DNA methylation is deregulated during oncogenesis. Since several major anti-cancer drugs act on topoisomerases, we investigated the effects of cytosine methylation on topoisomerase cleavage activities. Both topoisomerases I and II cleavage patterns were modified by CpG methylation in c-myc gene DNA fragments. Topoisomerase II changes, mainly cleavage reduction, occurred for methylation sites within 7 base pairs from the topoisomerase II breaks and were different for VM-26 and azatoxin. For topoisomerase I, cleavage enhancement as well as suppression were observed. Using synthetic methylated oligonucleotides, we show that hemimethylation is sufficient to alter topoisomerase I activity. Cytosine methylation on the scissile strand within the topoisomerase I consensus sequence had strong effects. Cleavage was stimulated by methylation at position -4 and was strongly inhibited by methylation at position -3 (with position -1 being the enzyme-linked nucleotide). This inhibitory effect was attributed to the presence of a methyl group in the major groove, since the transition uracil to thymine also inhibited cleavage. Altogether these results suggest an interaction of topoisomerase I with the DNA major groove at positions -3 and -4. In addition, DNA methylation may have profound effects on the activity of topoisomerases and may alter the distribution of cleavage sites produced by anticancer drugs in chromatin.

DNA methylation of cytosine in 5'-CpG-3' sequence is a vital (1) and precisely regulated phenomenon in mammalian cells (2). CpG methylation reinforces the developmental decision to shut down gene expression and is associated with a modification of chromatin structure from the euchromatin to the heterochromatin form, the latter being transcriptionally inactive (3, 4). Methylation can interfere with transcription, either directly through a reduction of the DNA binding affinity of the proteins involved in the transcription or indirectly by promoting the association of methyl-CpG-binding proteins (5, 6) which compete with the transcription factors. DNA methylation plays a role in DNA imprinting, inactivation of one X chromosome for the female and in the inactivation of some tissue-specific genes (7). DNA topoisomerases are essential for RNA transcription and DNA replication by relieving DNA torsional tension (swiveling) and resolving intertwined DNA molecules (unknotted- and de-catenation) (8-11). Topoisomerase reactions involve the trans-esterification of a DNA phosphodiester linkage to a tyrosine residue of the enzyme. The protein-associated strand breaks, called cleavable complexes, form transient gaps in the DNA backbone which allow DNA topoisomerization reactions. Topoisomerase inhibitors which are among the most active anticancer agents trap the enzyme cleavable complexes (12). The resulting lesions are probably responsible for the cytotoxicity of these drugs (13-15). Camptothecin derivatives selectively inhibit topoisomerase I (top1) (15, 16), whereas amsacrine, mitoxantrone, anthracycline, and epipodophyllotoxin derivatives inhibit topoisomerase II (top2) (16, 17).

The aim of the present study was to investigate the effects of DNA methylation on the activity of top1 and top2 using specific inhibitors, since chromatin modifications induced by methylation might change both the enzyme activities and the DNA sequence selectivity of topoisomerase inhibitors. We have examined the in vitro effects of cytosine methylation on the cleavage activity of top1 and top2 in the first exon and first intron of the human c-myc gene, and investigated more precisely the interactions of top1 with DNA using synthetic methylated oligonucleotides.

EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Enzymes—Etoposide (VP-16) and teniposide (VM-26) were obtained from Bristol-Myers Co., Wallingford, CT. 20-S-Camptothecin and amsacrine were obtained from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD). Azatoxin was provided by Dr. T. Macdonald, Department of Chemistry of Virginia, Charlottesville, VA. Drug stock solutions were made in dimethyl sulfoxide at 10 mM, and further dilutions were made in distilled water immediately before use. Human c-myc DNA inserted in pBR322, restriction enzymes, T4 polynucleotide kinase, Taq DNA polymerase, calf thymus DNA topoisomerase I, and polyacrylamide/bisacrylamide were purchased from Life Technologies, Inc., from the American Type Culture Collection (Rockville, MD), from Perkin-Elmer, or from New England Biolabs (Beverly, MA). [γ-32P]ATP and γ-32P-cordycepin were purchased from DuPont NEN. Top2 was purified from mouse leukemia L1210 cell nuclei as described previously (16). Oligonucleotides were prepared using a 982 DNA synthesizer from Applied Biosystem (ABI, Foster city, CA) and purified using oligonucleotide purification cartridges (ABI). Phosphoramidite of modified deoxynucleotides were purchased from Glen Research (Sterling, VA).

