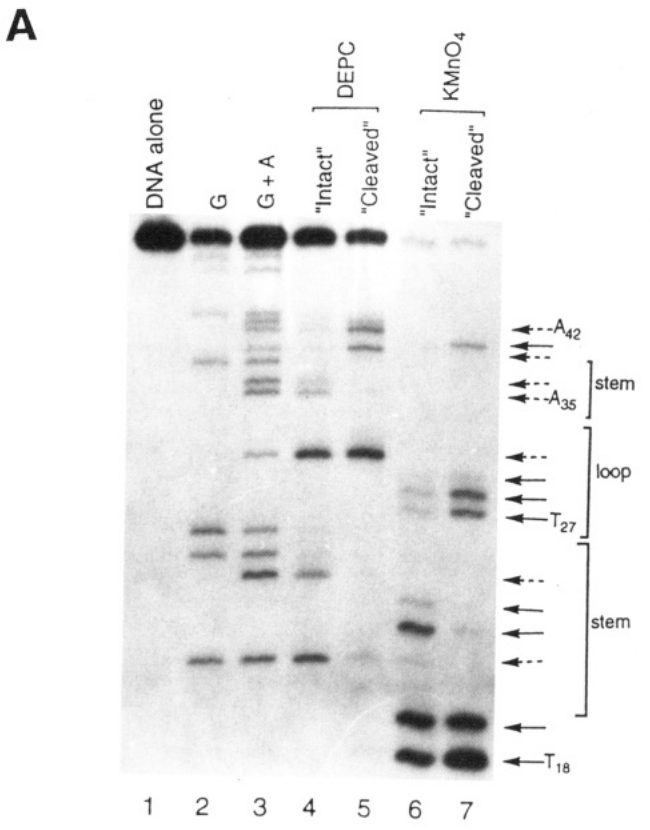


FIGURE 6: Proposed mechanism for topoisomerase I-mediated ligation across an 18-nt gap. The overall transformation is shown as a single process, along with the proposed enzyme−DNA intermediates.

was obtained by conformational mapping of the noncleaved strand with substrates containing an "intact" or "cleaved" scissile strand. The reagents utilized were $KMnO_4$, which reacts selectively with thymidine in single-stranded DNA, and diethyl pyrocarbonate (DEPC), which reacts selectively with purines (A > G) in single-stranded DNA (Furlong & Lilley, 1986; McCarthy et al., 1990). The sites modified by these reagents were detected following strand scission with piperidine and sequenced by comparison with the observed cleavage bands in Maxam−Gilbert sequencing reactions (Maxam & Gilbert, 1980).

Shown in Figure 7 is the conformational mapping of the gapped substrate that underwent ligation. The observed cleavage at the sequences 5′-T_{27}TTA-3′ and 5′-A_{39}TAA-3′ and the protection of the 5′-T_{22}TA-3′ and 5′-A_{35}A-3′ sequences of the gap and the flanking sequences suggest the



