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Effects of Uracil Incorporation, DNA Mismatches, and Abasic Sites on Cleavage and Religation Activities of Mammalian Topoisomerase I*

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Abasic sites and deamination of cytosine to uracil are probably the most common types of endogenous DNA damage. The effects of such lesions on DNA topoisomerase I (top1) activity were examined in oligonucleotides containing a unique top1 cleavage site. The presence of uracils and abasic sites within the first 4 bases immediately 5' to the cleavage site suppressed normal top1 cleavage and induced new top1 cleavage sites. Uracils immediately 3' to the cleavage site increased cleavage and produced a camptothecin mimicking effect. A mismatch with a bulge or abasic sites immediately 3' to the top1 cleavage site irreversibly trapped top1 cleavable complexes in the absence of camptothecin and produced a suicide cleavage complex. These results demonstrate that top1 activity is sensitive to physiological, environmental, and pharmacological DNA modifications and that top1 can act as a specific mismatch- and abasic site-nicking enzyme.

Abasic sites are the most common endogenous lesions found in DNA, with an estimated 10,000 lesions per human cell per day (1). They arise spontaneously by hydrolysis of the glycosidic bond primarily to purine bases. They are also produced during the course of excision repair of base damage due to oxidation or alkylation during normal metabolism and during the repair of exogenous damage by ionizing radiation, environmental carcinogens, or drugs used in cancer chemotherapy. Ubiquitous uracil *N*-glycosylase also processes uridines in DNA to abasic sites (for review, see Refs. 1–3). The occurrence of uracil, generated from the spontaneous deamination of cytosine, has been estimated at 100–500 per human cell per day (1).

Mammalian DNA topoisomerases (including top1¹) are ubiquitous enzymes involved in multiple processes, including DNA replication, transcription, and illegitimate recombination (4). top1 acts as a monomer, binds to duplex DNA, and creates transient single-strand breaks via the formation of covalent adducts between the 3'-phosphate of the cleaved strand and a tyrosyl residue of the enzyme. These intermediates are commonly referred to as cleavable complexes (4–6). Under physiological conditions, top1 catalyzes the religation of the 5'-hydroxyl group of the broken DNA. Camptothecin (CPT), a potent

anticancer agent, inhibits the religation step and transforms transient top1-linked DNA breaks into more persistent breaks. Although the intimate mechanism of action of CPT and its derivatives is not totally resolved, CPT probably forms a ternary complex with the enzyme and the DNA (5, 7, 8). In the cell, cleavable complexes can be converted into permanent DNA damage during replication and transcription (9, 10). These irreversible reactions have been referred to as "suicide reactions" because top1-cleavable complexes cannot religate their normal acceptor under these conditions (11, 12). top1 can then promote illegitimate recombination with various double-stranded DNAs bearing a 5'-hydroxyl terminus (11–15). All of these events may be responsible for cell death, converting top1 from an essential enzyme to a cell poison (5, 7).

In this study, we examined the effect of uracil incorporation and abasic site generation on top1 cleavage activity and demonstrate that these frequent DNA lesions can, depending on their location relative to the top1 cleavage site, inhibit the catalytic activity of the enzyme or trap top1 on DNA. Recently, *in vitro* cleavage of another mammalian topoisomerase, the type II enzyme, was found to be enhanced by the introduction of abasic sites into DNA substrates (16).

EXPERIMENTAL PROCEDURES

Chemicals—Oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX). α -³²P-cordycepin 5'-triphosphate was purchased from New England Nuclear (Boston, MA). Polyacrylamide was purchased from Bio-Rad, Inc. (Richmond, CA). Calf thymus type I DNA topoisomerase was purchased from Life Technologies, Inc. CPT was provided by Drs. Wani and Wall (Research Triangle Institute, Research Triangle Park, NC). 10 mM aliquots of CPT were stored at –20 °C, thawed, and diluted to 1 mM in dimethyl sulfoxide just before use.