Preparation of End-labeled DNA Fragments by PCR—Three sets of labeled DNA fragments were prepared from the human c-myc gene by PCR. A 254-base pair DNA fragment from the first intron was prepared

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1 The abbreviations used are: top1, DNA topoisomerase I; top2, DNA topoisomerase II; CPT, camptothecin; MeC, methylcytosine; MIF, c-myc intron factor; PCR, polymerase chain reaction; VM-26, teniposide; VP-16, etoposide.
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**FIG. 1.** DNA methylation by SssI methylase affects top1 and top2 cleavage sites in the c-myc first intron. DNA fragments were prepared by PCR using one primer labeled with $^{32}$P at the 5' terminus. Cleavage reactions were at 37 °C for 30 min with purified enzymes and stopped by adding SDS and proteinase K. DNA electrophoresis was in 7% denaturing acrylamide gels (7 M urea) in TBE buffer. Treatments are indicated above each lane, including DNA methylation at CpG sequences using SssI methylase (+ and -); CPT: top1 + 10 μM CPT; VM-26: top2 + 10 μM VM-26; FA: purine ladder obtained after formic acid reaction. Numbers on the right correspond to genomic position of the nucleotide covalently linked to top2 and letters on the left to top1 cleavage sites. Stars correspond to sites for the SssI methylase. A, c-myc sense strand labeled + VM-26; FA: purine ladder obtained after formic acid reaction. Numbers on the right correspond to genomic position of the nucleotide at position 3035. CpG methylation induced a top1 site, site C, in the presence of CPT. B, c-myc antisense strand labeled at position 3035 and 3288, with numbers referring to GenBank genomic positions, as described previously (19). A 481-base pair DNA fragment from the junction between the first intron and first exon was prepared between positions 2671 and 3073 using oligonucleotides: 5'-TGCCGCATCCACGAAACTMC-3' as sense primer and 5'-GAATTCTTCCAGTTTACCCCGG-3' as antisense primer. A 481-base pair DNA fragment from the first exon was prepared between positions 2265 and 2745 using oligonucleotides: 5'-GCATCTCTCCCGGTAACTTCCGCG-3' as sense primer and 5'-TCCTTCTGTCGGGTGGTTAAGTTCG-3' as antisense primer. Single end labeling of these DNA fragments was obtained by 5'-end labeling of the adequate primer oligonucleotide (19). Approximately 0.1 μg of the c-myc DNA that had been restricted by SmaI and PvuII (fragment 2265-2745 which contained the exon 1), XhoI and XbaI (fragment 2671-3073 corresponding to the junction exon I-intron I and fragment 3035-3288 corresponding to the first part of intron I) was used as template for the PCR (19).

