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Induction of cleavage in topoisomerase I c-DNA by topoisomerase I enzymes from calf thymus and wheat germ in the presence and absence of camptothecin

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ABSTRACT

In this study, we further examined the sequence selectivity of camptothecin in mammalian topoisomerase I cDNA from human and Chinese hamster. In the absence of camptothecin, almost all the bases at the 3′-terminus of cleavage sites are T for calf thymus and wheat germ topoisomerase I. In addition, wheat germ topoisomerase I exhibits preference for C (or not T) at −3 and for T at −2 position. As for camptothecin-stimulated cleavage with topoisomerase I, G (or not T) at +1 is an additional strong preference. This sequence selectivity of camptothecin is similar to that previously found in SV40 DNA, suggesting that camptothecin preferentially interacts with topoisomerase I-mediated cleavage sites where G is the base at the 5′-terminus. These results support the stacking model of camptothecin (Jaxel *et al.* (1991) *J. Biol. Chem.* 266, 20418–20423). Comparison of calf thymus and wheat germ topoisomerase I-mediated cleavage sites in the presence of camptothecin shows that many major cleavage sites are similar. However, the relative intensities are often different. One of the differences was attributable to a bias at position −3 where calf thymus topoisomerase I prefers G and wheat germ topoisomerase I prefers C. This difference may explain the unique patterns of cleavage sites induced by the two enzymes. Sequencing analysis of camptothecin-stimulated cleavage sites in the surrounding regions of point mutations in topoisomerase I cDNA, which were found in camptothecin-resistant cell lines, reveals no direct relationship between DNA cleavage sites *in vitro* and mutation sites.

INTRODUCTION

DNA topoisomerase I (topo I) has been reported to have an important role in DNA replication and RNA transcription (1, 2). By inducing transient single-strand breaks, it allows one strand to go through the break point, relaxes torsional tension and reseals the break (3). Addition of a denaturing agent stops this catalytic reaction and traps the intermediate which has been referred to as the cleavable complex (1, 2). Several factors, such as histone or salt condition, have been reported to affect the interaction between topo I and DNA (4–6). *In vitro* sequencing analysis of topo I-induced cleavage sites revealed that topo I binds to DNA non-randomly. Although base sequence may not be the only determinant of topo I-mediated DNA cleavage sites, several consensus sequences have been reported by using vaccinia virus, Tetrahymena, wheat germ, mouse, rat, calf and human topo I (7–15). Reported consensus sequences are not definitively consistent. Discrepancies may be due to differences in source of topo I, reaction conditions, substrate DNA and data analysis.

Camptothecin (CPT) is a plant alkaloid which inhibits the rejoining step of topo I activity (16, 17). Since CPT stabilizes cleavable complexes, base sequence preferences of topo I-mediated cleavage sites in the presence of CPT have been compared with those in the absence of drug (4, 10, 12, 15, 18–20). Based on the effect of CPT on the intensity of cleavage sites, topo I-mediated DNA cleavage sites can be classified into three groups: sites which are enhanced by CPT, sites which disappear and sites which show no alteration of intensity in the presence of CPT. One of the difficulties in investigating sequence selectivity of drugs is how to analyze the data. We have developed a probability analysis method for sequence selectivity and showed preferential base sequences for CPT as well as topoisomerase II inhibitors (21). Recent studies on base preference at the sites of CPT-stimulated cleavage by topo I indicate a strong bias for the bases immediately flanking the cleavage sites in SV40 DNA and synthesized oligonucleotides (15). CPT preferentially enhances topo I-mediated cleavage sites which have G at the 5′-terminus of break. Interestingly, two molecular forms from calf thymus topo I exhibit substantially different site specificity once CPT has been added, although cleavage pattern in the absence of CPT were similar (22). The position of the catalytic tyrosine has been identified in several topo I enzymes (2, 3). However, it has not been elucidated which parts of the enzyme

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is responsible for recognition of the base sequence and interaction with CPT.

To confirm the preferential base sequences for CPT which had been found in SV40 DNA using mouse leukemia topo I (15), we chose human and Chinese hamster topo I cDNAs as substrate DNA for cleavage reactions. Since base homology between the human and Chinese hamster topo I cDNAs is 90%, one of the advantages of these substrates is that it is possible to compare the intensity of cleavages sites where a few bases are different between human and Chinese hamster cDNAs. Another advantage is that site analysis of CPT-induced cleavage on topo I cDNA might provide some insights into relationship between drug-induced cleavage sites *in vitro* and mutation sites in topo I cDNAs from CPT-resistant cell lines. At present, three cell lines have been reported to have base mutations in topo I cDNA. Two of them are human cell lines in which CPT-resistance was achieved after stepwise increase of CPT-11 in the culture medium [CPT-K5 (23), PC-7/CPT (24)]. CPT-K5 and PC-7/CPT have two and one mutation in topo I cDNA when compared to their parental cell lines, respectively (25, 26). The other cell line (DC3F/C-10) was selected in Chinese hamster cells by a 10-day exposure to 1 μM CPT after mutagenic treatment with ethylmethane sulfonate (27, 28). It is unknown whether these mutation sites are hot spots for CPT-induced cleavage or not. Since CPT stabilizes cleavable complex, persistent existence of cleavable complex may work as a trigger for mutations.

The present study was undertaken in order to investigate further the sequence selectivity of CPT on topo I-mediated cleavage sites by using mammalian top 1 cDNA (from human and Chinese hamster) as substrates. We examined the DNA sequence selectivity of calf thymus and wheat germ topo I in the presence and absence of CPT. We analyzed the base preference of CPT by alignment of cleavage sites and probability analysis and by comparison of cleavage intensity at corresponding sites in human and Chinese hamster cDNAs which differ by a few bases. We also compared the CPT-induced cleavage sites and mutation sites in topo I cDNA from CPT-resistant cell lines to determine if the mutation sites are relevant to CPT-stimulated cleavages sites *in vitro*.

MATERIALS AND METHODS

Materials, enzymes, and drug

Partial Chinese hamster topo I cDNA was cloned in pBluescript SK(−) (Stratagene, La Jolla, CA) (28). Human topo I cDNA (T1B) was kindly provided by Dr Earnshaw (Johns Hopkins University) (29). Restriction endonucleases and proteinase K were obtained from New England Biolabs (Beverly, MA) and Merck (Darmstadt, Germany), respectively. Klenow fragment of DNA polymerase I, T_4 polynucleotide kinase, S_1 nuclease and calf thymus topo I were obtained from Gibco-Bethesda Research Laboratories (Gaithersburg, MD). Wheat germ topo I was purchased from either Epicentre Technologies (Madison, WI) or Promega Corporation (Madison, WI). CPT from Sigma Chemical Company (St Louis, MO) was dissolved in dimethyl sulfoxide at 10 mM and kept at −70°C. [α-^{32}P]deoxynucleotides were from Du Pont-New England Nuclear Research Products (Boston, MA).

Radiolabeling

In most cases, ^{32}P-end labeled topo I cDNA fragments for cleavage assays were prepared as follows. Topo I cDNA was first digested with one of the restriction enzymes listed in Figure 1. The 3′-ends of the restricted fragments were labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]deoxynucleotide (6000 Ci/mmol). The DNA was incubated with the second restriction endonuclease and separated by agarose gel electrophoresis. Labeled DNA was extracted from the gel and used for cleavage assay.