Oligonucleotides Labeling and Annealing—The scissile (upper) strand of the duplex oligonucleotides (see Figs. 1–5) were labeled with α -³²P-labeled cordycepin using terminal deoxynucleotidyl transferase (Stratagene, La Jolla, CA) as described previously (15). The reaction mixture was subsequently centrifuged through a G25 Sephadex column to remove the excess of unincorporated cordycepin. Labeled scissile strand was then annealed to the same concentration of unlabeled lower strands containing uracils at different positions, or a cytosine bulge as described in Fig. 4A (15).

Preparation of Oligonucleotides Containing Abasic Sites—Double-stranded oligonucleotides containing uracils at different positions were treated for 2 h at 30 °C with 1 unit (1 μ l) of uracil DNA glycosylase (Life Technologies, Inc.) to create an abasic site at the equivalent position (31). The buffer used was the same as for annealing (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA). The reaction mixture was then centrifuged through a G25 Sephadex column and used in the top1 reactions. The efficiency of abasic site formation by uracil DNA glycosylase was verified by the nicking of over 80% of the oligonucleotide at the abasic site in the presence of 10 mM NaOH (1 h at 25 °C) (see Fig. 5B, lane d). The tetrahydrofuran oligonucleotide used in Fig. 6 was purchased from Midland Certified Reagent Co.

top1 Reactions—DNA substrates (approximately 50 fmol/reaction) were incubated with 5 units of top1 for 15 min at 25 °C with or without

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¹The abbreviations used are: top1, topoisomerase 1; CPT, camptothecin.

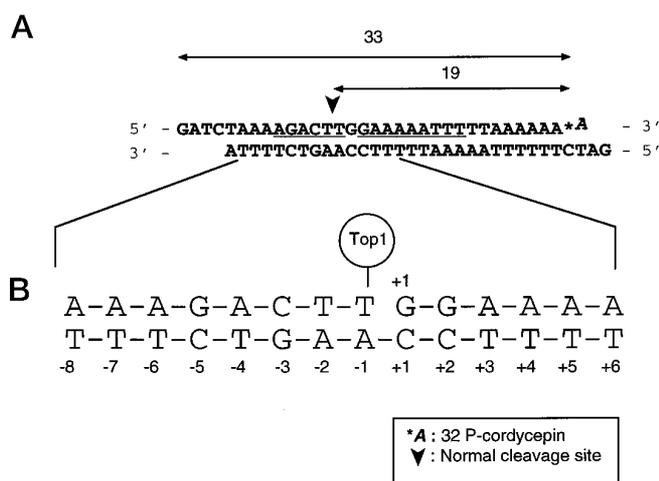


FIG. 1. DNA substrate for topoisomerase-I cleavage. *A*, a modified tetrahymena hexadecameric rDNA sequence (underlined) with a strong top1 cleavage site, indicated by the arrowhead, was used. Labeling was performed with 32 P-labeled cordycepin (*A) at the 3'-terminus of the scissile (upper) strand as described under "Experimental Procedures." Cleavage at the normal site gives rise to a 19-mer product as indicated. *B*, uracil replacements and abasic sites were introduced at each of several positions (from -8 to +6) on the lower strand and at position +1 on the upper strand. top1-mediated cleavage is associated with the covalent linkage of the enzyme to the 3'-terminus of the broken DNA.

CPT in standard reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 μg/ml bovine serum albumin). Reactions were stopped by adding either sodium dodecyl sulfate (SDS) (final concentration 0.5%) or NaCl (unless otherwise indicated, 0.5 M for 30 min at 25 °C followed by addition of 0.5% SDS). Kinetics of reversal were performed by adding NaCl (0.25 M final concentration) to the reactions and incubating the samples at 10 °C for indicated times. Time-course reactions were stopped with 0.5% SDS.

Gel Electrophoresis and Analysis of Cleavage Products—3.3 volumes of Maxam Gilbert loading buffer (98% formamide, 0.01 M EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromophenol blue) were 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 3MM Whatman paper sheets. Imaging and quantitations were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Effects of Uracil Incorporation and Mismatches—The substrates used in these studies were derived from a Tetrahymena oligonucleotide (17) containing a top1 cleavage site where adenine in +1 position on the scissile (upper) strand was changed to a guanine to increase the effect of camptothecin and its derivatives (Fig. 1A) (8). We first investigated the effect of uracil incorporation at various positions in the non-scissile (lower) strand on the DNA cleavage/religation equilibrium induced by top1 in the presence or absence of CPT.

