DNA Duplexes Containing 3'-Deoxynucleotides as Substrates for DNA Topoisomerase I Cleavage and Ligation*

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The DNA cleavage-ligation reaction of DNA topoisomerase I was investigated employing synthetic DNA substrates containing 3'-deoxyadenosine or 3'-deoxythymidine at specific sites and acceptor oligonucleotides of different lengths. The modified nucleotides were substituted systematically within the putative enzyme-binding domain and also next to the high efficiency cleavage site to determine the effect of single base changes on enzyme function. Depending on the site of substitution, the facility of the cleavage and ligation reactions were altered. The bases at positions −1 and −2 on the noncleaved strand were found to be important for determining the site of cleavage. Inclusion of 3'-deoxythymidine in the scissile strand at position −1 permitted the demonstration that topoisomerase I can cleave and form a 2′ → 5′-phosphodiester linkage. Partial duplexes doubly modified at positions −4 or −6 in the noncleaved strand and at positions +1 or −1 within scissile strand were not good substrates for topoisomerase I, showing that cleavage can depend importantly on binding interactions based on structural alterations at spatially separated sites. Substitution of a 3'-deoxyribonucleotide on the scissile strand at position −6 enhanced formation of the ligation product resulting from cleavage at site 1 and suppressed cleavage at site 2.

The DNA topoisomerases alter DNA topology via the introduction of transient breaks in the phosphodiester backbone of this biopolymer (1, 2). These enzymes participate in the essential cellular processes of DNA replication, transcription, and recombination (3). DNA topoisomerases are classified into two groups based on their mode of DNA strand scission: the type I topoisomerases mediate the transient single-strand breakage of a DNA substrate, whereas the type II topoisomerases break both strands. Because of their essential role in the cell, the eukaryotic topoisomerases have become important targets for the development of antitumor agents (4).

The mechanism of DNA cleavage involves nucleophilic attack of a tyrosine OH group in the active site of topoisomerase I on the phosphate ester backbone, resulting in the attachment of tyrosine to the DNA through an oligonucleotide 3′-phosphate with concomitant release of an oligonucleotide having a free 5′-OH terminus (5). After strand passage of the free DNA strand around the unbroken strand, religation of the broken strand occurs by a process believed to involve reversal of the cleavage reaction. Under normal circumstances the cleavage and ligation reactions are tightly coupled with low steady-state concentrations of the covalent intermediate (6). However, the two partial reactions can be uncoupled in vitro, e.g. by site-specific cleavage of partially double-stranded substrates containing a high efficiency cleavage site (see Fig. 1) (7). Cleavage of the “suicide substrate” occurs without sequential religation due to the instability of the duplex involving the truncated strand downstream from the site of cleavage; loss of this oligonucleotide traps the topoisomerase I-DNA covalent intermediate. The covalently bound enzyme is catalytically competent, as may be judged by formation of the intact duplex upon admixture of an oligonucleotide complementary to the single-stranded region in the enzyme-DNA covalent binary complex (see Fig. 1B). The covalent binary complex also undergoes ligation with partially complementary acceptors containing a free 5′-OH group (8).

The topoisomerases I from all species characterized thus far recognize specific nucleotide sequences in their DNA substrates, which are cleaved with high efficiency (see Fig. 1) (9, 10). Such sites can be identified by footprinting analysis (11). Although the cleavage-ligation reactions have been studied in some detail, further investigation is needed to define the possible roles of single nucleotides within high efficiency sequences on enzyme binding, as well as cleavage and ligation (12). Different approaches have been utilized to determine the role of individual nucleotides, including phosphate ethylation (13) and deoxyguanosine N7-methylation and subsequent depurination to create abasic sites (11). Abasic sites within the scissile strand on the 5′-side of the high efficiency cleavage site rendered the DNA substrate refractory to cleavage by topoisomerase I (11). In addition, Pourquier et al. (14) have incorporated deoxyuridine and abasic sites into the noncleaved strand of a DNA substrate and investigated the effect on topoisomerase I-mediated cleavage. They found that the position of cleavage was altered and that the new site of cleavage was dependent on the location of the site modified.

