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IMPORTANCE OF DNA END PHOSPHORYLATION AND CAMPTOTHECIN EFFECTS*

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We used purified mammalian topoisomerases I (top1) and oligonucleotides containing a unique top1 cleavage site to study top1-mediated cleavage and recombination in the presence of nicks and short gaps mimicking DNA damage. In general, top1 cleavage was not induced opposite to the nicks, and nicks upstream from the top1 cleavage site suppressed top1 activity. Irreversible top1 cleavage complexes (suicide products or aborted complexes) were produced in DNA containing nicks or short gaps just opposite to the normal top1 cleavage site. Camptothecin enhanced the formation of the aborted top1 complexes only for nicks downstream from the cleavage site. These aborted (suicide) complexes can mediate DNA recombination and promote illegitimate recombination by catalyzing the ligation of nonhomologous DNA fragments (acceptors). We report for the first time that top1-mediated recombination is greatly enhanced by the presence of a phosphate at the 5′ terminus of the top1 aborted complex (donor DNA). By contrast, phosphorylation of the 3′ terminus of the gap did not affect recombination. At concentrations that strongly enhanced inhibition of intramolecular religation, resulting in an increase of top1 cleavable complexes, camptothecin did not reduce recombination (intermolecular religation). Nicks or gaps with 5′-phosphate termini would be expected to be produced directly by ionizing radiations or by processing of abasic sites and DNA lesions induced by carcinogens or drugs used in cancer chemotherapy. Thus, these results further demonstrate that DNA damage can efficiently trap top1-cleavable complexes and enhance top1-mediated DNA recombination.

DNA rearrangements such as deletions, translocations, inversions, or gene amplifications are involved in genetic diseases and carcinogenesis (1–4). These modifications are based on the cell’s ability to promote recombinations. This process can be initiated after exposure to exogenous DNA damaging agents such as ionizing radiations, environmental carcinogens, and drugs used in cancer chemotherapy (5). Even though their molecular mechanisms are not yet fully understood, two types of recombinations have been described depending on the homology of the DNA molecules joined. In eukaryotes, the nonhomologous (or illegitimate) recombination occurs more frequently than the homologous (or legitimate) recombination (6).

Topoisomerases are ubiquitous enzymes involved in multiple processes including replication, transcription, chromosome segregation, and recombinations (7, 8). DNA topoisomerase I (top1) acts as a monomer and creates transient DNA single-strand breaks via the formation of a covalent bond between the 3′-phosphate of the cleaved strand and a tyrosine residue of the enzyme. Under physiological conditions, these intermediates are referred to as cleavable complexes. The cleavable complexes are readily reversible by top1-mediated religation of the 5′-hydroxyl termini (7, 9, 10).

In mammalian top1, top1 is involved in illegitimate recombination in vitro (11–15) and probably in vivo (16–18). top1 inhibitors such as camptothecin (CPT) and its derivatives, which have recently been introduced in cancer treatment, inhibit the religation step and lead to the conversion of cleavable complexes into DNA double-strand breaks after replication fork collisions (19, 20). We recently reported that top1 can be trapped irreversibly by common DNA endogenous lesions such as DNA mismatches and abasic sites (21). Previous studies also reported that nicks in the vicinity of the top1 cleavage site were able to generate double-strand breaks, leading to suicide or aborted products, where covalently bound top1 is able to relegate various nonhomologous acceptors bearing a 5′-hydroxyl terminus and thus participates into illegitimate recombination (10, 12, 13, 22–24).

In this study, we used purified mammalian top1 and DNA oligonucleotides containing a unique top1 cleavage site derived from the rDNA sequence of Tetrathymenta (25, 26) to study top1-mediated DNA damage and recombination induced in DNA substrates containing nicks or gaps in the non-scissile strand (i.e. opposite to the cleaved strand). Such nicks and gaps mimic endogenous DNA lesions or modifications resulting from DNA damage (such as ionizing radiations) or DNA repair (base elimination or excision). Such lesions were introduced at various positions relative to the top1-mediated cleavage site in the oligonucleotide shown in Fig. 1. The results demonstrate for the first time the importance of a phosphate at the 5′ terminus of the DNA break opposite to the top1 linkage for top1-mediated recombinations. We also report the influence of DNA nicks on CPT activity and discuss these results in the context of the known potentially effect of ionizing radiations on camptothecin activity.

EXPERIMENTAL PROCEDURES

Chemicals—High performance liquid chromatography-purified oligonucleotides were purchased from The Midland Certified Reagent Co.