Cleavage activity of top1 was significantly altered, depending on the uracil position. As shown in Fig. 2, three different effects were observed: (i) suppression of top1 cleavage when uracil was incorporated at positions -3, -2, and -1, and to a lesser extent at position -4. This suppression was observed even in the presence of 10 μM CPT. These results show that uracil misincorporation within the 4 bases immediately upstream from the top1 site (5' to the top1 cleavage site) suppresses DNA cleavage. Thus, modifications of DNA in this region can markedly alter top1 catalytic activity. (ii) Enhancement of top1 cleavage in the absence of CPT at the preexisting site was observed when uracil was incorporated at either the -6, -5, or +1 position (Fig. 2, compare lanes 2 for these positions and lanes 2 for the controls). This enhancement was 7–10-fold compared with control, and this effect was still observed for positions as far as -7 and -8 from the cleavage site

(data not shown). In all cases, cleavage was reversible upon addition of 0.5 M NaCl (Fig. 2, lanes 3 and 5). (iii) Induction of a new top1 cleavage site was observed when uracils were incorporated at positions -2 and -1 (Fig. 2A, white arrow). In the case of the -2 mismatch, the new top1 cleavage site was independent of CPT and was located immediately upstream from the mismatch, which is consistent with the enhancement produced by a mismatch at the +1 position. These data indicate that base mismatches can trap top1 cleavable complexes (18).

Effects of Abasic Sites—Modified oligonucleotides were used to investigate the effect of abasic sites at given positions on the top1 cleavage activity in the presence or absence of CPT. Depending on the position of the abasic site, top1 cleavage activity was differentially affected (Fig. 3). Abasic sites at positions -4, -3, -2, and -1 suppressed top1 cleavage at the normal site. New sites were also induced immediately upstream from the abasic site when the abasic site was at position -5, -4, or -2. top1 cleavage was enhanced (4–5-fold) in the absence of CPT when the abasic site was at positions -6, -2, and +1. This enhancement was associated with an inhibition of religation when the abasic site was at position -2 or +1. This can be seen in Fig. 3 as a persistent cleavage band (60–80% of the initial cleavage) after addition of salt (Fig. 3, compare lanes 4 and 5 and lanes 2 and 3). It should be noted that the persistent site observed with the oligonucleotide containing an abasic site at position -2 corresponds to an abasic site immediately downstream from the cleavage site. The results observed both with the abasic sites at positions +1 and -2 indicate that the presence of an abasic site immediately downstream from a top1 cleavage site enhances cleavage in the absence of CPT by inhibiting DNA religation and induces suicide-type reaction.

top1 Suicide Product Generated by a Single-stranded DNA Loop Immediately 3' from the top1 Cleavage Site—Enhancement of top1 cleavage in the absence of CPT was also observed using an oligonucleotide containing a displaced loop (bulge) next to the cleavage site on the non-scissile strand (Fig. 4A). Using different conditions of reversal, such as increased concentration of sodium chloride (*N*) or proteinase K (*P*) or heat treatment (*H*), we found that top1 cleavage persisted under these conditions. Using this mispair loop substrate, CPT had no further effect. With the control substrate, CPT-induced DNA cleavage was completely reversed by salt, proteinase K, or heat (Fig. 4B, left). Together, these results demonstrate that top1 can cleave efficiently DNA with a bulge but fails to religate the DNA, probably because of the stretching out of the loop and separation of the acceptor DNA from the top1. This would generate a suicide-type reaction.