We have reported previously that topoisomerase I can catalyze the ligation to the covalent topoisomerase I-DNA binary complex of complementary acceptor oligonucleotides having modified nucleophiles at the 5′ terminus; the resulting duplexes have altered connectivity at the site of ligation (15). Recently, we have extended these findings by employing a modified acceptor oligonucleotide having the 5′-terminal deoxyadenosine linked to the remainder of the oligomer through a 2′ → 5′-phosphodiester bond (see Fig. 1C). When employed in the presence of a partial DNA duplex that afforded a single-stranded region complementary to the acceptor upon cleavage by topoisomerase I, the modified oligonucleotide was incorporated into a newly formed duplex (16). The extent of (modified) duplex formation was only ~10% of that achieved with the respective unmodified acceptor oligonucleotide after 1 h, but the yield of the modified duplex could be increased to 20% after a 13-h incubation (16). In addition, modified partial duplexes
were utilized as substrates; each contained a single 3’-deoxyadenosine moiety within the DNA topoisomerase I-binding region on the scissile strand. The facility of cleavage or ligation was altered in a fashion dependent on the location of the 3’-deoxyadenosine moiety.

To further characterize the role of single nucleotides within the recognition domain on substrate recognition and cleavage by topoisomerase I, additional partial duplex substrates containing modifications in the scissile and noncleaved strand have been prepared. Presently we demonstrate that (i) substitutions on the scissile or noncleaved strands can affect the topoisomerase I-mediated cleavage and ligation reactions, (ii) certain combinations of modified nucleotides within the substrate can substantially enhance overall cleavage and ligation efficiency, (iii) topoisomerase I can effect the cleavage and ligation of a 2’ → 5’-phosphate ester linkage within the DNA substrate, and (iv) alterations in efficiency of enzyme action caused by the introduction of a 3’-deoxyribonucleotide at a specific site can sometimes be compensated by introduction of the complementary 3’-deoxyribonucleotide at the same site on the opposite strand.

**EXPERIMENTAL PROCEDURES**

T4 polynucleotide kinase and proteinase K were purchased from U. S. Biochem Corp., exonuclease III was from Life Technologies, Inc. β-Cyanoethyl phosphoramidites, activator solution, and the solid support for oligonucleotide synthesis were obtained from Craelium Inc. Nensorb prep nucleic acid purification cartridges were from NEN Life Science Products, and γ-[^32]P]ATP (7000 Ci/mmol) was obtained from ICN Pharmaceuticals. Scintillation counting was performed on a Beckman LS-100C instrument using Beckman Ready Safe scintillation fluid.

Gel electrophoresis was carried out on 20% polyacrylamide gels (19% (w/v) acrylamide, 1% (w/v) N,N-methylenebisacrylamide, 8 μm urea) in 90 mM Tris borate buffer, pH 8.3, containing 1 mM EDTA. Polyacrylamide gel loading solution included 10 μl urea, 1.5 mM EDTA, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromphenol blue. Gels were visualized by autoradiography at −80 °C with Kodak XAR-2 film and quantified utilizing a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 3.2 software. DNA sequence analysis was performed by modification of the traditional Maxam-Gilbert method (17) for short, single-stranded deoxyoligonucleotides. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

**Oligonucleotide Substrates—**Synthetic oligonucleotides were purchased from Craelium Inc. or synthesized on a Biosearch 8600 series DNA synthesizer using standard phosphoramidite chemistry (18). The oligonucleotides synthesized on the Biosearch DNA synthesizer were deblocked and cleaved from the solid support by treatment with concentrated NH₄OH at 55 °C for 12 h. The oligomers were purified by Nensorb column chromatography and then by preparative 20% denaturing polyacrylamide gel electrophoresis. The formation of modified duplexes was verified by exonuclease III digestion, which accurately reflected the position of the modified nucleotide in each of the partial duplexes (15) (data not shown).

**Effect of Modifications within the Noncleaved Strand—**The topoisomerase I-mediated cleavage and ligation reactions were investigated using partial duplexes containing high efficiency topoisomerase I cleavage sequences (22) and a single 3’-deoxyribonucleotide within the noncleaved strand at various positions. The 17- and 19-nucleotide acceptor oligonucleotides were fully complementary to those regions of the duplex that became single-stranded upon cleavage at sites 1 and 2, respectively (Fig. 1). We first investigated the topoisomerase I-mediated cleavage-ligation reaction using partial duplexes singly modified within the noncleaved strand at positions −1, −2, −4, or −6. The 5’-[^32]P end-labeled partial duplexes containing these modifications were treated with calf thymus DNA topoisomerase I in the presence of the 17-mer acceptor oligonucleotide at 37 °C for 60 min. The reaction mixtures were quenched by treatment with 1% SDS and proteolysed with proteinase K to digest the covalently bound enzyme. The reactions were analyzed on a 20% denaturing polyacrylamide gel. As shown in Fig. 2A, the topoisomerase I-mediated cleavage and ligation in the presence the 17-mer acceptor oligomer afforded ligated products when the noncleaved strand was modified at positions −4 or −6. Modifications at position −1 or −2 in the noncleaved strand diminished ligation with the 17-mer acceptor, although the presence of some cleavage products was apparent. To determine whether the modifications at positions −1 and −2 in the noncleaved strand created a duplex that was a poor substrate for the enzyme or simply created new preferred cleavage sites, the same set of reactions under the same conditions was carried out in the presence of the 19-mer acceptor. As shown in Fig. 2B, the topoisomerase I-mediated cleavage-ligation reaction with the unmodified partial duplex using a 19-mer acceptor afforded only a very limited amount of ligation product (cf. lanes 3 and 6), in good agreement with a previous study (23). In contrast, incubation of topoisomerase I with partial duplexes containing a modified nucleoside at position −1 or −2 gave