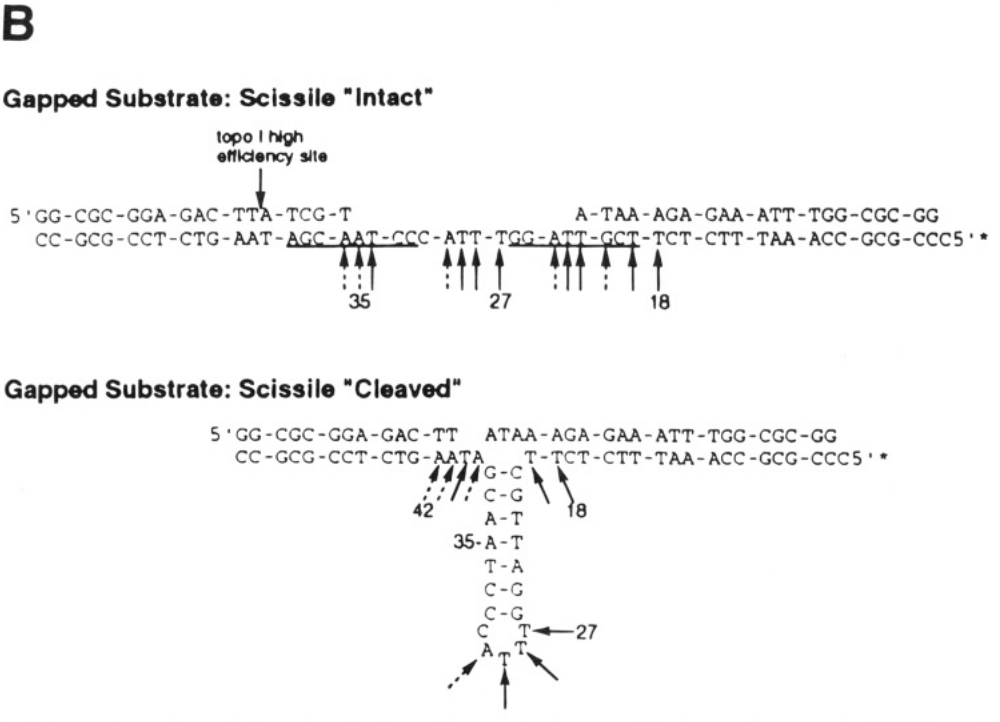


FIGURE 7: Analysis of the conformations of "intact" and "cleaved" gapped substrates. (A) Autoradiogram of a 20% denaturing polyacrylamide gel showing the conformational mapping of "intact" or "cleaved" gapped DNA. Lane 1, DNA control; lanes 2 and 3, Maxam−Gilbert sequencing lanes; lanes 4 and 5, DEPC treatment; lanes 6 and 7, $KMnO_4$ treatment. (B) Schematic representation of sites of modification by DEPC (dashed arrows) and $KMnO_4$ (solid arrows).

formation of a hairpin by the "cleaved" gapped substrate (Figure 7A, lanes 5 and 7), i.e., a synthetic duplex that mimics the topoisomerase I−DNA cleavage product. In comparison, the "intact" gapped substrate must be precluded from hairpin formation, as judged by the lack of protection afforded to sequences 5′-T_{22}TA-3′ and 5′-A_{35}A-3′ (Figure 7A, lanes 4 and 6). As anticipated, analysis of the gapped substrates containing the 19-nt acceptor oligomer yielded an analogous pattern of cleavage and protection, consistent with the interpretation that this substrate forms a hairpin, but the 5′-end of this acceptor is too short to reach across the junction (Welch et al., 1993). Conversely, neither the "cleaved" nor "intact" gapped substrates containing the fully complementary 21-nt acceptor gave a pattern suggesting the formation

of a hairpin structure (supplementary material, Figure 5); the lack of spatial proximity of the acceptor to the covalently bound enzyme was consistent with the lack of ligation observed.

DISCUSSION

DNA molecules containing branches in the scissile strand are known to be particularly good substrates for topoisomerase I-mediated irreversible cleavage (Christiansen & Westergaard, 1992; Christiansen et al., 1993). Comparison of topoisomerase I-mediated cleavage of branched substrates with cleavage of an incomplete duplex demonstrated that the presence of an acceptor strand capable of branch formation could alter the preferred site of topoisomerase I cleavage (Figure 1). This type of observation has not been made previously, although it is known that DNA secondary structure can influence topoisomerase I activity, with bent and supercoiled DNAs being preferred substrates (Camilloni et al., 1986, 1991). A role for secondary structure was also suggested by the observation that both salt and temperature, two factors that can influence DNA conformation, altered the sequence specificity of cleavage by topoisomerase I (Been & Champoux, 1981). Further support for an influence of DNA structure on DNA cleavage specificity can be inferred from the observation that topoisomerase I sites containing the same sequence can sometimes be cleaved with unequal efficiencies (Been et al., 1984). Therefore, even when defined substrates are utilized, caution should be used in assigning the apparent sites of topoisomerase I-mediated strand transfer in the absence of DNA sequence analysis because the site of cleavage can change unexpectedly and in ways that are not presently predictable.

With the branched DNA substrate containing an 18-nt acceptor oligomer that positioned the 5′-OH nucleophile spatially between the two observed cleavage sites, two topoisomerase I-mediated ligation products were observed (Figure 1). Products resulted from topoisomerase I intramolecular ligation at each of the two cleavage sites; one product involved a 1-nt deletion, the other a 1-nt insertion. While topoisomerase I 1-nt insertions and 1-nt deletions have been observed previously (Shuman, 1992), this is the first example of topoisomerase I-mediated strand transfer producing two "recombinant" duplexes from a single set of reactants.