DNA cleavage assays

Reactions were performed in 30 μl of reaction buffer [10 mM Tris−HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1.7 mM dithiothreitol and 15 μg/ml bovine serum albumin]. DNA was incubated with 30 units of topo I in the presence and absence of 10 μM CPT at 37°C for 15 min, unless otherwise indicated. Reactions were stopped by adding sodium dodecyl sulfate and proteinase K (final concentration; 1% and 100 μg/ml, respectively) and further incubated at 37°C for an additional 30 min.

Phosphorylation of 5′-DNA termini generated by topo I was performed as follows. Topo I + camptothecin reaction products were by ethanol precipitated, dried and resuspended in T_4 polynucleotide kinase buffer containing T_4 polynucleotide kinase (New England Biolabs, Beverly, MA) and unlabeled ATP (0.45 mM) for 30 min at 37°C. Reactions were stopped by adding EDTA (25 mM final concentration) at heat treatment at 70°C for 10 min.

For agarose gel analysis (see Figure 2), DNA was purified by phenol and chloroform extractions, and precipitated with ethanol. Resuspended DNA was treated with S, nuclease to convert single-stranded DNA breaks to double-stranded breaks and electrophoresed in 1.2% agarose gel (30). Autoradiographies of the gels were scanned with a Beckman DU-8B spectrophotometer in order to determine the positions of cleavage sites and quantify the intensity of each signal (18, 31).

For sequencing gel analysis, samples were precipitated with ethanol, resuspended in Maxam−Gilbert loading buffer and run into 6% wedge-shaped denaturing polyacrylamide gels. Cleavage sites were determined by comparison with Maxam−Gilbert sequencing markers as described previously (18).

Statistical analysis

Topo I-induced cleavage sites on the autoradiographies of the sequencing gels were classified into three groups according to their relative intensities (strong, moderate and weak). For a given autoradiography, most intense sites were classified as strong, sites just clearly detectable above background as weak, and sites of intermediate intensity as intermediate. The bases at the 3′- and 5′-termini of the analyzed break points were numbered −1 and +1, respectively.

The nucleotide sequence bias for topoisomerase sites resides in the difference between the number of instances of each base observed at each position relative to the cleavage site and the number of instances of that base to be expected from chance occurrence. We estimate this difference taking into account any bias of nucleotide triplet frequencies that may exist in the DNA being analyzed (because of a typographical error in one of the equations as printed in reference 21, the relevant equations are given below, and we review the basis of this method of calculation). Our method of calculation takes into account bias for each type of base relative to the identity of the next base on each flank. The probability of finding a base x between upstream base a and downstream base b is given by

$$f(a,x,b) = n(a,x,b) / \sum_{i=1}^{4} n(a,i,b) \qquad (1)$$

$n(a,x,b)$ is the number of occurrences of base x between bases a and b throughout the region of DNA analyzed. The denominator in equation (1) is the total occurrences the pairs a and b independent of the central base (the 4 possibilities, A, C, G or T, are assigned the integers $i = 1, 2, 3$ or 4, respectively). The matrix of 64 values of $f(a,x,b)$ was calculated separately for each strand of the topo I gene; these values showed some substantial strand-dependent differences (Table 1). In this way, the cleavage sites observed in each strand were evaluated relative to the expected base triplet patterns for that strand.

The first approximately 1000 base pairs of the topo I gene constitutes a CpG island with markedly different base frequencies compared to the remainder of the gene. The DNA fragments used in the current work did not include the first 1000 base pairs. We calculated the $f(a,x,b)$ matrix with or without exclusion of the first 1000 base pairs, and found only minor consequent differences.

The expected number of occurrences of base x at a given position relative to the point of cleavage, $e(x)$, is the sum of the $f(a,x,b)$ values for the set of cleavage sites analyzed. The probability of finding base x by chance at a given position relative to the point of cleavage in the set of cleavage sites is

$$p = e(x)/n \qquad (2)$$

where n is the total number of cleavage sites in the set analyzed.

The probability, P, of chance occurrence of a deviation from expectation greater or equal to that observed was calculated as follows. Let m be the number of sites that are found to have a given base at some position relative to the point of cleavage, the expected number at that position being pn.

If $m > pn$, the chance occurrence of m or more instances is

$$P = \sum_{i=m}^{n} \frac{n!}{i!(n-1)!} p^{i} (1-p)^{n-i} \qquad (3a)$$

If $m < pn$, the chance occurrence of m or fewer instances is

$$P = \sum_{i=o}^{m} \frac{n!}{i!(n-i)!} p^{i} (1-p)^{n-i} \qquad (3b)$$

Factorials were computed to 10-digit precision as their logarithms using the Lanczos approximation (32).

RESULTS

First, we examined the overall topo I-mediated cleavage patterns in human topo I cDNA. In order to radiolabel sense and antisense strands of human topo I cDNA, the cDNA (T1B) in pUC9 plasmid was first digested with EcoRI and labeled at both 3′-ends with [α-^{32}P]dATP by Klenow fragment of DNA polymerase I. The 3′-end labeled DNA was redigested with either BsaHI or BsaBI and purified after agarose gel electrophoresis. The long fragments of the EcoRI/BsaHI and EcoRI/BsaBI restriction digests corresponded to cDNA whose sense and antisense strands were radiolabeled, respectively (see Figure 1). Figure 2 shows calf thymus topo I and wheat germ topo I-mediated cleavage patterns in human topo I cDNA in the absence and presence of 10 μM CPT. Panel A shows typical cleavage patterns in the topo I cDNA using the 3′-end-labeled sense strand. Panel B corresponds to a computation of the densitometer scanning of autoradiography films (Panel A and data not shown) for CPT-induced DNA cleavage. Several major cleavages were induced in the same regions by both topo I enzymes. However, spectrophotometric analysis demonstrated that relative intensities of each peak were clearly different, for example intensity of peak E was very strong in the case of wheat germ topo I while it was relatively weak in the case of calf thymus topo I. The densitometer scanning of cleavage reactions and autoradiography performed with the antisense labeled-strand yielded similar patterns of CPT-induced DNA cleavage as expected, with computed positions of major peaks within 200 bases (data not shown). Since overall cleavage was stronger for wheat germ topo I than for calf thymus topo I (Panel A), we examined the cleavage patterns under different conditions. Increasing the amount of calf thymus enzymes from 30 up to 120 units in the presence of 10 μM CPT enhanced cleavage but did not alter its pattern. Similarly, decreasing CPT down to 1 μM in the presence of 30 units of wheat germ topo I did not change cleavage patterns (data not shown). These results demonstrate that CPT-induced cleavage exhibits different selectivity for calf thymus and wheat germ topo I and suggest that topo I enzymes from different sources have different base preference.