1 The abbreviation used is: TEAA, triethylammonium acetate.
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The effect of single nucleotide modifications within the noncleaved strand. The 5'-32P end-labeled modified partial duplexes were treated with calf thymus DNA topoisomerase I in the presence of an acceptor oligonucleotide at 37 °C for 60 min. The reaction mixtures were quenched by treatment with 1% SDS, followed by proteolysis with proteinase K to digest the covalently bound enzyme. The reaction mixtures were analyzed on a 20% denaturing polyacrylamide gel.

Table I

Effect of modification of the noncleaved strand on the efficiency and site selectivity of DNA cleavage by topoisomerase I

| Site of modification | Cleavage efficiency* | Site 1:Site 2 |
|----------------------|----------------------|--------------|
| −1                   | 100                  | 5:1          |
| −2                   | 80                   | 1:3          |
| −4                   | 22                   | 13:1         |
| −6                   | 35                   | 55:1         |
|                     | Extent of total cleavage obtained at sites 1 and 2, relative to the value obtained for the unmodified partial duplex.

Effect of modification of the scissile strand at position −2—As described previously, modification at position −4 of the scissile strand resulted in decreased ligation of the 17-mer acceptor oligonucleotide by shifting the substrate cleavage site (Fig. 7). Inclusion of 3'-deoxyadenosine at position −4 in the scissile strand in addition to modification at positions −1 or −2 in the noncleaved strand altered cleavage from site 1 to site 2 essentially completely (Fig. 7). Interestingly, when there was a "compensatory" modification at −4 on the noncleaved strand, the product of cleavage and ligation at site 1 was restored, consistent with possible stabilization of the duplex, which could enable effective ligation by topoisomerase I. Modification at −6 on the noncleaved strand disrupted ligation by the enzyme with both the 17- and 19-mer. Although topoisomerase I is clearly cleaving the substrate at site 2 in the presence of the position −6 modification on the noncleaved strand, this modification presumably alters binding of the enzyme, thereby affecting ligation relative to the normal duplex.

Effect of Modification of the Scissile Strand at Position −6—The substitution at position −6 resulted in cleavage largely at site 1 and concomitant enhancement of ligated product formation (Fig. 8). Interestingly, modifications at positions −1 and −2 on the noncleaved strand that previously altered cleavage by topoisomerase I to site 2 when present as the sole modification (16) or in combination with the presence of 3'-deoxyadenosine at position −4 on the scissile strand (Fig. 7), afforded cleavage predominantly at site 1 when combined with modification at position −6 on the scissile strand (Fig. 8). Additional
a substrate containing a 2′ → 5′ linkage at the high efficiency cleavage site. Topoisomerase I-mediated coupled cleavage-ligation reactions were carried out with partial duplexes containing 3′-deoxyadenosine within the scissile strand at position −1 and modified noncleaved strands as indicated. A, using a 17-mer acceptor. B, using a 19-mer acceptor. The control in the third lane of each panel contained an unmodified partial duplex incubated with a 17-mer acceptor. The reactions were carried out as indicated in the legend to Fig. 2.

modifications on the noncleaved strand at positions −4 or −6 resulted in cleavage virtually exclusively at site 1 (Fig. 8). The substrate having modifications on both strands at position −6 gave enhanced ligation at site 1 relative to the unmodified substrate (Fig. 8). In the presence of a 19-mer acceptor, the modification at position −2, combined with modification at position −6 on the scissile strand, exhibited the greatest amount of ligation due to partial cleavage at site 2.