**Enzymatic Methylation of c-myc DNA Fragments**—PCR products prepared for the mapping of the topoisomerase cleavage sites were methylated for 2 h using 10 units of SssI methylase (New England Biolabs, Beverly, MA). The unmethylated DNA control was incubated in parallel without methylase. DNA samples were purified by ethanol precipitation after two cycles of protein extraction using Stratagene Resin™ (Stratagene, La Jolla, CA), respectively. Duplex oligonucleotides derived between positions 3035 and 3288, with numbers referring to GenBank genomic positions, as described previously (19). A 481-base pair DNA fragment from the junction between the first intron and first exon was prepared between positions 2671 and 3073 using oligonucleotides: 5'-TGCCGCATCCACGAAACTMC-3' as sense primer and 5'-GAATTCTTCCAGTTTACCCCGG-3' as antisense primer. A 481-base pair DNA fragment from the first exon was prepared between positions 2265 and 2745 using oligonucleotides: 5'-GCATCTCTCCCGGTAACTTCCGCG-3' as sense primer and 5'-TCCTTCTGTCGGGTGGTTAAGTTCG-3' as antisense primer. Single end labeling of these DNA fragments was obtained by 5'-end labeling of the adequate primer oligonucleotide (19). Approximately 0.1 μg of the c-myc DNA that had been restricted by SmaI and PvuII (fragment 2265-2745 which contained the exon 1), XhoI and XbaI (fragment 2671-3073 corresponding to the junction exon I-intron I and fragment 3035-3288 corresponding to the first part of intron I) was used as template for the PCR (19).

**Enzymatic Methylation of c-myc DNA Fragments**—PCR products prepared for the mapping of the topoisomerase cleavage sites were methylated for 2 h using 10 units of SssI methylase (New England Biolabs, Beverly, MA). The unmethylated DNA control was incubated in parallel without methylase. DNA samples were purified by ethanol precipitation after two cycles of protein extraction using Stratagene Resin™ (Stratagene, La Jolla, CA), respectively. Duplex oligonucleotides derived

**Sequence 1:**

S: 5' GCTGGGGGTTGGGCTTTGGGCTGGGACGAAAAGCCCCTTGCATCTGAGCTCCTTG

**Sequence 2:**

A: 3' CGACCCCCAAGAAAGCCGCTTCTCTTCCGGGAACTCGACTCCGAGGAACCTCA

| 3221 | 3237 | 3240 | 3256 |
|----|----|----|----|
| SEQUENCE 1: | S: 5'-GCTGGGGGTTGGGCTTTGGGCTGGGACGAAAAGCCCCTTGCATCTGAGCTCCTTG |
| SEQUENCE 2: | A: 3'-CGACCCCCAAGAAAGCCGCTTCTCTTCCGGGAACTCGACTCCGAGGAACCTCA |

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FIG. 2. Relationship between VM-26-induced cleavage sites and SsI DNA methylation sites. The gel presented in Fig. 1A was quantified using a betascope analyzer. Profiles of the lanes for the top2 cleavage sites induced in the presence of VM-26 are shown. Upper profile corresponds to DNA pretreated with SsI methylase (Me). The boxed sequence corresponds to a palindrome containing a prominent cleavage site at the beginning of the first intron. It is also part of the MIF-2 binding site (24). Numbers correspond to genomic position of the nucleotide covalently linked to the top2, and stars correspond to SsI methylase sites. The cleavage sites induced in the presence of VP-16 were identical except for the faint band at position 2744 indicated by (#); FA, purine ladder obtained after formic acid reaction. Arrowheads indicate the main modifications induced by DNAmethylation (upper three lanes). Numbers correspond to the genomic position of the nucleotide covalently linked to the top2 and stars to SsI methylase sites.

FIG. 3. Differential effect of DNA methylation on top2 cleavage sites. Top2 cleavage reactions were run as described in the legend to Fig. 1 using the sense DNA strand. A, top2 + 100 μM azatoxin; V, top2 + 100 μM VP-16; C, top2 control lane for the methylated DNA (the unmethylated DNA control was identical except for the faint band at position 2744 indicated by #); FA, purine ladder obtained after formic acid reaction. Arrowheads indicate the main modifications induced by DNA methylation (upper three lanes). Numbers correspond to the genomic position of the nucleotide covalently linked to the top2 and stars to SsI methylase sites.

from the c-myc first intron and starting at the genomic position 3221 (GenBank numbering) were modified at the underlined positions. The sense strand (S) is indicated S m when methylated at position 3237. The antisense strand (A) is indicated A m when methylated at position 3238. Preparation of 3'-labeled oligonucleotides on the scissile sense strand (upper strand above) was performed as described previously (20).