Next we compared calf thymus and wheat germ topo I-mediated DNA cleavage sites in human topo I cDNA by sequencing gel analysis. Cleavage sites of calf thymus topo I were

Table 1. Expected base triplet patterns for sense and antisense strands of human topoisomerase I cDNA[1]

Sense strand							*a/b*									
x	A/A	A/C	A/G	A/T	C/A	C/C	C/G	C/T	G/A	G/C	G/G	G/T	T/A	T/C	T/G	T/T
A	.39	.30	.44	.30	.40	.28	.40	.30	.41	.30	.47	.36	.24	.15	.19	.20
C	.14	.19	.04	.20	.30	.33	.11	.28	.17	.23	.07	.22	.24	.27	.10	.15
G	.33	.26	.26	.20	.11	.05	.11	.09	.31	.26	.21	.15	.29	.22	.37	.18
T	.15	.26	.27	.30	.19	.33	.39	.34	.11	.20	.25	.26	.23	.36	.35	.48
Antisense strand							*a/b*									
x	A/A	A/C	A/G	A/T	C/A	C/C	C/G	C/T	G/A	G/C	G/G	G/T	T/A	T/C	T/G	T/T
A	.48	.26	.34	.30	.35	.25	.39	.27	.36	.24	.33	.26	.23	.11	.19	.15
C	.18	.15	.09	.20	.37	.21	.11	.26	.22	.26	.05	.26	.29	.31	.11	.33
G	.15	.22	.28	.20	.10	.07	.11	.04	.27	.23	.33	.19	.24	.17	.30	.14
T	.19	.36	.30	.30	.19	.47	.40	.44	.15	.30	.28	.30	.24	.41	.40	.39

[1]The tabulated numbers are $f(a,x,b)$, the probability of finding base x, given that the immediately flanking bases on the 5′ and 3′ sides are a and b, respectively, calculated according to equation (1) for the sense and antisense strands between positions 1000 and 3644 of the human topo I cDNA.

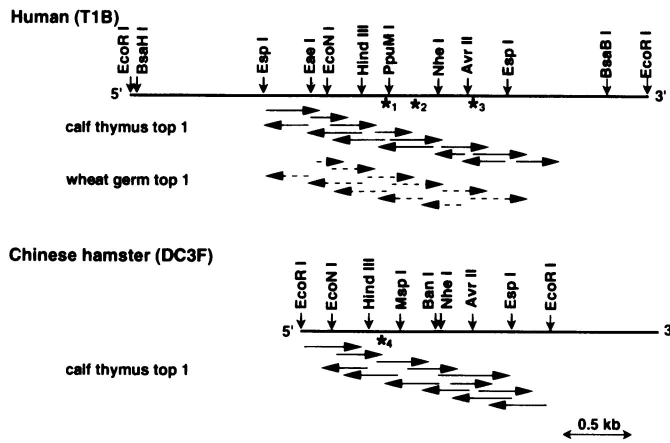


Figure 1. Strategy for analysis of CPT-induced cleavage sites in topo I cDNA. Vertical arrows indicate restriction sites. The 3'-ends of restricted fragments were labeled with [α-^{32}P] deoxynucleotides using the Klenow fragment of DNA polymerase I. The enzymes used to recut the labeled DNA fragment used for sequencing gel analysis prior to purification are not shown. Horizontal arrows correspond to the analyzed regions. Asterisks indicate the position of base mutations in topo I cDNA from CPT-resistant cell lines. *1, *2, *3 and *4 correspond to mutation sites in CPT-K5, CPT-K5, PC-7/CPT and DC3F/C-10 cell lines, respectively.

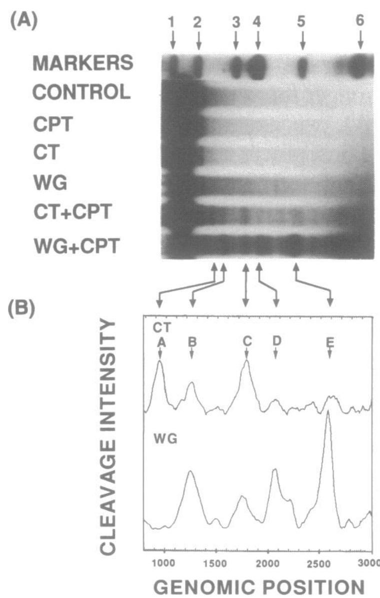
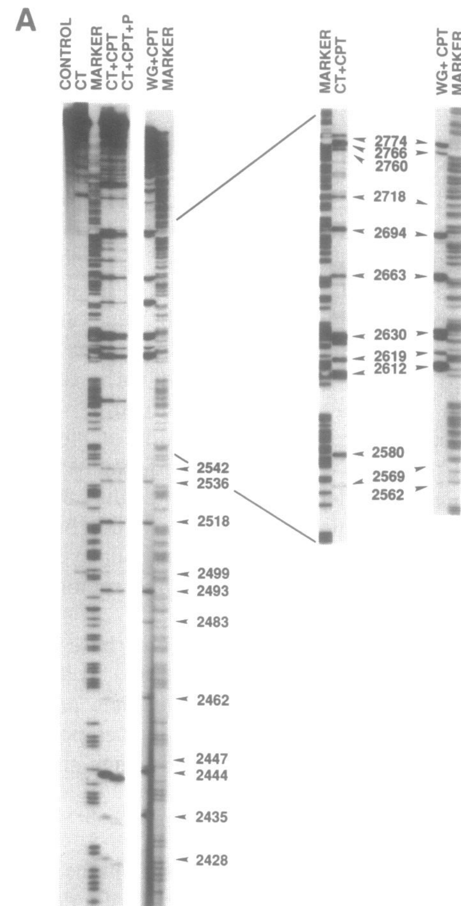


Figure 2. Overall cleavage patterns an human topo I cDNA. The 3'-end labeled human topo I cDNA was incubated with calf thymus topo I (CT) or wheat germ topo I (WG) in the absence and presence of 10 μM CPT. **Panel A** shows an agarose gel autoradiography and the cleavage patterns in the topo I cDNA using the radiolabeled sense strand. Untreated DNA is shown as control. Numbers at top indicate the migration position of markers (in kb): 1 through 5 are 3.06, 2.04, 1.64, 1.02, and 0.52, respectively. **Panel B** shows the relative intensity of cleavage sites after computation of autoradiography (Panel A) using a spectrophotometer. The two profiles correspond to the cleavage patterns of CT and WG topo I in the presence of CPT. The autoradiography used for CT was exposed 2-fold longer than the one used for WG. Genomic positions from the EcoRI site correspond to numbers in horizontal axis. Arrows and letters indicate the major peaks.