**DISCUSSION**

Eukaryotic DNA topoisomerase I participates in the control of DNA topology. The enzyme can relax supercoiled DNA by transiently cleaving one strand of the DNA substrate and then passing and religating the cleaved strand via a 3′-linked DNA-enzyme covalent intermediate containing a phosphorothioate linkage (15, 24). Previously, we reported that DNA topoisomerase I can promote the rearrangement of DNA structure; nucleotide insertions and deletions were noted in addition to alteration of the DNA backbone (15, 23). The enzyme trapped using “suicide substrates” can undergo ligation with exogenous DNA acceptors, affording structural transformations of the DNA (25, 26).

The minimum DNA duplex region required for topoisomerase I-mediated reaction in vitro was determined as an optimized sequence containing nine nucleotides on the scissile strand and five nucleotides on the noncleaved strand (9) (cf. Fig. 1A). This sequence is cleaved with the same specificity by eukaryotic topoisomerase I from various organisms (26) and functions as a high efficiency site for DNA relaxation (27). To determine which nucleotides contribute to the sequence specificity and facility of catalysis by the eukaryotic enzyme, previous studies have utilized DNA containing methylated nucleotides, abasic sites, and uracil substitutions. Abasic sites at positions −2 to −7 on the scissile strand rendered the substrate refractory to cleavage by topoisomerase I. An abasic site at position −1 had little effect on enzyme function (11). Recently, it was reported that an abasic site on the noncleaved strand at positions −1 to −4 suppressed DNA cleavage at the normal site and created new cleavage sites. An abasic site at position −6 on the noncleaved strand increased the extent of cleavage (14). Neither study involved a substrate having two modifications within the topoisomerase I-binding region, although double modifications could potentially facilitate a better understanding of the role of DNA structure on topoisomerase I-mediated cleavage, relaxation, and ligation.

Two abasic sites would be expected to destabilize the DNA duplex dramatically (28, 29). In contrast with the effects of abasic sites on DNA stability, it has been reported that the insertion of two 3′-deoxyadenosines within a 16-nucleotide duplex reduced the $T_m$ by only 11–12 °C, depending on the sites of substitution (30, 31). In an earlier study, we utilized as substrates a few modified partial duplexes, each of which contained a single 3′-deoxyadenosine within the topoisomerase I-binding region on the scissile strand. Depending on the location of the 3′-deoxyadenosine substitution, the facility of the topoisomerase I-mediated cleavage or ligation reaction was altered (16). Presently, we have modified the partial duplex systematically to assess the importance of specific nucleotides by incorporating their 3′-deoxynucleotide counterparts. Depending on the site of substitution, this particular modification can significantly modulate the effects of the enzyme by altering cleavage site or facility of cleavage or ligation, presumably reflecting alterations in substrate binding by the enzyme.

Initially, 3′-deoxynucleotides were incorporated into the noncleaved strand to observe the effect on cleavage and ligation. These data (Fig. 2) indicate that modifications at positions −1 and −2 alter the cleavage site, because ligation was observed in presence of the 19-mer acceptor but not in presence the 17-mer acceptor. The same trend was observed in the absence of any acceptor (Table I). It could be argued that these modifications affect accessibility of the enzyme to cleavage site 1 rather than the affinity of the enzyme for this substrate per se, given the increase in cleavage at site 2. Introduction of 3′-deoxynucleotides into positions −4 and −6 had a lesser effect on cleavage and ligation at site 1; little change in the amount of ligation was noted for the substrate modified at positions −4 or −6. This was true despite the fact that the extent of cleavage for these modified partial duplexes in the absence of acceptor oligonucleotides was only a small fraction of that observed for the unmodified partial duplex. This clearly indicates that the acceptors can facilitate the overall process of cleavage and ligation. It is interesting that the formation of ligation products from the substrates modified at positions −4 and −6 resulted essentially exclusively from cleavage at site 1. Suppression of cleavage at site 2 might be thought to be logical because, for example, position −4 on the noncleaved strand bears the same relationship to site 2 that position −2 does to site 1 (cf. Figs. 2 and 6).

Alternatively, 3′-deoxynucleotides were incorporated within the scissile strand. Beginning with a modification at position +1, 3′-deoxynucleotides were also introduced systematically at positions −1, −2, −4, and −6 within the scissile strand (Fig. 3). These modified oligonucleotides were then utilized in coupled cleavage-ligation reactions. Subsequently, the modified scissile
strands were combined with modified noncleaved strands to obtain more information about topoisomerase I function.