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† The abbreviations used are: top1, topoisomerase I; CPT, camptothecin; DTT, dithiothreitol.
Pellets were washed four additional times: once with RSB1, twice with sodium deoxycholate, followed by another wash with RSB1. Pellets were resuspended in 37°C in the presence of the same amount of a 3'9' nicks or gaps as indicated by the sequences shown in the figures.


diagram

**Fig. 1. DNA substrates used.** A modified *Tetrahymena* hexadecameric rDNA sequence with a strong top1 cleavage site indicated by the arrowhead (25, 26) was generally labeled with [32P]Cordycepin (\(\text{A}^3\)) at the 3' terminus of the scissile (upper) strand as described under "Experimental Procedures." Cleavage at the normal site (arrowhead) gives rise to a 23-mer product as indicated. Nicks and gaps are defined using the numbering shown at the bottom relative to the top1 cleavage site. Nicks added to the reaction mixtures before loading. 16% denaturing polyacrylamide gels (7 M urea) were run at 40 V/cm at 50 °C for 2–3 h and dried on No. 3MM Whatman paper sheets. Imaging and quantitations were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Position-dependent Effects of Nicks**—Nicks were introduced at various positions in the non-scissile strand of the oligonucleotide shown in Fig. 1. Nicks at positions −5/−4 and −4/3 suppressed top1-induced cleavage even in the presence of CPT (Fig. 2A). Top1 cleavage was also decreased by the nick at position −3/−2. These results are consistent with the effects of uracil and abasic site incorporations at these positions (21) and confirm the importance of DNA-top1 interactions in this upstream (5') region of the DNA substrate for top1 activity.

Nicks at position −1/1 + and +1/+2 enhanced top1-mediated DNA cleavage independently of CPT (26- and 13-fold, respectively). When a nick was present at the −2/−1 position, cleavage at the normal cleavage site was suppressed, but cleavage at two bases upstream (Fig. 2, open arrowhead) was observed. Cleavage was not reversed by salt for nicks at positions −1/1 + and +1/+2 and was partially reversed for nick at position −2/−1 (Fig. 2, see lanes 3 and 5 for the corresponding panels). These results are consistent with the formation of double-strand breaks associated with suicide (or aborted) top1 intermediate when top1 cleavage occurs opposite to a nick.

When nicks were introduced more downstream (3') from the expected top1 cleavage site, from positions +2 to +6, salt-resistant top1-mediated DNA cleavage was induced by CPT (Fig. 2B, lanes 5). Therefore, nicks at these positions enhanced top1 activity and were converted to double-strand breaks (and suicide products) in the presence of CPT.

**DNA End Phosphorylation Affects top1-mediated DNA Cleavage and Recombination in Nicked and Gapped oligonucleotides**—Processing of physiological, environmental, or pharmacological DNA damage frequently leads to loss of bases. In the case of ionizing radiation or abasic site processing, DNA top1 activity in such oligonucleotides bearing either a phospho- or an hydroxyl group at the 5' terminus (Fig. 3).

When a hydroxyl was present at the 5' terminus, top1-mediated cleavage at the predicted site was greatly enhanced and was salt-resistant for the oligonucleotides containing a nick.
and a one-base gap (Fig. 3, arrowhead). However, cleavage was suppressed for the two-base gap oligonucleotide bearing a 5’-hydroxyl. The presence of a phosphate at the 5’ terminus increased top1 cleavage in the oligonucleotides with a nick or a one-base gap. 5’ end phosphorylation restored top1 cleavage activity for the two-base gap oligonucleotide, probably by repositioning the enzyme on the DNA recognition site (Fig. 3, far right panel; see "Discussion"). In all cases, cleavage was irreversible and led to suicide products and DNA double-strand breaks (Fig. 3, compare lane 2 with lane 3). Interestingly, presence of a phosphate at the 5’ terminus markedly stimulated the formation of top1-mediated recombination products with all three substrates (Fig. 3, R band).

To determine the effect of 3’ end phosphorylation on top1-mediated cleavage and recombination, the one-base gap oligonucleotide was used as a substrate with the four different combinations for phosphorylation of the DNA termini (Fig. 4).

Recombination was readily detected when a phosphate was present at the 5’ terminus of the gap (same result as Fig. 3), and several products of different sizes were detected. By contrast, presence of hydroxyl or phosphate at the 3’ end of the gap had no effect on top1 activities. Taken together, these results indicate that a phosphate at the 5’ end of a gap mimicking DNA damage or DNA repair intermediates increases both the cleavage and the recombinogenic activity of top1.