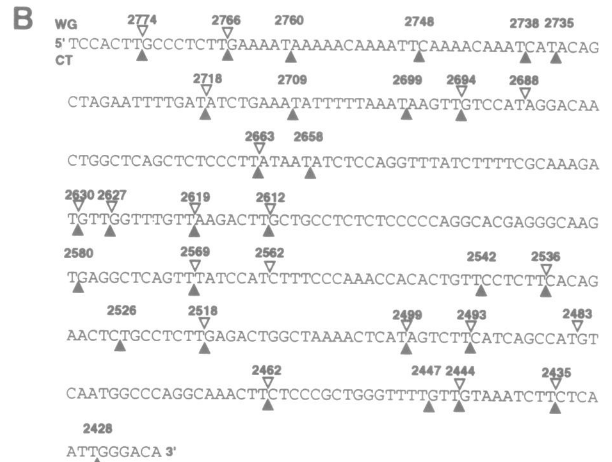
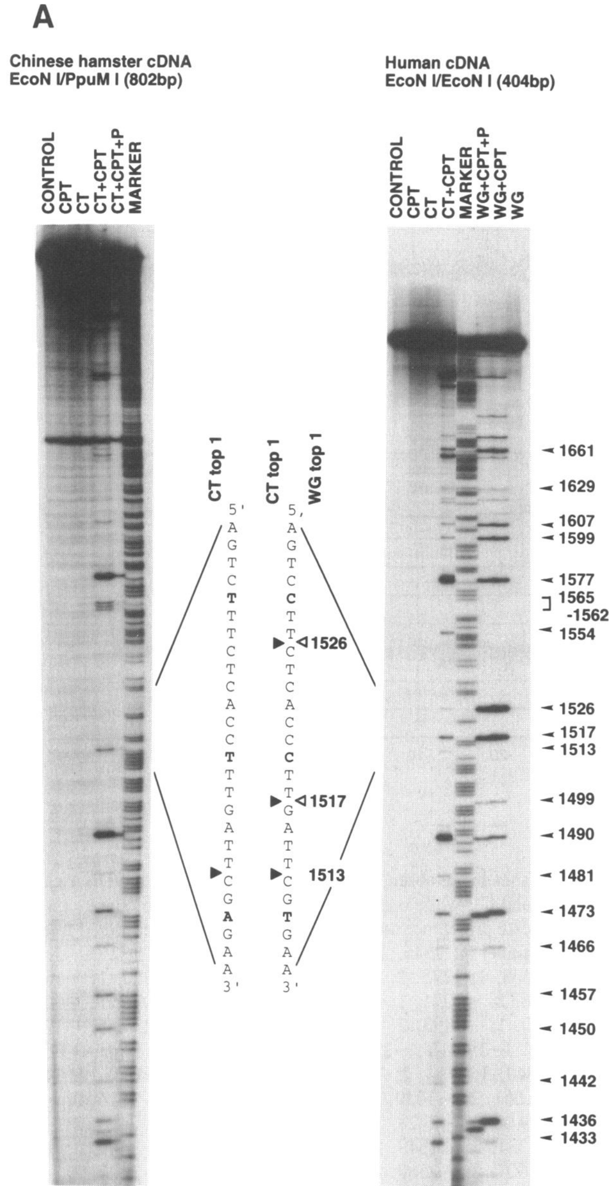


Figure 3. Comparison of CPT-induced cleavage sites by calf thymus and wheat germ. The short fragment of AvrII/EcoRI digests of human cDNA (1257 base pair) was incubated with calf thymus (CT) or wheat germ (WG) topo I in the absence and presence of 10 μM CPT. Aliquots which had been treated with T_4 polynucleotide kinase after topo I cleavage reaction are shown as +P. Cleavage sites of the antisense strand were analyzed using 6% denaturing polyacrylamide gels. DNA fragments after formic acid reactions (A + G markers) were electrophoresed in the same gel (Autoradiography: **panel A**). Numbers indicate the genomic position of the base at the 3'-terminus of several cleavage sites (position −1). **Panel B** compares CPT-induced cleavage sites by CT (solid symbols) and WG topo I (open symbols). They are indicated below and above the sequence of human topo I cDNA, respectively.



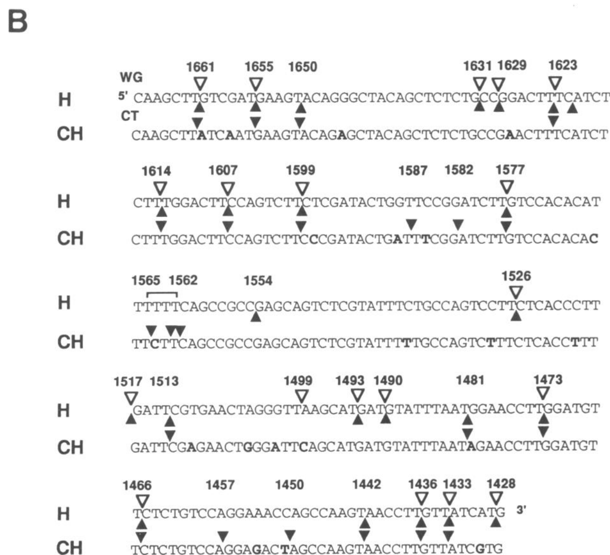


Figure 4. Comparison of CPT-induced cleavage sites in human and Chinese hamster cDNA by calf thymus and wheat germ topo I. The EcoNI/PpuMI fragment of Chinese hamster cDNA was incubated with calf thymus topo I (CT) in the absence and presence of 10 μM CPT. The EcoNI/EcoNI fragment of human cDNA was reacted with either calf thymus (CT) or wheat germ (WG) topo I under the same conditions. Cleavage sites of the antisense strands were analyzed using 6% denaturing polyacrylamide gels. Aliquots which had been treated with T_4 polynucleotide kinase after topo I cleavage reaction are shown as +P. DNA fragments after formic acid reactions (A + G markers) were electrophoresed in the same gel. **Panel A** shows autoradiographies and numbers indicate the genomic position of the base at the 3'-terminus of several cleavage sites (position −1). The lower intensity of the cleavage bands in lane CT + CPT + P than in lane CT + CPT is due to a loss of DNA after the phosphorylation and DNA purification step. **Panel B** shows the sequence of human (H) top 1 cDNA between positions 1667 and 1427 and corresponding region of Chinese hamster cDNA (CH). Bold letters indicate base differences between human and Chinese hamster topo I cDNA. Cleavage sites by CT (solid symbols) and WG (open symbols) topo I in the presence of CPT are indicated. The indicated base sequence of Chinese hamster topo I cDNA was checked by dideoxynucleotide chain-termination method.

also analyzed in Chinese hamster topo I cDNA. Figure 1 shows the fragments used for mapping the cleavage sites. Figure 3 demonstrates CPT-induced cleavage sites in antisense strand of human topo I cDNA which had been labeled at AvrII site. This fragment includes the region which corresponds to peak E in Figure 2B. An aliquot of cleavage reaction mixture with CPT and topo I was additionally incubated with T4 polynucleotide kinase to add a phosphate to the 5'-hydroxy termini of cleaved fragments and electrophoresed in parallel, so that cleavage sites could be identified unambiguously (7). Although many cleavages were induced at the same sites, relative intensities of each site were not similar. One of the differences between calf thymus and wheat germ topo I-mediated cleavages in the presence of CPT was at position 2663 where cleavage intensity was much greater in the case of wheat germ topo I than in the case of calf thymus topo I. This difference at position 2663 might contribute to the significant difference at peak E in Figure 2.