Deletions were also observed with a nicked DNA substrate (Figure 3); the action of topoisomerase I near a pre-existing nick resulted in deletions of up to three nucleotides. Topoisomerase I-mediated 1- and 2-nt deletions have previously been demonstrated by Shuman (1992), with the 2-nt deletion occurring with decreased efficiency compared to the 1-nt deletion. In our system, the larger deletion (3-nt) was produced in greater yield than the 1-nt deletion, suggesting that the formation of deletions need not decrease with increasing distance. A logical inference to be drawn from this observation was that still larger deletions might be possible. In the observed topoisomerase I-mediated deletions, the acceptor oligonucleotide had the potential to base pair in proximity to the covalent DNA−topoisomerase I complex; correct base pairing at the site of ligation was not a requirement, although the increased efficiency of the 3-nt deletion may be facilitated by the ability of the acceptor to form a base pair at the site of ligation.

In the proposed mechanism of nucleotide deletion, the participation of the acceptor oligonucleotide as part of a duplex structure is supported by both the alteration of the preferred site of cleavage in the branched substrate (Figure 1B) and the increased cleavage efficiency of the nicked substrates (Figure 3B). As illustrated for both the branched and nicked DNA substrates, topoisomerase I-mediated intramolecular ligation led to the introduction of DNA mismatches via deletions or insertions of nucleotides. The potential biochemical relevance of these transformations may be appreciated from the recent finding that topoisomerase I recognizes DNA mismatches (Yeh et al., 1994).

The ability of topoisomerase I to delete larger segments of DNA was demonstrated by the ligation of two DNA strands across an 18-nt gap (Figure 5). The proposed model suggests that the two DNA ends are brought into close proximity by the formation of a hairpin (Figure 6). Previously it was demonstrated that topoisomerase I catalyzed strand transfer to heterologous acceptors with a requirement of at least 4 base pairs of complementarity at the site of ligation. However, if the acceptor was physically linked to the covalent complex, sequence specificity was eliminated (Shuman, 1992). In the present case, the acceptor in the gapped substrate has only two sites of complementarity adjacent to the covalent complex (5′-AT) but is capable of hybridizing to the covalent complex farther downstream, suggesting strongly that ligation across the gap occurs intramolecularly. Consistent with the putative requirement for intramolecular ligation, an acceptor containing 5′-AT..., but unable to base pair with the single-stranded region of the noncleaved strand, failed to undergo ligation analogous to that shown in Figure 5. Additional support for the hybridization of the acceptor to the DNA substrate, and also for the intermediacy of a hairpin in the process of topoisomerase I-mediated nucleotide deletion, was obtained by conformational mapping of "intact" and "cleaved" DNA substrates (Figure 7; supplementary material, Figure 5). The obligatory intermediacy of a hairpin was further secured by alteration of the sequence of the noncleaved strand to eliminate self-complementarity; this abolished efficient ligation (not shown).

It is well known that DNA polymerases can produce frame shift errors *in vitro* during DNA synthesis. Current models suggest that DNA structure contributes to mutagenesis through misalignment of the primer and template, facilitated by iterated and palindromic DNA sequences (Streisinger et al., 1966; Glickman & Ripley, 1984). It has also been observed that T4 DNA ligase catalyzes at low efficiency the joining of gapped DNA up to approximately 100 base pairs in length (Nilsson & Magnusson, 1982). While there is evidence that enzymes such as DNA polymerases and ligases can promote the formation of deletions either by ligating across a gap or by slipped mispairing, the ability of topoisomerase I to effect efficient deletions at these sites is novel.

Illegitimate recombination is an inclusive term used to denote processes in which chromosomal rearrangements occur in regions having little or no sequence homology, thereby generating insertions, deletions, duplications, and inversions of genetic material (Low, 1988). While illegitimate recombination is the most frequent mechanism of recombination in mammalian somatic cells and is linked to genetic diseases, cancer, and evolution (Schimke et al., 1986;

Meuth, 1989; Solomon et al., 1991), the underlying molecular mechanism(s) remains unclear. It is quite apparent, though, that such chromosomal rearrangements must require the production and joining of DNA ends. As such, topoisomerases and other cellular enzymes that catalyze the cleavage or ligation of DNA are candidates for promoting nonhomologous DNA exchange (Froelich-Ammon et al., 1994).