Topoisomerase-induced DNA Cleavage Reactions—DNA fragments were equilibrated with or without drug in 1% dimethyl sulfoxide, 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 2 mM dithiothreitol, 0.1 mM NAD, 1 mM EDTA, 1 mM ATP, and 15 μg/ml bovine serum albumin for 5 min before addition of purified top2 (40–70 ng) in 20-μl final reaction volume. For reactions with top1, ATP was omitted, and 7–30 units of enzyme were used. Unless otherwise specified, reactions were performed at 37 °C for 30 min. Reactions were stopped by adding 1% sodium dodecyl sulfate (SDS) and 0.4 mg/ml proteinase K (final concentrations) followed by an additional incubation at 50 °C for 30 min. Samples were ethanol-precipitated before separation of the topoisomerase-cleaved fragments on denaturing polyacrylamide gels.

Electrophoresis and Data Analysis—Sequencing gels were made of 7 or 16% polyacrylamide in 1 x TBE buffer (90 mM Tris borate, 2 mM EDTA, pH 8.3). Electrophoresis was at 2500 V (60 watts) for 2–5 h. Gels were quantified using a Phosphorlmager (Molecular Dynamics, Sunnyvale, CA) or a Betascope 603 Blot Analyzer (Betagen Inc., Waltham, MA). For oligonucleotides, the percentage of cleavage at a given site was calculated as the radioactivity signal at this site minus the radioactivity signal in the oligonucleotide control at the corresponding position (background counts) divided by the total amount of radioactivity signal in the lane.

RESULTS

Effect of CpG Methylation on Drug-induced Top2 Cleavage Sites in c-myc DNA—Drug-induced top2 cleavage sites were mapped in the human c-myc gene between the P1 promoter and the beginning of the first intron. This region is important for the c-myc gene transcription regulation and contains strong
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Topoisomerase cleavage sites in vivo (21). It is also rich in CpG sequences that can be methylated in vitro using SssI methylase. Fig. 1 shows the top1 and top2 cleavage sites obtained in a 5'-region of the first intron on the sense and the antisense strands. Top2 sites were mapped and designated by the genomic position of the covalently linked to the top1 and stars to methylation sites.

Methylation on Drug-induced Top1 Cleavage Sites—Top1 cleavage sites induced in the presence of the CPT were affected by methylation sites nearby (Fig. 1). However, the 5'-labeling used in the experiments shown in Fig. 1 did not allow precise mapping of top1 cleavage sites. Indeed, despite proteinase K digestion, top1 polypeptides remain covalently linked to the 5'-DNA termini. As a result, the electrophoretic migration of the DNA fragment is retarded. Therefore, cleavage sites were labeled with letters. Reductions of the cleavage intensities were seen (Fig. 1A, site E, and several sites in the exon 1; results not shown). Interestingly, CpG methylation could also induce top1 cleavage sites (Fig. 1A, site C). This induction was unexpected regarding the association of top1 activity with the DNA transcription and the inhibitory effect of DNA methylation on transcription. Further investigations were performed using methylated oligonucleotides modified during the synthesis by incorporation of methyleytoine. This approach allows, at will, MeC positioning around the cleavage site.

Mapping of sites B and C (this latter induced by CpG methylation) was performed using an oligonucleotide prepared from c-myc genomic positions 3221-3278. MeC were included in the CPG sequence at position 3237 on the sense strand and 3238 on the antisense strand, and the oligonucleotide was 3'-end-labeled with ^32P-cordycepin on the sense strand. Genomic positions of top1 cleavage site were determined by comparison with a pure lane bearing a similar 5' terminus. Site B was mapped at position 3256 and site C at position 3240 (Fig. 4). Utilization of synthetic oligonucleotides also enabled us to study the effect of hemimethylation. We compared CPT-induced cleavage in unmethylated, hemimethylated, and fully methylated oligonucleotides (Fig. 5). Site 3240 was barely visible in the unmethylated DNA (SA), and cleavage was most enhanced by full methylation on the sense and antisense strand (S,A,A). Hemimethylation of the scissile strand (S,A) had a greater enhancing effect than hemimethylation of the uncleaved strand (S,A,m).