Figure 4 demonstrates the effect of base substitution on CPT-induced cleavage in addition to the difference of sequence selectivity between calf thymus and wheat germ topo I. The EcoNI/PpuMI fragment of Chinese hamster topo I cDNA (802 base pairs [bp]) was incubated with calf thymus topo I in the absence and presence of 10 μM CPT. The EcoNI/EcoNI fragment of human topo I cDNA (404 bp) was reacted with either calf thymus or wheat germ topo I under the same condition. As shown in Figure 4, comparison of calf thymus topo I-induced cleavage with CPT in human and Chinese hamster cDNA revealed that many sites were detectable in both human and Chinese hamster cDNA but that some sites were unique to one or the other target DNA (Figure 4B). Base substitution from C to T at −3 position seemed to inhibit cleavage in the presence of CPT (positions 1517 and 1526). As shown in Figures 3 and 4, calf thymus and wheat germ topo I enzymes demonstrated different cleavage patterns in human topo I cDNA, which is consistent with the agarose gel analyses (Figure 2). Although intensities of CPT-stimulated cleavages at positions 1517 and 1526 in the case of wheat germ topo I were strong, they were relatively weak in the case of calf thymus topo I.

Analysis of topo I cleavage sites induced by the calf thymus and the wheat germ enzymes in the absence and presence of CPT was performed in the set of DNA fragments shown in Figure 1. Probabilities of the observed base frequency deviation from

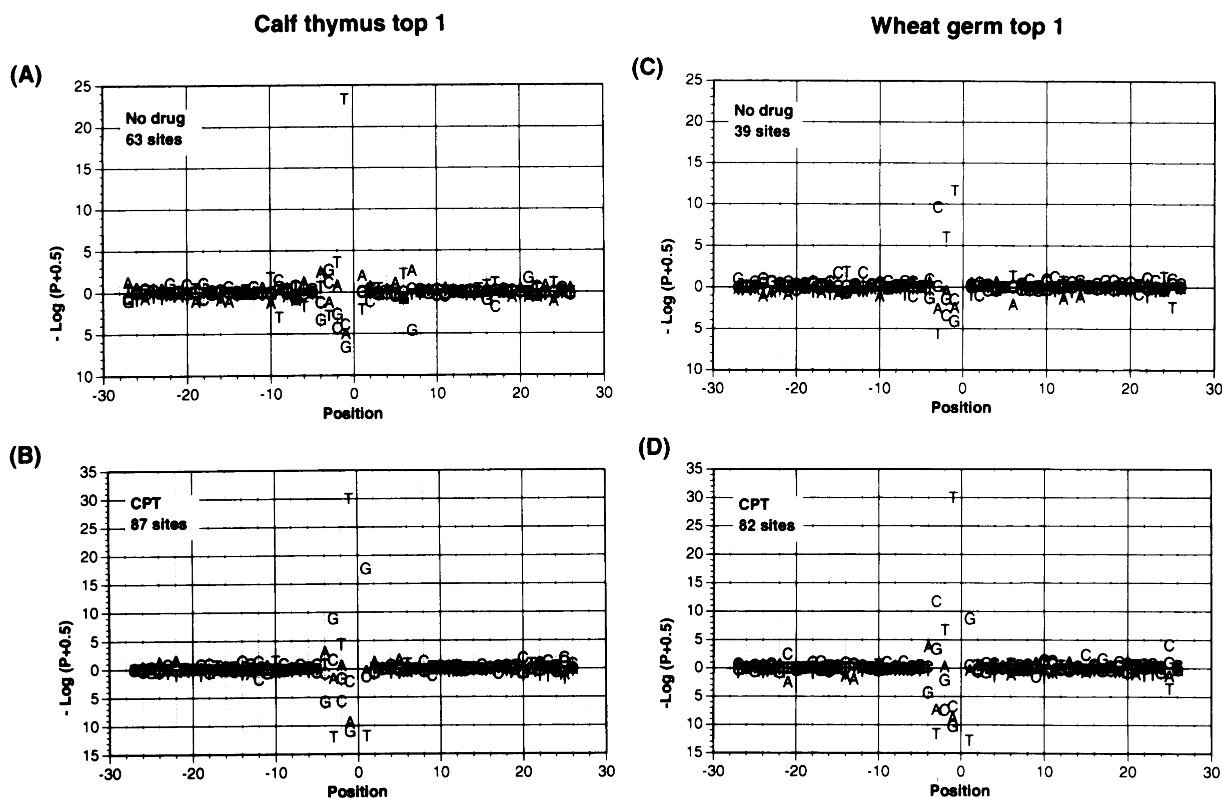


Figure 5. Probabilities of the observed base frequency deviations from expectation at topo I cleavage sites in the absence and presence of CPT. All the DNA cleavage sites of calf thymus and wheat germ topo I induced in the absence of CPT were analyzed (panels A and C), while only the strongest (strong and moderate intensity) induced by 10 μM CPT were analyzed in panels B and D. Values above and below the base lines indicate preference and deficiency relative to the expected frequency of each base and were calculated according to equations 3a and 3b, respectively. The sites were: **panel A**: 2645, 1; 2635, 2; 2618, 2; 2563, 1; 2530, 1; 2499, 1; 2497, 1; 2491, 2; 2440, 1; 2431, 1; 1909, 2; 1840, 1; 1820, 1; 1791, 1; 1653, 1; 1620, 1; 1408, 1; 1275, 2; −1866, 1; −1870, 2; −1880, 1; −1899, 2; −1916, 1; −1934, 1; −1938, 1; −2106, 1; −2135, 2; −2141, 1; −2159, 2; −2178, 1; −2216, 2; −2271, 1; −2412, 1; −2462, 1; −1022, 1; −1050, 2; −1077, 2; −1082, 2; −1085, 1; −1088, 1; −1091, 2; −1113, 1; −1124, 1; −1134, 1; −1140, 1; −1193, 2; −1217, 2; −1226, 1; −1235, 1; −1250, 2; −1265, 1; −1271, 2; −1359, 1; −1361, 1; −1364, 1; −1374, 1; −1409, 1; −1419, 1; −1433, 1; −2542, 1; −2760, 1; −2858, 2; −2974, 1; **panel B**: 2563, 2; 2491, 3; 2431, 3; 2307, 3; 2304, 3; 2284, 2; 2151, 3; 2145, 2; 2089, 2; 2029, 2; 1995, 2; 1840, 3; 1764, 2; 1653, 2; 1620, 3; 1568, 2; 1508, 2; 1445, 3; 1432, 2; 1378, 2; 1320, 3; 1275, 2; 1269, 2; 1167, 3; 1125, 2; 1109, 2; 1075, 2; −1655, 2; −1661, 2; −1729, 2; −1745, 3; −1814, 2; −1826, 2; −1848, 2; −1866, 3; −1880, 2; −1899, 2; −1938, 2; −2092, 2; −2106, 3; −2337, 2; −2411, 2; −2414, 2; −2428, 2; −2444, 3; −2457, 3; −2493, 2; −2518, 2; −1022, 2; −1050, 3; −1077, 2; −1091, 3; −1094, 2; −1193, 2; −1265, 2; −1271, 2; −1331, 3; −1349, 2; −1361, 3; −1364, 3; −1409, 2; −1433, 2; −1436, 2; −1473, 2; −1481, 2; −1490, 3; −1517, 2; −1554, 2; −1577, 3; −1599, 2; −1607, 2; −2580, 2; −2612, 3; −2619, 2; −2627, 2; −2630, 3; −2663, 2; −2694, 2; −2718, 2; −2760, 2; −2766, 3; −2774, 2; −2903, 2; −2912, 2; −2955, 2; −2988, 2; −2997, 2; **panel C**: 2062, 1; 2058, 1; 1791, 1; 1744, 1; 1732, 1; 1660, 2; 1653, 2; 1620, 1; 1483, 1; 1445, 2; 1408, 2; −1623, 2; −1688, 2; −1899, 1; −1934, 1; −1963, 1; −1970, 1; −2019, 2; −2098, 1; −2123, 1; − 2135, 2; −2159, 1; −2171, 1; −2174, 1; −2216, 2; −2322, 2; −2346, 2; −2398, 1; −2435, 1; −1 436, 1; −1 473, 2; −1 517, 2; −1 526, 2; −1577, 1; −1599, 1; −1607, 1; −1614, 2; −2663, 1; −2766, 1; **panel D**: 2304, 2; 2151, 3; 2089, 2; 2066, 2; 2062, 2; 1985, 2; 1962, 2; 1942, 2; 1840, 2; 1820, 2; 1797, 2; 1785, 2; 1764, 3; 1746, 2; 1732, 3; 1653, 3; 1620, 3; 1508, 2; 1491, 2; 1445, 3; 1408, 2; 1391, 2; 1378, 2; 1320, 2; 1275, 2; 1269, 3; 1167, 3; 1125, 2; 1109, 2; 1075, 2; 1068, 2; −1655, 2 −1661, 3; −1673, 2; −1695, 2; −1745, 3; −1814, 2; −1829, 3; −1856, 2; −1866, 3; −1899, 3; −1934, 2; −1938, 3; −1952, 2; −1970, 3; −1997, 2; −2022, 2; −2042, 2; −2106, 2; −2135, 3; −2216, 3; −2228, 2; −2337, 3; −2255, 2; −2316, 2; −2322, 3; −2414, 3; −2435, 3; −2444, 3; −2462, 2; −1364, 3; −1382, 2; −1409, 3; −1436, 2; −1473, 3; −1490, 2; −1517, 3; −1526, 3; −1577, 2; −1599, 2; −1607, 2; −2483, 2; −2493, 3; −2518, 2; −2536, 2; −2612, 3; −2627, 2; −2630, 3; −2663, 3; −2694, 2; −2766, 2; −2774, 2. The first number indicates genomic position of the +1 nucleotide (the EcoRI site was taken as position 1 [GenBank convention]). Positive and negative values indicate that cleavage was on the sense and antisense strands, respectively. The second number correspond to intensity (3: most intense, 1: least intense, and 2: intermediate intensity).