Effects of Mismatches and Abasic Sites at the +1 Position of the Scissile Strand on top1 Cleavage Activity—Because the guanine at position +1 on the scissile strand has been shown to increase the specificity of CPT derivatives for top1 cleavable complexes (19), we tested the effect of an uracil or an abasic site at this position. Uracil mismatch (U:C) or wobble base pair (U:G) increased top1 cleavage in the absence of CPT 8- and 7-fold, respectively (Fig. 5A, lanes 11, 12, 17, and 18). On the other hand, when an adenine was incorporated at the +1 position on the lower strand, leading to a U:A base pairing, no cleavage difference was noted as compared with the control and CPT was still active (Fig. 5A, lanes 5 and 6). All corresponding substrates containing an abasic site also increased top1 cleavage in the presence or the absence of CPT (Fig. 5A, lanes 8, 9, 14, 15, 20, and 21). Reversal of cleavage was studied in the presence of 0.25 M NaCl to investigate the irreversible nature of the cleavage. The reaction rate of the top1-mediated religation process was decreased for the control oligonucleotide in the presence of 10 μM CPT, but reversal was complete after 15 min

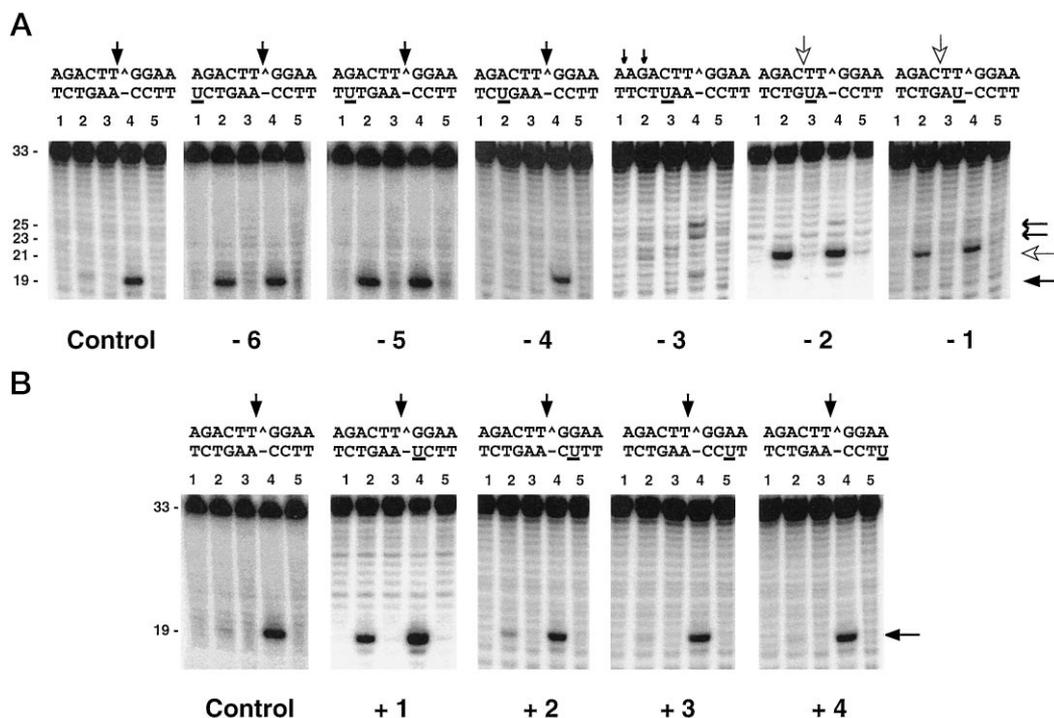
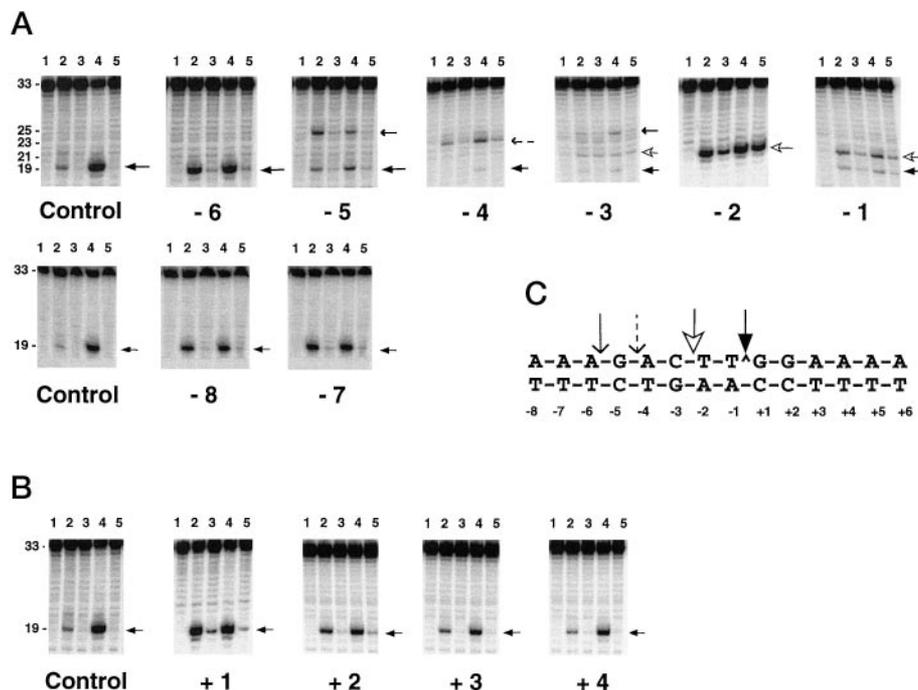