Kinetics of Formation and Resealing of the Top1 Sites 3240 and 3256—Previous studies have suggested an inverse relationship between the stability of a given cleavage site and its intensity (26). Accordingly, the induction of top1 site 3240 after cytosine methylation may result from its greater stability. This hypothesis was tested using unmethylated, fully methylated, and hemimethylated oligonucleotides (Fig. 6). Cleavage sites were allowed to form at room temperature for 5 min before their resealing was initiated by adding sodium chloride at 0.35 M final concentration. The rapid kinetics of resealing for site 3256 is characteristic of most top1 cleavage sites (26). Unexpectedly, the kinetics of resealing for site 3240 was extremely slow regardless of the methylation status (Fig. 6B). Kinetics of formation of top1 cleavage was then investigated in the unmethylated (SA) and fully methylated (S,A,A) oligonucleotides (Fig. 7, A and B). Reactions were performed at 10 °C to slow down reaction kinetics and to allow more accurate quantifications. Site 3256 was induced very rapidly in both oligonucleotides, whereas site 3240 was only induced in the S,A,A oligonucleotide. Its formation kinetics was slower than that of site 3256 (Fig. 7B). Therefore, the effect of methylation on site 3240 appeared to be essentially an inductive effect rather than an inhibition of religation.

A. Tanizawa and Y. Pommier, unpublished observation.
FIG. 6. Kinetics of resealing of top1 cleavage sites 3240 and 3256 as a function of DNA methylation. Top1 cleavage was induced in the presence of 10 μM CPT for 15 min at 22 °C. Resealing was induced by adding 0.35 M (final concentration) sodium chloride at time 0. Reactions were stopped at the indicated times by adding SDS and proteinase K. A, gel autoradiograph. B, the gel shown in A was quantified using a PhosphorImager. The percentage of cleavage at each site was calculated for each time point as indicated under "Materials and Methods." Open and filled symbols correspond to cleavage sites 3256 and 3240 (induced by cytosine methylation), respectively. Circles are for the unmethylated DNA (SA: ○), triangles are for the DNA hemimethylated on the scissile sense strand (SmA: △), and squares are for the fully methylated DNA (SmAAm: ●).

Relationship between the Position of the Methylcytosine Group around the Top1 Cleavage Site and Top1 Cleavage Intensity—We took advantage of the presence of 4 consecutive cytosines 5' to the 3256 top1 site to test the effect of a MeC at positions -3 to -6 (Fig. 8). These hemimethylated substrates are not physiological, but they allow further study of the DNA-enzyme interactions. MeC at position -3 strongly reduced cleavage (by 95%), whereas MeC at position -4 increased cleavage (by 50%), consistent with the effect of hemimethylation at position -4 from the cleavage site 3240. Methylation at positions -5 or -6 had a modest inhibitory effect (around 30%). Mutations at positions +1 on the scissile strand or +1 and +2 on the uncleaved strand of an oligonucleotide containing the strong top1 site isolated in the tetrahymena rDNA (27) did not reveal any cleavage alteration when C was replaced by MeC at these positions (data not shown). Since T-1 is strongly preferred for top1 cleavage (20, 26), MeC at positions -1 and -2 were not investigated. Taken together, our results indicate that cytosine methylation within the top1 consensus sequence (the 4 bases upstream the cleavage site) has profound effects on top1 cleavage activity.