expectation were computed as described previously (21, see Methods). Analyses for all the cleavage sites induced by calf thymus and wheat germ topo I in the absence of CPT are shown in panels A and C, respectively (Figure 5). Analyses of the strong and moderate cleavage sites induced in the presence of 10 μM CPT are shown in panels B and D. *P* values above the base line indicate preference while values under base line indicate deficiency relative to the expected frequency of each base. Panel A shows that −1 T was strongly preferred for topo I. At position −2, the bias was for T or not C. Also, the observed frequency of G at +7 was remarkably below expectation. As for CPT-induced DNA breaks, probabilities of G or not T at +1 and −3 were clearly significant in addition to that of T at −1 and T or not C at −2 (Figure 5B). These findings are basically in agreement with those found in SV40 DNA except for not G at +7 position (15, 18). However, this difference is probably due to random occurrence rather than true base preference since it was not detectable in the other data sets. On the other hand, in

Table 2. Frequencies of observed bases at cleavage sites

Intensity		Position	Calf thymus				Wheatgerm				
			A	C	G	T	A	C	G	T	
With camptothecin											
Strong		−4	44	0	0	56	55	23	6	16	
		−3	4	32	64	0	0	68	32	0	
	25 sites	−2	44	4	0	52	19	3	0	77	31 sites
		−1	0	4	0	96	0	0	0	100	
		+1	44	88	4	10	32	58	0		
Moderate		−4	45	16	5	34	39	22	4	35	
		−3	19	35	44	2	6	55	37	2	
	62 sites	−2	29	5	8	58	35	2	4	59	51 sites
		−1	2	6	0	92	0	0	0	100	
		+1	19	21	58	2	16	31	51	2	
Weak		−4	34	19	12	35	26	18	17	38	
		−3	31	32	25	12	11	46	38	5	
	171 sites	−2	33	11	13	43	40	11	7	42	104 sites
		−1	5	11	5	79	4	3	4	89	
		+1	29	29	27	15	22	34	31	13	
All		−4	38	16	9	37	34	20	12	34	
		−3	26	33	33	9		8	52	37	3
	258 sites	−2	33	9	11	47	35	7	5	53	186 sites
		−1	4	9	3	84	2	2	2	94	
		+1	24	24	41	10	18	33	41	8	
Without drug											
		−4	46	10	5	40	31	33	8	28	
		−3	16	35	37	13	57	4	18	3	
	63 sites	−2	35	5	2	59	18	3	0	79	39 sites
		−1	3	3	0	94	3	8	0	90	
		+1	33	24	25	17	26	26	28	21	

Frequencies of observed bases at position −4, −3, −2, −1 and +1 of cleavage sites are expressed as percentages.

Table 3. Consensus sequences for calf thymus and wheat germ topoisomerases I in the absence and presence of camptothecin

Position	−4	−3	−2	−1	+1
Without drug					
Calf thymus	A/not G	G/not T	T/not C	T	*
Wheat germ	*	C/not T	T/not C	T	*
With CPT					
Calf thymus	not G	G/not T	T/not C	T	G/not T
Wheat germ	A/not G	C/not T	T/not C	T	G/not T

*indicates no preference or deficiency.

the case of wheat germ topo I-mediated cleavage without CPT, T at −1, T at −2 and C/not T at −3 were preferential bases (Figure 5C). In the presence of CPT, *P* values for T at −1, T/not C at −2 and C/not T at −3 were significant. Cleavage sites with G/not T at +1 were preferentially selected by CPT (Figure 5D).

Base frequencies around cleavage sites in human topo I cDNA which were induced by both topo I enzymes in the absence and presence of CPT are expressed as percentage in Table 2. Base preference and deficiency were often enhanced at strong sites and reduced at weak sites. Comparison of base preferences of calf thymus and wheat germ topo I shows that both enzymes have very strong preference for T at −1 (approximately 84% and 94% of all sites, respectively), and for not T at −3 in the presence of CPT (Table 2, Figure 5). Differences between calf thymus and wheat germ topo I were; i) at position −3 where calf thymus topo I preferred G while wheat germ topo I preferred C, ii) preference for G at position +1 in the presence of CPT was stronger in the case of calf thymus topo I than in the case of wheat germ topo I.

Human and Chinese hamster topo I cDNAs exhibit 90% homology at the nucleotide level. Figure 6 shows the list of 28 CPT-stimulated cleavage sites which were suppressed either in human or in Chinese hamster cDNAs. Except for cleavage sites at positions 2337 and 2428, every site has base substitution between at positions −4 and at +1. Base substitution from T at either −1 or −2 to C reduces cleavage intensity by CPT. T at +1 has negative effect on cleavage intensity. These findings are in agreement with the results of the probability analysis (Figure 5).