FIG. 2. Effects of uracil replacements on topoisomerase I-mediated DNA cleavage. Oligonucleotides containing uracils (*U*) at distinct positions of the lower strand were annealed to the 3'-end-labeled upper strand (see DNA sequence above panels). Double-stranded DNA was reacted with top1 in the absence or presence of 10 μ M CPT at 25 $^{\circ}$ C for 15 min. Different arrows (see above panels) and numbers correspond to the position and the size of the cleavage products, respectively. Lane 1 of each panel, DNA alone; lanes 2 and 3, +top1; lanes 4 and 5, +top1 + 10 μ M CPT. Reactions were stopped with 0.5% SDS (lanes 2 and 4) or 0.5 M NaCl (lanes 3 and 5) for 30 min at 25 $^{\circ}$ C. A, uracil misincorporations upstream from the normal cleavage site (positions -6 to -1; see Fig. 1). B, uracil misincorporations downstream from the normal cleavage site (positions +1 to +4).

FIG. 3. Effects of abasic sites on topoisomerase I-mediated DNA cleavage. Oligonucleotide duplexes containing abasic sites on the lower strand were incubated with top1 as described in Fig. 2. Lane numbers are identical to those in Fig. 2. A, abasic sites upstream from the normal cleavage site (positions -8 to -1; see Fig. 1). B, abasic sites downstream from the normal cleavage site (positions +1 to +4). C, positions of the cleavages are indicated by the different arrows.



incubation (Fig. 5B), which is consistent with previous findings indicating that CPT reversibly inhibits the religation step of the top1 catalytic reaction (12, 20, 21). Uracil incorporation at the +1 position on the scissile strand did not affect the religation step in the absence of CPT, and reversal was complete after 5 min. In contrast, the presence of an abasic site at the same position inhibited the reversal of top1 cleavage, even when longer reversal times were used (Fig. 5B, right panel,

arrow), suggesting the formation of a suicide product.

We further tested the effect of the abasic site at the +1 position by using a modified oligonucleotide synthesized with a tetrahydrofuran abasic site analogue at this position (Fig. 6A). The same irreversible trapping of top1 was observed in the absence or presence of camptothecin (Fig. 6B). Together, these results indicate that the presence of an abasic site immediately 3' to the top1 cleavage (position +1) generates a suicide product.

DISCUSSION

The present study demonstrates that uracil incorporation, DNA mismatches, and abasic sites can have profound and contrasting effects on top1 activity, depending on their position relative to the top1 cleavage site. Modifications within the first 4 bases immediately upstream of the cleavage site (positions -1 to -4) generally suppressed top1 cleavage, whereas modifications immediately downstream (position +1) generally trapped top1 cleavable complexes.