The Presence of a Methyl Group in the Major Grove at Position -3 Appears to Suppress Top1 Cleavage—To confirm that the addition of a methyl group at position -3 was responsible for the inhibitory effect on top1 cleavage, we investigated the effect of different methylated pyrimidines. The base at position 3254 was changed from cytosine (C) to MeC and from uracil (U) to thymine (T). Because the introduction of a T or a U required the modification of the complementary strand, the U versus T and C versus MeC oligonucleotides should be compared (Fig. 9). Interestingly, the addition of a methyl group on the cytosine and the uracil was similarly responsible for inhibition of cleavage at site 3256. Therefore, the presence of a methyl group in the major groove at position -3 was responsible for the reduction of the 3256 top1 cleavage site. Additionally, the introduction of
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Fig. 7. Kinetics of formation of top1 cleavage sites 3240 and 3256 as a function of DNA methylation. The top1 kinetics of association was compared at sites 3240 and 3256 in the unmethylated (SA) and in the fully methylated (SmAm) oligonucleotides. Reactions were performed at 10°C in the presence of 10 μM CPT and were stopped at the indicated times with SDS and proteinase K. A, gel autoradiograph. B, the gel shown in A was quantified as in Fig. 6. Open and filled symbols correspond to cleavage sites at positions 3256 and 3240, respectively. Circles are for the unmethylated DNA (SA: ●), and squares are for the fully methylated DNA (SmAm: ■).

uracil or thymine shifted the top1 site to position 3255. These results are consistent with the top1 consensus sequence that shows an exclusion of T at position −3 (20, 28).

DISCUSSION

Topoisomerases interact preferentially with specific DNA sequences. Mapping of topoisomerase sites was first performed in vitro using purified DNA and enzymes in many different systems such as the SV40 DNA (23, 26, 29, 30), the c-myc gene (21, 31), and the rDNA (32). More recently, topoisomerase sites were also mapped in vivo using SV40 (28), the c-myc gene (21, 33, 34), the fos gene (35), the rDNA (32), the histone genes (36), and the heat shock genes (36, 37). Comparison of in vitro and in vivo cleavage sites mapping shows differences. Mainly in vivo sites are less numerous and correspond to a subset of the in vitro sites (38, 39). Remarkably, when mapping data are available at the nucleotide level, in vitro sites are visible in vitro, and, at least for top1, the same consensus sequences have been obtained in cellular and purified systems (20, 26, 28). The quantitative reduction of topoisomerase cleavage sites observed in vitro may have a variety of origins related to chromatin structure. Among the possible factors are the gene transcription activity (37, 40–43), the presence of nucleosomes, which protects the bound DNA and reinforces the cleavage activity in the linker region (38), and the DNA modifications, such as DNA glycosylation in phase T4 (44). In the present work, we demonstrate that DNA methylation at CpG sequences can alter the topoisomerase-DNA interactions and may contribute to the cleavage differences observed in vitro and in vivo.

We find that cytosine methylation alters CPT-induced top1 cleavage sites in the c-myc DNA proto-oncogene (GenBank identification for the human c-myc oncogene is H5MYCC) as well as in synthetic oligonucleotides. The most important effects resulted from the methylation on the 5'-flank of the cleavage site (Fig. 8), in agreement with the localization of essential bases for top1 binding (20, 26, 27). Cleavage was enhanced by MeC at position −4 on the scissile strand and to a lesser extent by MeC at position −3 on the uncleaved strand. In fully methylated CpG, the two methyl groups lay side by side at about 7.6 Å from each other symmetrically from the longitudinal major groove axis (45). Therefore, they may interact with a common hydrophobic pocket of top1. Enhancement of DNA-binding protein activity in the presence of MeC has also been reported for the human immunodeficiency virus integrase (46) and for the MeCpG binding proteins (5). By contrast, N7 guanine methylation at position −3 on the uncleaved strand inhibits top1 cleavage activity (27). Also, top1 cleavage was inhibited by cytosine methylation at positions −3, −5, or −6 from the cleavage site on the scissile strand. The most prominent effect was at position −3 (Fig. 8), within the consensus sequence for top1 DNA binding (20, 26, 27). It is unlikely that CpG methylation in the oligonucleotides could alter DNA structure (45) and facilitate a B to Z transition, since CpG methylation was neither in alternating CpG repeats nor in proximity of poly(A) (47). Rather, the effect of CpG methylation is a direct consequence of the addition of a methyl group in the major groove, because cleavage inhibition was also observed after the replacement of uracil by thymine at position −3 on the scissile strand (Fig. 9). The importance of DNA major groove interactions for top1 binding has recently been reported for the vaccinia enzyme (48). Hence, top1 interacts closely and precisely with the DNA major groove at positions −3 and −4 upstream from the cleavage site. Methylation at position −3 of the scissile strand suppresses cleavage, whereas methylation at −4 increases cleavage.