Characterization of the Recombination Products—To further characterize the nature of the different recombination products, a time course was performed with the one-base gap oligonucleotide containing a phosphate at the 5’ end (see Fig. 4). As shown in Fig. 5A, appearance of the recombination products was time-dependent and increased more slowly than the cleavage product. The first recombination product (51-mer) was detectable after a 1-min reaction, whereas the 65-mer recombination product was clearly observed after a 30-min reaction.
Trapping of top1 by DNA Damage and Recombination

and the 79-mer product, after 1 h. To determine the mechanism of the recombination, the bands were excised from the sequencing gel after 4 h reaction and submitted to purine sequencing (29). Results are shown in Fig. 5B, and a scheme of the putative mechanism leading to the recombination products is presented in Fig. 5C. Arrow 1 (panels B and C) indicates the intermolecular junctions between the 14-mer (donor) top1-linked DNA fragment generated after cleavage of the 37-mer substrate and the 5′ terminus of another uncleaved oligonucleotide (acceptor). The 51-mer resulting product can then serve as a new acceptor and be religated to a second donor, giving rise to the 65-mer product (Fig. 5, B and C, arrow 2). In agreement with this recombination scheme, we did not observe any recombination when the 5′ terminus of the scissile strand was phosphorylated (data not shown).

Differential Inhibition of top1-mediated Intra- and Intermolecular Reciliation by Camptothecin—We further tested whether CPT affected top1-mediated DNA recombination, as it is able to inhibit intramolecular reilation (9, 30). For this purpose, we compared the inhibition of intra- and intermolecular reiliation using increasing concentrations of CPT (Fig. 6). As expected, inhibition of intramolecular reiliation occurred with low CPT concentrations, resulting in an increase of top1 cleavage (Fig. 6A). To test the effect of CPT on intermolecular reiliation, we used an unlabeled donor containing the one-base gap (see Fig. 4) with a 5′-phosphate and incubated this sub- strate with top1 in the presence of a 3′-labeled 23-mer blunt-end acceptor and increasing concentrations of CPT (Fig. 6B). High concentrations of CPT were required to inhibit intermolecular recombination. Both intra- and intermolecular reiliation products were quantified, expressed as the percentage of the highest rate of reiliation obtained, and plotted as a func- tion of CPT concentration (Fig. 6C). Complete inhibition of intramolecular reiliation was observed between 0.3 and 1 μM CPT. By contrast, in this concentration range, intermolecular reiliation was not significantly inhibited. Inhibition of intermolecular reiliation required much higher concentrations of CPT, such as 10–100 μM. Previous experiments using a partial double-stranded top1-DNA post-cleavage complex also demonstrated that CPT concentrations in excess of 1 μM were required for inhibition of reiliation of an exogenous scissile strand (27). Together, these data are consistent with the selective binding of CPT at the enzyme-DNA interface and with its interaction with the +1 base pair 3′ to the top1 cleavage site (25, 29, 31).

DISCUSSION

Nicks and single-base gaps are frequent DNA lesions that can arise from both spontaneous and environmental DNA modi- fications. Nicks can be produced directly by ionizing radia- tions, and single-base loss can result from base mismatch, uracil misincorporation, or modified base processing via the base excision repair pathway. After recognition by a specific DNA glycosylase, misincorporated or modified bases are pro- cessed into abasic sites, which upon conversion by AP endo- nucleases, lead to base elimination (5).

In this study, we systematically investigated the effects of nicks and gaps on top1 activity by studying the effects of such DNA lesions as a function of their position relative to a preex- isting top1 site. A first conclusion is that the presence of nicks does not appear to induce novel top1 sites opposite to the nicks. This result is consistent with an earlier study (23) using plas- mid DNA and probability calculations showing that the con- version of nicks to double-strand breaks by eukaryotic top1 was due to the random distribution of the top1-cleavable complexes rather than to the induction of top1 breaks by the preexisting nicks. A second conclusion of the present study is that nicks immediately upstream (5′) (between positions −5 and −3) from a preexisting top1 site abolish top1 activity. This observation is consistent with our previous findings that top1-cleavable complexes are suppressed by DNA mismatches and abasic sites within the first 4 bases immediately upstream to the top1 cleavage site (21) and by cytosine methylation at the −3 position (32). Furthermore, the base sequence preference for top1 cleavage also indicates a strong preference for 4 base pairs immediately upstream (5′) of the top1 cleavage site (29, 33). Together, these observations indicate that DNA structure in- cluding DNA backbone integrity immediately upstream from the top1 cleavage site is critical for enzyme activity (34–37).