Uracil incorporation can result in true mismatches (C:U,

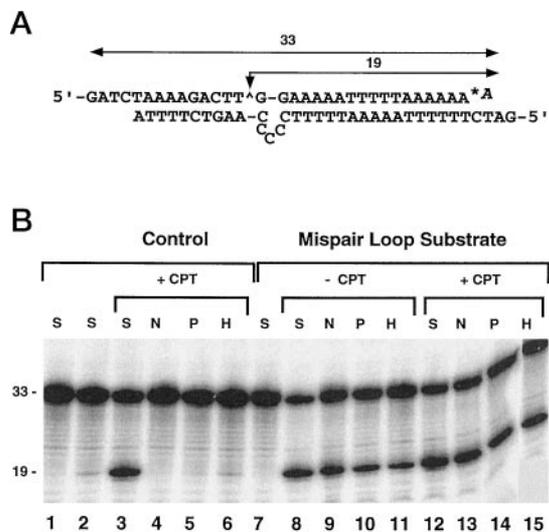
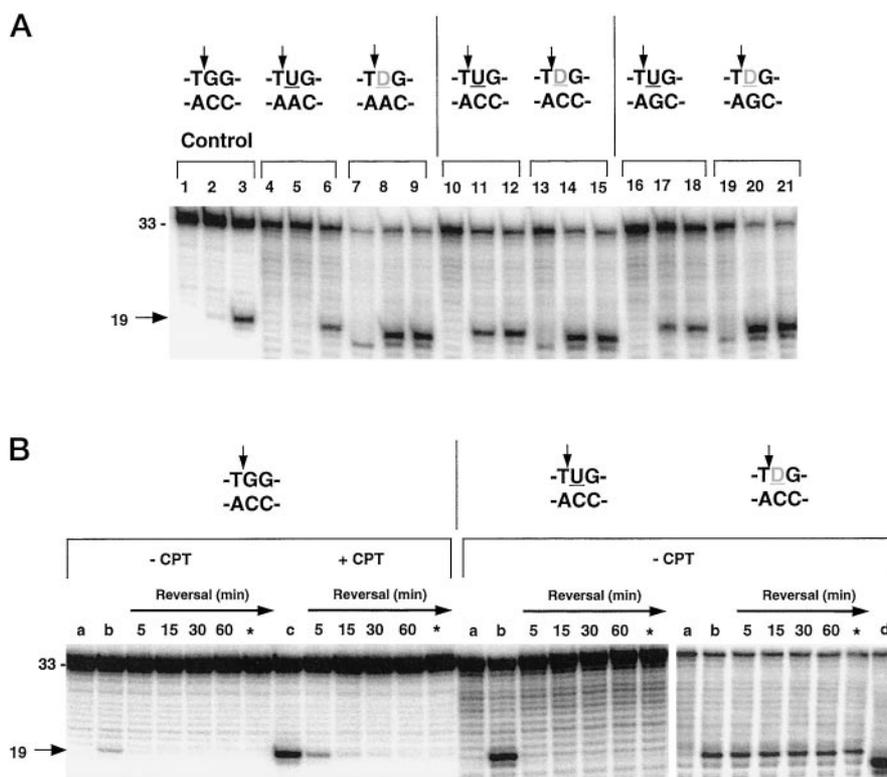


FIG. 4. Topoisomerase I cleavage activity using a displaced loop substrate. *A*, the 3'-labeled scissile strand was annealed to a nonscissile strand containing three extra cytosines between position +1 and +2, creating a loop in the double-stranded DNA substrate. *B*, substrates were reacted with top1 without (-CPT) or with 10 μ M CPT (+CPT) for 15 min at 25 $^{\circ}$ C. Lanes 1 and 8, DNA alone; lanes 2 and 8-11, +top1; lanes 3-6 and 12-15, +top1 + CPT. Reactions were stopped either with 0.5% SDS (S), 0.5 M NaCl for 1 h at 25 $^{\circ}$ C (N), proteinase K (0.5 mg/ml) for 1 h at 50 $^{\circ}$ C (P), or heat for 1 h at 50 $^{\circ}$ C (H).