The present results also show that the presence of T at position −3 on the scissile strand strongly suppressed top1 cleavage and shifted the cleavage site by one nucleotide in the upstream direction (Fig. 9). These data are consistent with those obtained by base sequence analysis in vitro (20, 26) or in vivo (28). Additionally, MeC and T share the same positive and negative effects on top1 cleavages. Their methyl groups are identically positioned in the major groove of B-DNA. Consequently, methyl group hindrance is responsible for the strong inhibitory effect of T or MeC at position −3. In contrast, the same methyl group at position −4 on the scissile strand (Figs. 5 and 8) or at position −3 on the uncleaved strand (Fig. 5) in-
Fig. 8. Effect of the position of cytosine methylation on top1 cleavage. Cytosines on the 5' side of the cleavage site 3256, 3-6 bases away from that cleavage site, were individually replaced by MeC 3' bases away from that cleavage site 3256. These results suggest that DNA-top1 interactions in the consensus sequence must be involved in transcription regulation (site 3145 in the first intron within the MIF-2 binding sequence (24), Fig. 2; and P2 promoter (21)). Hence, alteration of the topoisomerase functions may contribute to the effects of CpG methylation on transcription.

Fig. 9. Effect of a methyl group at position -3 on top1 cleavage. Cytosine 3254 was replaced by MeC, uracil (U), or thymine (T); for the two substitutions, base complementarity was preserved by performing simultaneously a transition G to A on the uncleaved strand. Reactions were performed as described in the legend to Fig. 8. Lanes 1, DNA alone; lanes 2, + top1 alone; lanes 3, + top1 + 10 μM CPT.

creases the top1-induced DNA cleavage. These results suggest that DNA-top1 interactions in the consensus sequence must take place in the major groove at least at -3 and -4 positions on both strands.

Current mechanisms for transcription inhibition by CpG methylation involve the fixation of specific proteins with (6) or without (5, 49) DNA sequence selectivity which interferes with the binding of transcription factors. Alternatively, DNA methylation may directly interfere with the DNA binding of transcription factors such as the c-myc-max heterodimer (50), the NF-κB factor on human immunodeficiency virus-long terminal repeat (HIV-LTR) (51), the retinoblastoma binding factor 1 (52), the cAMP response element-binding protein (53), AP-2 (54), and E2F (55). Hemimethylation can be sufficient to inhibit the binding of transcription factors (56) and cellular transcription (57). CpG hemimethylation was also sufficient to affect top1 cleavage activity (Fig. 5). Top2 cleavage activity was also altered by CpG methylation, including at palindromes that are likely to be involved in transcription regulation (site 3145 in the first intron within the MIF-2 binding sequence (24), Fig. 2; and P2 promoter (21)). Hence, alteration of the topoisomerase functions may contribute to the effects of CpG methylation on transcription.

Topoisomerase II inhibitors differ in their DNA sequence selectivity (11) which has been attributed to the preferential stacking of drugs with the bases that flank the cleavage sites (58). Among topoisomerase II inhibitors, activity differences observed in the clinics might be related to differences of top2-induced DNA genomic lesions. Consequently, modifications of topoisomerase cleavage patterns by DNA methylation might have profound effects on drug activity because methylation status changes with cell differentiation, aging (3, 59) and oncogenesis (60, 61). Since alterations of gene methylation might be reversed after efficient therapy (62, 63), and gene methylation might be involved in the evolution to chemotherapy resistance (64-66), we postulate that methylation might modulate the tissue selectivity and efficiency of topoisomerase inhibitor cytotoxicity.

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