Conversion of the nicks or short gaps into a DNA double-strand break by top1 was consistently observed for nicks at positions −1/−1 and +1/−2 (opposite from the preexisting top1 site) and when the +1 base was removed opposite to the cleavage site (one-base gap oligonucleotide). Such double-strand breaks represent a unique type of DNA damage, as top1 re- mains covalently attached to the 3′ DNA terminus as an aborted (suicide) intermediate. Thus, our data further demon- strate that top1 can be trapped by DNA damage. Recently, trapping of top1 by DNA mismatches, abasic sites (21), and UV damage (38) has also been reported. At the present time, it is not known whether such a trapping is part of DNA repair (39) or cell death pathways. Top1 suicide (or aborted product) is a very efficient donor for the reiliation of an exogenous DNA duplex bearing a 5′-hydroxyl end (Figs. 3–5) (12, 13, 18, 22, 24, 27, 40). Religation of blunt-ended DNA duplex molecules is not influenced by the terminal sequence of the acceptor (13). Thus, these data further demonstrate that top1-mediated intermolecular reiliation can produce illegitimate recombination.

Our data demonstrate for the first time that the efficiency of
the intermolecular religation (recombination) is markedly enhanced by the presence of a phosphate group at the 5' terminus of the break or gap opposite from the top1 linkage. This is physiologically relevant, as damaged DNA with nicks or gaps generally exhibits 5'-phosphate and 3'-hydroxyl termini. For example, nicks and gaps induced by ionizing radiations are usually 5'-phosphate and 3'-phosphate or phosphoglycolate (5).

Thus, taking into account the high frequency of top1 sites in most genes (for instance see Ref. 33), our study suggests that by random occurrence a fraction of the top1-cleavable complexes might be converted into DNA double-strand breaks and aborted (suicide) top1 intermediates with high recombinogenic potential in the presence of DNA-damaging agents that lead to backbone interruptions. Secondly, from an enzyme-DNA interaction standpoint, the fact that the −1/+1 phosphate opposite from the top1–DNA covalent bond facilitates top1 cleavage, and intermolecular religations suggests that this phosphate is involved in the interaction between the enzyme and the DNA. Further evidence for a structural role of this −1/+1 phosphate can be inferred from the observation that its removal inhibits top1 cleavage in the oligonucleotide with a two-base gap 3' to the cleavage site (Fig. 3). Transcription bypass by T7 RNA polymerase has also been shown to be sensitive to the phosphorylation of the termini of the gapped DNA template (41).

Finally, the present study provides further insights for the molecular interactions of camptothecin with top1. This is the first report showing that nicks downstream (between bases +2 and +5) from a top1 site can convert camptothecin-induced reversible cleavable complexes into DNA double-strand breaks and abortive (suicide) intermediates. Since this effect was not

**Fig. 5.** Top1-mediated recombination generated from the one-base gap oligonucleotide. The one-base gap oligonucleotide (−1/+2) was used (see Figs. 1 and 4 for sequence). A, time course for the formation of the recombination products. Oligonucleotides containing either a phosphate (P) or a hydroxyl (OH) at the 5' terminus of the gap were incubated with top1 as described in Fig. 2, except that the incubation times were 1, 3, 5, 10, 30, 60, 120, and 240 min (lanes 1–9, respectively). C, cleavage products; R, recombination products. Numbers correspond to the sizes of the products. B, purine sequencing of the first two recombination products (51 and 65 base pairs). Arrows (1 and 2) indicate the intermolecular religation sites. C, scheme of the top1-mediated recombination. Numbers indicate the product sizes. Asterisks indicate the 3'-end labeling.
 detectable in the absence of camptothecin, it seems plausible that camptothecin destabilizes the DNA immediately downstream (3') from the top1 cleavage site. This would be consistent with the selective binding of camptothecin at the enzyme-DNA interface (25, 31) and with its interaction with the +1 base pair immediately flanking the cleavage site (29). The importance of the +1 base pair for camptothecin binding inside the top1-cleavable complexes is further supported by the present data showing that camptothecin is markedly more potent for inhibition of intramolecular versus intermolecular religation. The low efficiency of camptothecin for inhibition of the intermolecular religation could be due to the inefficient interaction of camptothecin with DNA-enzyme complexes lacking the +1 base pair. The present results may help to explain the synergism between ionizing radiations and camptothecin derivatives (42, 43) and contribute to a better understanding of the mutagenic effects of top1 in the absence and presence of camptothecin and its derivatives (44–47).

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