FIG. 5. Topoisomerase I-mediated DNA cleavage is affected by uracil misincorporation and abasic sites in the scissile strand. *A*, the oligonucleotide duplex shown in Fig. 1 was modified at the +1 position by the replacements shown; *D*, abasic site. These substrates were reacted with top1 for 30 min at 25 $^{\circ}$ C, and reactions were stopped with 0.5% SDS. Lanes 1, 4, 7, 10, 13, 16, and 19, DNA alone; lanes 2, 5, 8, 11, 14, 17, and 20, +top1; lanes 3, 6, 9, 12, 15, 18, and 21, +top1 + 10 μ M CPT. Arrows and numbers indicate the positions and the sizes of the cleavage products. *B*, kinetics of reversal were studied with substrates containing either an uracil or an abasic site at +1 position on the scissile strand (right panel) as compared with the regular oligonucleotide (left panel). Substrates were incubated with top1 in the absence (-CPT) or presence of 10 μ M CPT (+CPT) for 15 min at 25 $^{\circ}$ C. Lane *a* in each panel, DNA alone; lane *b*, +top1; lane *c*, +top1 + 10 μ M CPT. Reactions were reversed for 5-60 min in 0.25 M NaCl at 10 $^{\circ}$ C. *, additional 15 min incubation time at 37 $^{\circ}$ C following 60 min reversal. Lane *d*, the abasic oligonucleotide was treated for 1 h at 25 $^{\circ}$ C with 10 mM NaOH (final concentration) to verify the presence of the abasic site after uracil DNA glycosylase digestion. Under these conditions, 80% of the abasic oligonucleotide was converted into the nicked product.

T:U) or abnormal base pairs (G:U or A:U). Yeh *et al.* (18) have reported that the mammalian all-type mismatch nicking enzyme forms a cleavable complex with the 3'-DNA terminus 5' to the eight possible types of DNA mismatches. They found that this mismatch nicking activity was in fact an intrinsic activity of top1 (18). Nash and Robertson (22) have also demonstrated that λ -Int topoisomerase specifically cleaves heteroduplex attachment sites containing mismatches. Consistent with these results, we found that the true mismatch U:C (Fig. 5A) resulted in enhanced top1 cleavage activity. However, we also found the same enhancing effect when base pairing was retained as in the case of the wobble base pairs G:U (Fig. 2B) or U:G (Fig. 5A). However, normal base pairing as in A:U had no effect on the enzyme activity (data not shown). These results demonstrate that abnormal DNA structure at the +1 position, immediately 3' to the top1 cleavage site, is more important than the presence of uracil *per se* at this position. The study of Yeh *et al.* (18) also demonstrated the influence of DNA sequence immediately flanking the mismatch but did not investigate mismatches at specific sites relative to the top1 cleavage sites. Our study suggests that the top1 mismatch nicking activity exhibits selectivity for base mismatches immediately downstream of the preexisting top1 cleavage site: primarily at the +1 position and to a lesser extent at the +2 position because no cleavage enhancement was observed for mismatches at positions -1, -2, or -3 (23). This indicates that top1 can act as a mismatch-nicking enzyme only at limited sites on the DNA and that such sites are primarily determined by the enzyme. We also show for the first time that a mispaired single-stranded loop (bulge) immediately 3' to the cleavage site leads to an irreversible cleavage complex.

The effects of uracil incorporation 5' to the cleavage site depended on its position and on the structure of the resulting base pair (true mismatches C:U or T:U, wobble base pair G:U, or normal A:U base pair). When uracil was close to the top1 cleavage site (positions -1, -2, or -3) and resulted in T:U or C:U mismatches, top1 cleavage was suppressed. The lack of



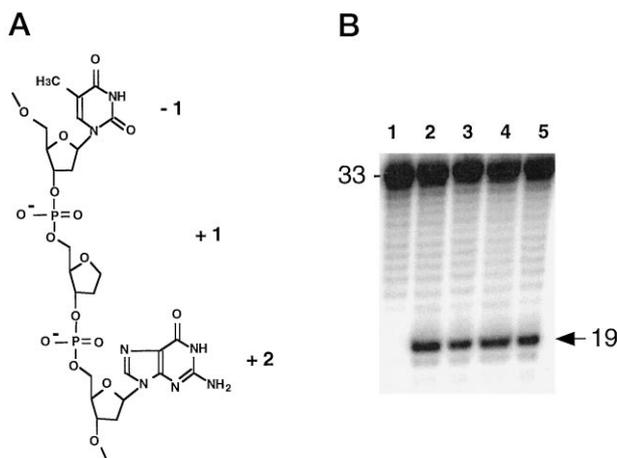


FIG. 6. Effects of tetrahydrofuran abasic oligonucleotide on top1-mediated DNA cleavage. A, structure of the tetrahydrofuran abasic site at the +1 position on the scissile strand. B, top1 reactions were performed as described in Fig. 2. Lane 1, DNA alone; lanes 2 and 3, +top1; lanes 4 and 5, +top1 + 10 μ M CPT. Reactions were stopped with 0.5% SDS (lanes 2 and 4) or 0.5 M NaCl (lanes 3 and 5) for 30 min at 25 $^{\circ}$ C.