3'-Deoxynucleotide incorporation at position +1 in the scissile strand significantly altered substrate cleavage from site 1 to site 2, contrary to the enhancement of cleavage demonstrated when uracil and abasic sites were incorporated at these positions (14). The results were also contrary to experiments in which depurination of +1 caused accumulation of cleaved intermediates (11). In the presence of the 19-mer acceptor, topoisomerase I cleaved the partial duplex predominantly at site 2; the intermediate underwent ligation to form the 30-mer product. Further modifications at positions −1 or −2 had no effect on ligation when the 19-mer acceptor was present, whereas further modification at position −4 or −6 on the noncleaved strand decreased ligation even in the presence of the 19-mer acceptor (Fig. 3).

The substitution of a 3'-deoxynucleotide at position −1 was of special interest because it permitted investigation of the ability of the enzyme to cleave the DNA backbone at the site of an unnatural linkage. Topoisomerase I did effect cleavage at site 1 in this substrate (Fig. 4), although most of the cleavage was redirected to site 2. The intermediate cleavage products formed at sites 1 and 2 both underwent ligation in the presence of the 17- and 19-mer acceptor oligonucleotides, respectively. As noted for the substrates lacking any scissile strand modification (Fig. 2), additional modification of the partial duplexes at positions −1 and −2 on the noncleaved strand suppressed cleavage at site 1; modifications at position −4 and −6 suppressed cleavage at site 2.

In contrast, the substrate having 3'-deoxythymidine at position −2 (scissile strand) exhibited unaltered cleavage and ligation at site 1 (Fig. 6). However, cleavage at site 2 was completely suppressed in analogy with the effects of substitution at position +1 on cleavage at site 1 (cf. Figs. 3 and 6). However, additional modification on the noncleaved strand did alter the cleavage-ligation patterns at sites 1 and 2. The substrate additionally modified at position −1 on the noncleaved strand underwent cleavage and ligation poorly, and only at site 2, whereas additional modification at position −2 on the noncleaved strand also resulted in limited cleavage and ligation but only at site 1. Additional substitutions at positions −4 or −6 on the noncleaved strand afforded substrates whose cleavage and ligation at site 1 was enhanced (Fig. 6, cf. lanes 3, 15, and 18).

Introduction of 3'-deoxyadenosine at position −4 of the noncleaved strand resulted in cleavage and ligation almost exclusively at site 2 (Fig. 7). This is consistent with the interpretation that the presence of the 3'-deoxynucleotide actually has a facilitating effect on cleavage at the closer cleavage site, fully consistent with the effects noted upon modification at position −2 on the scissile strand (cf. Figs. 6 and 7). Additional modification at site −1 or −2 on the noncleaved strand had minimal
further effects on the ratio of cleavage-ligation at site 1 versus site 2.

The most dramatic effects occurred in the presence of a modification at position −6 on the scissile strand. Cleavage occurred exclusively at site 1 in the presence of the substrate lacking any modification on the noncleaved strand. The same was also true for partial duplexes having 3’-deoxynucleotides at position −4 or −6. Event in the presence of modifications at positions −1 or −2 on the noncleaved strand, which frequently alter the cleavage from site 1 to site 2, topoisomerase I cleaved the substrate predominantly at site 1. Further, most of the substrates afforded a greater extent of cleavage and ligation than the unmodified partial duplex substrate (Fig. 8). This constitutes strong evidence that alteration of DNA structure at a site distant from the actual cleavage site can have a strong facilitating effect on DNA cleavage and ligation.

Of particular interest was an analysis of the effects of introducing complementary 3’-deoxynucleotides at the same site on both strands. Substitutions at positions −1 and −2 on both strands (Figs. 4 and 6, respectively) had the effect of shifting topoisomerase I-mediated cleavage to those sites (2 and 1, respectively) farther from the position of modification. Interestingly, when the modifications were present at sites distant from both cleavage sites, the compensatory modifications enhanced both the facility and specificity of cleavage. This was apparent for modification at positions −4, which restored virtually full cleavage-ligation to site 1 (Fig. 2) and especially for modification at positions −6, which resulted in strong enhancement of cleavage-ligation specifically at site 1 (Fig. 8).

Overall, substitution at position −2 on the noncleaved strand generally directs cleavage to site 2 and thereby greater ligation at this site. The exceptions occur where the modification occurs opposite a complementary modification and in the presence of a substrate modified at position −6 on the scissile strand. Interestingly, modification at position −1 on the noncleaved strand alters cleavage to site 2 in almost every case. The single exception involves substrates modified at position −6 on the scissile strand. Because the modification of DNA at a specific site must alter overall DNA structure, the present results do not permit firm conclusions to be drawn about the specific sites of topoisomerase I-DNA interaction. However, the patterns of response to DNA structural alterations argue strongly that specific common enzyme-DNA contacts do control the specificity and efficiency of DNA substrate cleavage and ligation by topoisomerase I.

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