Finally, we compared CPT-induced cleavage sites in topo I cDNA and mutation sites in topo I cDNA from CPT-resistant cell lines (Figure 7). The DC3F/C-10 cell line has been derived from a Chinese hamster lung fibroblast cell line (DC3F) (27). DC3F/C-10 has one point mutation which corresponds to position 1718 in human topo I cDNA (28). CPT-induced cleavage sites in Chinese hamster topo I cDNA are more than 9 bases away from the mutation site. In human topo I cDNA, CPT induces cleavages between T at 1806 and C at 1807, and between G at

```
T at -1
S1660  5'CTTCATTGAT AAGCTTGCTC3'  CH
               C  (C)             H
S1744    AGTGGAACAT ATCAATCTAC    CH
         T     G  (C)             H
S2284    GAAGGCTGTT CAGAGACTGG    H
                  (A)       A     CH
S2332    AGCTACAGAT CGAGAGGAAA    CH
           C      (C)             H
S2374    CTCTAAACTT AATTATCTGG    CH
           C      (C)             H
S2482    GGCCATTGAT ATGACCGACG    CH
                  (C)   G T  T    H
S2491    CATGGCTGAT GAAGACTATG    H
         T  A C   (C)  G      C   CH
A1729    CACACGAAGT GAGCAGCAGC    H
           T T    (A)  A     A    CH
A1963    CTTGGCAGTT AATCCCTCCA    CH
               T  (C)  G          H

G or not T at +1
A1349    TTATATCCTC GGGCATGATT    H
          G  G      (A)        C  CH
A1355    TGATAATGAT GTCCTCAGGC    CH
            G  T    (A)     G     H
A1358    AGTTGATAAT GATGTCCTCA    CH
                G   (T)  A     G  H
A1814    AGTATCTGAT GGAGTCCTTC    H
                    (T)        T  CH
A2457    AACTTCTCCC GCTGGGTTTT    H
                    (T)           CH
A2157    TCAATCTTAG ACTGCAAGTT    CH
                    (T)           H

G or not T at -3
A1355  5'TGATAATGAT GTCCTCAGGC3'  CH
               G (T)  A    G      H
A1409    CTTTCCACTT ATGTCCTGGA    H
                 (T)              CH
A1517    TCTCACCCTT GATTCGTGAA    H
                 (T)       A      CH
A2249    CCTTCTTGGT CTTTGCATCC    CH
                 (C)              H

T or not C at -2
S1329    TGGGTATGTT GAAGAGAAGG    CH
              C   (C)       C A   H
S1863    TTAAGAACTT ACAACTATTT    CH
                  (C)             H
S2304    AGGAACAGTT GATGAAGCTG    H
          A  G    (C)             CH
A2493    TCATAGTCTT CATCAGCCAT    H
            G     (C)  G  G T     CH

not G at -4
S1522    AATCAAAGGT GAGAAAGACT    CH
                (G)        G      H
S1744    AGTGGAACAT ATCAATCTAC    CH
         T      (G) C             H
S2038    AGCCCCTGAT GAGAATGTAC    CH
                (G)      CA C     H

Unknown
S1432    CCGGCATGAT AACAAGGTTA    H
         T  A  C                  CH
A2106    GCCCTCTGAT GGTTACAAAG    H
                  G         T     CH
A2337    TTATTTTCCT CTCGGTCTGT    H
                        A         CH
A2428    CTTCTCAATT GGGACACCCC    H
                        C         CH
```

Figure 6. Relationship between reduction of intensity at CPT-stimulated cleavage sites and base substitution. Arrows indicate the position of CPT-stimulated cleavage sites and numbers the genomic position of the base at the 3'-terminus of the breaks in the sense (S) and antisense (A) strands induced by calf thymus topo I which are strong or moderate in one of the topo I cDNA and become weak or not-existent in the other. Base sequences of strong or moderate cleavage sites in one cDNA are shown. Each symbol below the sequence is the base substituted in the other cDNA. Based on the results from Figure 5, each site was classified into 6 groups. Each circle indicates the possible base which is responsible for reduction of cleavage intensity. H and CH correspond to human and Chinese hamster cDNAs, respectively.

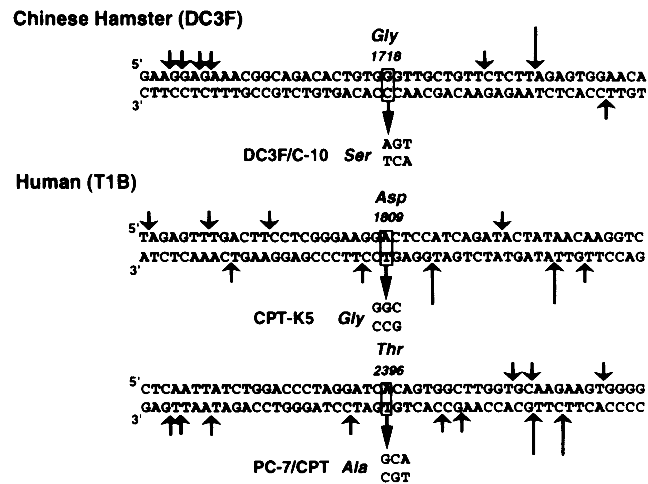


Figure 7. CPT-induced cleavage sites in the surrounding regions of CPT-resistant mutations. Sequences of topo I cDNA from Chinese hamster (DC3F) and human (T1B) are shown. Topo I cDNA from CPT-K5 cells has two point mutations when compared to that from the parental cells. One of them (Asp→Gly), which is supposed to be responsible for resistance to CPT, is shown. The mutation site in topo I cDNA from CPT-resistant DC3F/C-10 cells is numbered as the corresponding base in human cDNA. Short and long arrows indicate weak and moderate CPT-induced cleavage sites, respectively.

1813 and T at 1814 on the antisense strand, which are in the vicinity of the mutation site (genomic position 1809) in CPT-K5 (25). However, CPT does not induce a detectable cleavage at position 1809. As for the other mutation site (position 1959) in CPT-K5 cDNA, cDNA from the parental cells of CPT-K5 has a base substitution at position 1959 when compared to T1B cDNA. As far as we investigated by using T1B cDNA, the CPT-induced cleavage sites are more than 3 bases away from the mutation sites (data not shown). In the case of the mutation in topo I cDNA from PC-7/CPT cell line (genomic position 2396), CPT induces weak cleavage sites more than 3 bases away from the mutation site.