Evidence for the involvement of topoisomerase I in the process of recombination includes the observations that site specific recombinases such as Int protein of bacteriophage λ, Tn*3* family of transposons, and FLP recombinase all exhibit type I topoisomerase activity (Landy, 1989; Ikeda, 1990). The ability of topoisomerase I to subserve the function of certain recombinases has been demonstrated by vaccinia virus topoisomerase I-promoted Int prophage excision *in vivo* (Shuman, 1991). A role for topoisomerase I in illegitimate recombination is also supported by both *in vivo* and *in vitro* studies. For example, Bullock et al. (1984, 1985) have studied chromosomal excision of SV40, which is known to involve illegitimate recombination events. They found that the DNA sequences in proximity to the crossover points for excision were associated with preferred cleavage sites for eukaryotic topoisomerase I. Further supporting evidence includes the demonstration that camptothecin and other antineoplastic drugs that stabilize the topoisomerase−DNA covalent intermediate exhibit increased levels of chromosomal aberrations and sister chromatid exchange (Liu, 1989). In the absence of camptothecin, cells treated with β-lapachone, a proposed activator of topoisomerase I, also have increased frequencies of chromosomal alterations (Degrassi et al., 1993).

Illegitimate recombination may be defined operationally as the joining of nonhomologous DNA sequences (Low, 1988). This can include several types of DNA rearrangements, including integration of foreign DNA, translocations, duplications, insertions, and deletions. In the present study we have utilized three types of modified DNA substrates to model partial reactions that seem likely to occur during nonhomologous DNA strand exchange. The ability of topoisomerase I to efficiently catalyze the joining of two nonhomologous DNA strands *in vitro* provides direct biochemical evidence that topoisomerase I may participate in some illegitimate recombination events (Been & Champoux, 1981; Halligan et al., 1982; McCoubrey & Champoux, 1986; Svejstrup et al., 1991; Shuman, 1992; Christiansen et al., 1993). While earlier investigations using substrates that separated the cleavage and ligation reactions demonstrated that topoisomerase I covalently bound to the broken DNA strand could catalyze the joining of new DNA molecules, these studies utilized a heterogeneous population of donors (i.e., topoisomerase I−DNA complexes) so the possible sequence specificity of the process could not be defined (Been & Champoux, 1981; Halligan et al., 1982; McCoubrey & Champoux, 1986). More recently, investigations have been carried out using defined DNA substrates to investigate intra- and intermolecular ligation reactions mediated by topoisomerase I; however, the nature of the products was determined only by their electrophoretic mobilities (Svejstrup et al., 1991; Shuman, 1992; Christiansen et al., 1993). In the present study, we demonstrate that branched and nicked substrates can undergo cleavage and subsequent ligation, at sites not previously anticipated, thus underscoring the need to define the formed products by DNA sequence analysis. We also demonstrate directly that three DNA substrates undergo facile insertions and deletions of nucleotides mediated by topoisomerase I.

The processes described herein require DNA substrates having strand interruptions in the scissile strand to separate the cleavage and ligation reactions, ultimately resulting in nonhomologous DNA exchange. DNA structures containing partial duplexes, nicks, and gaps are present *in vivo* during the cellular processes of DNA replication and transcription, and DNA damage induction and repair, demonstrating the potential biological relevance of the topoisomerase I-mediated nonhomologous ligations studied here. The present results provide further support for the involvement of topoisomerase I in illegitimate recombination, as well as plausible mechanisms for the insertion and deletion of nucleotides.

ACKNOWLEDGMENT

We thank Dr. L.-K. Wang for the calf thymus topoisomerase I used in this study and Dr. R. Manderville for assistance with the synthesis of DNA substrates.

SUPPLEMENTARY MATERIAL AVAILABLE

Sequencing analysis of topoisomerase I-mediated ligation products and conformational analysis of "intact" and "cleaved" gapped substrates (6 pages). Ordering information is given on any current masthead page.

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BI942748Y