suppression by uracil incorporation at position -4 might be due to an insignificant structural modification of the DNA because it resulted in an A:U base pair. When uracil was further upstream, at positions -6 , -7 , or -8 , also resulting in A:U base pairs, top1-induced DNA cleavage was stimulated. Thus, major groove contacts in this region upstream from the cleavage site appear to be critical for enzyme activity (23–26). This result is consistent with a previous study (27) showing that cytosine methylation at position -3 on the scissile strand suppressed top1 cleavage, whereas no such suppression was observed at position -4 . Together these observations indicate that both base pairing and major groove structure at each position upstream from the top1 cleavage site are critical for enzyme activity (23–26).

This is the first report of a top1-abasic site nicking activity that is strongly dependent on the specific position of the abasic site relative to the top1 cleavage site. A recent study of Osheroff and coworkers (16) showed that mammalian topoisomerase II exhibits nicking activity in DNA containing random chemically generated abasic sites. However, no such activity was demonstrated for top1 in their conditions using plasmid DNA. This could be attributed to the critical importance of the position of the abasic site relative to the top1 cleavage sites. Thus, the enhancing effects could have been masked by the suppressive effects in their global analysis using a large DNA fragment. An abasic site immediately 5' (position +1 on the scissile or uncleaved strand) to the cleavage site trapped irreversible top1-cleavable complexes (suicide products). Enhanced top1 cleavage in the absence of camptothecin was also observed with abasic sites at the +2 position and to a lesser extent at the +3 position. However, enhancement was less pronounced and cleavable complexes were more reversible than for the abasic sites at position +1. This is a camptothecin-mimetic effect (5). Under these conditions, the religation step afterward is hindered by the abasic site. This could be interpreted as a requirement for base pairing immediately downstream from the top1-DNA linkage to align the cleaved strand for religation.

The presence of abasic sites had opposite (suppressive) effects when they were located 5' to the top1 cleavage site, from position -1 to position -4 . This observation is consistent with the requirement of optimum enzyme-DNA contact with a tetramer oligonucleotide immediately upstream from the top1 site (23). Evidence for the close interaction of mammalian top1 with

the 4 base pairs immediately upstream from the top1 cleavage site has already been suggested from the uracil incorporation data discussed above and from previous studies demonstrating that base preferences for top1 cleavage sites is strongest for the -1 , -2 , -3 , and -4 positions (19, 21, 28, 29).

As indicated in the introduction, spontaneous formation of uracil bases by hydrolysis of cytosines and abasic sites by depurination, alkylation, or glycosylase action are among the most frequent DNA lesions, with thousands of such lesions formed daily in any given human cell (1). Loop mispairs have been implicated in mismatch repair (3). The existence of top1 cleavable complexes associated with such lesions has not been demonstrated *in vivo* to date except for the observation of Yeh *et al.* (18) that top1 may correspond to the all-type mismatch nicking enzyme. Assuming that damaged DNA can trap cleavable complexes, several scenarios can be envisaged. First, trapping of top1 cleavable complexes may play a role in DNA repair by tagging the mismatches, recruiting DNA repair enzymes, and/or arresting transcription and replication, and subsequently preventing errors. This situation might be analogous to poly(ADP-ribose) polymerase, which binds to single-strand breaks and may initiate DNA repair (30). Alternatively, top1 trapping may exert lethal effects, as in the case of top1 cleavable complexes trapped by camptothecin (5). This may represent a way for cells with damaged DNA to be tagged for programmed cell death. However, a fraction of the damaged cells may survive, and the irreversible (suicide), as well as the reversible cleavable complexes, as in the case of camptothecin, may lead to DNA recombinations (15).

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