DISCUSSION

We have analyzed topo I-mediated DNA cleavage sites in the absence and presence of 10 μM CPT and identified, 63 and 39 sites without CPT, and 258 and 186 sites with CPT in human topo I cDNA by calf thymus and wheat germ topo I, respectively. Frequency of detectable cleavage sites for calf thymus topo I is approximately one site per 59 nucleotides without CPT and one per fourteen with CPT. The former value is relatively low when compared to the cleavage site frequencies without CPT in previous works, 68 sites in 827 nucleotides for human topo I (7), 223 and 245 sites in 1781 nucleotides for rat liver and wheat germ topo I, respectively (8). It is probably due to the reaction condition, especially the ratio of enzyme and substrate DNA. Since reduction of enzyme concentration induces less overall breakage without alteration in the cleavage pattern (8), most cleavage sites in our study correspond probably to the strong sites of other reports (7, 8). However, there is an apparent difference between our results and previous studies. Edwards *et al.* analyzed the human and calf thymus topo I-mediated cleavage sites in SV40 DNA in the absence of CPT and deduced the following consensus sequence, not G at −4, not T at −3, not G at −2, not G/not A at −1 and not G at +1 (7). These authors did not find the very strong preference for T at −1 position as we did. However, these authors used single-stranded DNA rather than duplex DNA. Our results are more consistent with those by Been *et al.* who used rat liver and wheat germ topo I for cleavage reaction (8). When comparing the consensus sequences of wheat germ topo I-induced strong cleavage sites in their report and in ours, preference for T at −1 is similar. One of the difference is the preference at −3. G or C are preferred in their study although C shows a stronger bias than G in ours. Perez−Stable *et al.* and Jaxel *et al.* also found preference for T at the −1 position with human and mouse topo I, respectively (10, 15, 18).

As for the cleavage sites in the presence of CPT, some reports found preference for G at +1 position and the others did not. Champoux *et al.* showed slight preference for G at +1 for rat liver topo I and not for wheat germ topo I, and found that CPT-induced cleavage sites exhibit significant preference for the nucleotides at position +1 to +3 (not T), +9 (A/T), +10 (A/G), and −10 (T) (4, 12). However, only 14 cleavage sites were analyzed with wheat germ topo I and no statistical analysis was performed (12). Bronstein *et al.* analyzed calf thymus topo I-mediated cleavage sites with and without CPT. They found preference for T at −1 and +1, G at +2 and alteration of preference for A at −3 to −5 in the presence of CPT (11). Perez−Stable *et al.* showed the consensus sequence as follows; preference for T at −1 and A or T at −2 (10). *In vivo* mapping

of cleavage sites in SV40 DNA from monkey cells exhibits preference for G at +1 in contrast to *in vitro* mapping which did not show such preference (13). Kjeldsen *et al.* and Tsui *et al.* also revealed that CPT frequently cleaves between T at −1 and G at +1, and between T at −1 and G/A at +1, respectively (19, 33). Different substrate DNAs used for analysis might lead some discrepancies among reported consensus sequences. As shown in Table 1, expected base triplet patterns are different even between sense and antisense strands. This asymmetrical distribution of 4 bases suggests that statistical analysis of cleavage sites is necessary to lead any consensus sequence. Therefore, we believe that the present method of analysis is the most objective at the present time since it takes into account any base frequency bias in the target sequence and adjusts the probability values to the number of sites analyzed.

In this study, we found the strong and statistically very significant preference for G/not T at +1 in the case of both calf thymus and wheat germ topo I enzymes cleavage sites induced by CPT. These results are in agreement with our previous work with mouse topo I and SV40 DNA (15, 18). Moreover, frequencies of observed bases at break points clearly shows that preference and deficiency correlate with cleavage intensity (Table 2). Our consensus sequences for topo I-mediated cleavage without and with CPT are shown in Table 3, which indicates preference at +1 as the major specific difference for CPT-induced cleavage. One discrepancy between our previous work (15, 18) and this study is the deficiency for G at +7 position. We believe that this deficiency is within experimental error and should not be considered significant since it is not observed in the presence of CPT (Figure 5B) and was not detectable in our previous analysis in SV40 DNA (15). The analyzed region of the substrate human topo I cDNA has 77 sites whose base sequence is 5′-TTG-3′. Forty-five out of 77 sites (58%) are actually cleaved by calf thymus topo I in the presence of CPT. Twenty-nine sites out of the 32 uncleaved sites have either G at −4 or T at −3. Thus, forty-five out of 48 sites (94%), that are consistent with consensus, 5′-(not G)-(not T)-T-T^G-3′, exhibit cleavage between T and G in the presence of CPT (caret). On the assumption that CPT-stimulated cleavage sites are endogenous topo I sites, it is not surprising that the consensus sequence for CPT-stimulated cleavage sites upstream from the cleavage sites basically reflects that for topo I-mediated cleavage without CPT. For combination of wheat germ topo I and CPT, preference for C at −3 may be due to wheat germ topo I itself and it may not be essential for interaction between CPT and wheat germ topo I-DNA complex, because percentage of C at −3 without CPT is higher than that with CPT (Table 2). Taken together, these observations demonstrate that the major difference between preferential bases with and without CPT is G/not T downstream from the cleavage sites at the +1 position.

We have previously postulated that inhibitors of both topoisomerase I and II may increase cleavable complexes by preventing DNA religation because of their stacking inside the enzyme cleavage sites (15, 21). This hypothesis was derived from the observation that agents from different chemical classes exhibit a selective preference for one of the bases flanking the cleavage sites. Adenine at the 3′-terminus (−1 position) for doxorubicin, cytosine for etoposide (VP-16), adenine at the 5′-terminus (+1 position) for m-AMSA (21). Since all compounds are planar conjugated ring systems, stacking with the bases would be facilitated. Preference for G at +1 position supports the stacking model of CPT (15). Although G at +1 may be essential for the interaction between CPT and topo I-DNA complex, topo I enzyme or base sequence other than G at +1 may affect cleavable complex formation since we found that: i) in this study, preference for G at +1 is stronger in the case of calf thymus topo I than in the case of wheat germ enzyme; ii) preference for G/not T at −3 appears greater in the presence of CPT than in the absence of the drug. Enzymatic characteristics of calf thymus and wheat germ topo I are not identical. For instance, the DNA relaxing activity of wheat germ topo I is not inhibited by CPT when compared to that of mammalian topo I (12). Thus, interaction between CPT and G at +1 may not be of equal importance for different topo I enzymes.

Hot spots for acridine-induced frame shift mutations corresponded to topoisomerase II-mediated DNA cleavage sites (34−36). Topo I-mediated DNA cleavage sites have been considered to be relevant to the recombination sites, because many of the recombination sites show the consensus base sequence for topo I-induced cleavage and association with topo I cleavage sites *in vitro* (37−39). Since CPT-induced cleavage exhibits base sequence selectivity, it is possible that CPT attacks specific sites in the genes and induces mutation(s) (40, 41) which may cause drug-resistance. However, there is no report which describes the relationship between topo I inhibitor-induced cleavage and point mutations as far as we know. Cleavage site analysis on human topo I cDNA showed that CPT induces the cleavage in the vicinity of the mutation site (positions 1809 and 2396). Although it remains possible that these cleavages worked as a trigger to induce the point mutation which resulted in CPT-resistance, CPT does not induce detectable breaks at the mutation sites themselves. Porter *et al.* investigated *in vivo* CPT-stimulated cleavage sites on SV40 DNA isolated from monkey kidney cells and found notable differences between *in vitro* and *in vivo* sites (13). Thus, mapping the cleavage sites *in vivo* will be more conclusive, even though base sequence at mutation sites are not compatible with consensus sequence for CPT-stimulated cleavage. Further analysis of other mutation sites induced in cells which had been treated with CPT should provide more definitive conclusions.

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