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Molecular Analysis of Yeast and Human Type II Topoisomerases

ENZYME-DNA AND DRUG INTERACTIONS*

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The DNA sequence selectivity of topoisomerase II (top2)-DNA cleavage complexes was examined for the human (top2α), yeast, and *Escherichia coli* (*i.e.* gyrase) enzymes in the absence or presence of anticancer or antibacterial drugs. Species-specific differences were observed for calcium-promoted DNA cleavage. Similarities and differences in DNA cleavage patterns and nucleic acid sequence preferences were also observed between the human, yeast, and *E. coli* top2 enzymes in the presence of the non-intercalators fluoroquinolone CP-115,953, etoposide, and azatoxin and the intercalators amsacrine and mitoxantrone. Additional base preferences were generally observed for the yeast when compared with the human top2α enzyme. Preferences in the immediate flanks of the top2-mediated DNA cleavage sites are, however, consistent with the drug stacking model for both enzymes. We also analyzed and compared homologous mutations in yeast and human top2, *i.e.* $Ser^{740} \rightarrow Trp$ and $Ser^{763} \rightarrow Trp$, respectively. Both mutations decreased the reversibility of the etoposide-stabilized cleavage sites and produced consistent base sequence preference changes. These data demonstrate similarities and differences between human and yeast top2 enzymes. They also indicate that the structure of the enzyme/DNA interface plays a key role in determining the specificity of top2 poisons and cleavage sites for both the intercalating and non-intercalating drugs.

DNA topoisomerases are enzymes that catalyze changes in the topology of DNA via a mechanism involving the transient breakage and rejoining of phosphodiester bonds in the DNA backbone (1, 2). Studies in both prokaryotic and eukaryotic cells have demonstrated the importance of topoisomerases in transcription, DNA replication, and chromosome segregation. The type II topoisomerases (top2)[1] make transient DNA double-strand breaks and change the linking number of DNA in steps of two. They play key roles in DNA metabolism and chromosome structure and are essential in eukaryotic cells (2, 3). In order to maintain the integrity of the cleaved DNA during this process, the top2 enzymes form a proteinaceous bridge that spans the DNA break. This bridge is anchored by covalent phosphotyrosyl bonds established between each of the active site tyrosine residues of the homodimeric enzyme and the 5′-DNA termini of the newly created DNA double-strand break (2). Under physiological conditions, these covalent top2-DNA complexes (referred to as cleavage or cleavable complexes) are normally short lived intermediates in the catalytic cycle of the enzyme.

Beyond its vital cellular functions, top2 is the primary cytotoxic target for some of the most active drugs for the treatment of human cancers (4–8). Top2 inhibitors can be divided into two groups, top2 catalytic inhibitors and top2 poisons (8). Top2 catalytic inhibitors do not stabilize DNA cleavage complexes. Bisdioxopiperazines (ICRF 159, 187 (dexrazoxane), and 193) belong to this category (9). Top2 poisons inhibit the enzyme by increasing the steady-state levels of DNA cleavage complexes (8, 10, 11). Hence they convert top2 into a physiological toxin that creates DNA double-strand breaks in the genome of treated cells (5, 8, 10, 12). Top2 poisons can be further subdivided into two groups as follows: the DNA intercalators that include doxorubicin, mitoxantrone, amsacrine, ellipticines/olivacines, and the non-intercalators whose main representatives are the demethylepipodophyllotoxins etoposide (VP-16) and teniposide (VM-26), the quinolones among which CP-115,953 acts as a dual eukaryotic and prokaryotic top2 poison (13, 14), and some azatoxin derivatives (15).

Although top2 cleaves DNA at preferred sequences, little is known regarding the mechanism by which the enzyme selects its sites of action. Recent studies with etoposide suggested that etoposide interacts with top2 rather than with the DNA (7). On the other hand, studies with a photoactivated amsacrine derivative and with bisantrene/amsacrine congeners indicated that for these agents, drug-DNA interactions are critical for the formation of top2-DNA cleavage complexes (16, 17). Analyses of drug-induced top2 cleavages revealed drug-specific base preferences in the immediate vicinity of the cleavage sites. In the case of amsacrine, A at position +1 was preferred, whereas in the case of etoposide, teniposide, mitoxantrone, and quinolones the highest preference is for C at position −1 (see diagram in Fig. 3). From these results, it has been proposed that drugs bind at the enzyme-DNA interface and form a ternary complex with top2 and the DNA. This model has been referred to as the drug stacking model (8, 18) or position poison model (19, 20).

Yeast is a powerful model system to study topoisomerase inhibitors (3, 21, 22). However, no detailed comparison has been reported for DNA cleavage complexes formed by the yeast and the human top2 enzymes. Furthermore, since detailed fundamental information is available for the yeast enzyme (2, 23), but not for the human enzymes, direct comparison of the human and yeast proteins is useful for a structural under-

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[1] The abbreviations used are: top2, topoisomerase II; PCR, polymerase chain reaction.

standing of the human enzymes as a drug target. Yeast top2 is also a potential target for antifungal treatment, and structural differences between the yeast and human top2 may allow selective targeting of the yeast top2 over its human counterpart. In this way, a clear and detailed comparison between yeast and human top2 is warranted and necessary. Since mutation of a conserved serine residue ($Ser^{740} \rightarrow Trp$) in yeast top2 was recently reported to alter both enzyme-DNA and drug interactions (24), the homologous mutation ($Ser^{763} \rightarrow Trp$) in human top2$\alpha$ was analyzed in this study.

EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Enzymes—Etoposide (VP16) was obtained from Bristol-Myers Squibb Co. Amsacrine and mitoxantrone were from the Drug Synthesis and Chemistry Branch (NCI, Bethesda, MD). Azatoxin and its derivatives were provided by Dr. T. Macdonald, Department of Chemistry of Virginia, Charlottesville, VA (15). CP-115,953 was the gift of Drs. P. R. McGuirk and T. D. Gootz of Pfizer. Drug stock solutions were made in dimethyl sulfoxide (Me_2SO) at 10 mM. Further dilutions were made in distilled water immediately before use. Human c-*MYC* inserted into pBR322, restriction enzymes, T4 polynucleotide kinase, polyacrylamide/bisacrylamide, and *Taq* DNA polymerase were purchased from Lofstrand Laboratories (Gaithersburg, MD), Life Technologies, Inc., New England Biolabs (Beverly, MA), or Qiagen Inc. (Valencia, CA). [γ-^{32}P]ATP was purchased from NEN Life Science Products. PCR oligonucleotide primers were obtained from Life Technologies, Inc.

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 between positions 3035 and 3288, with numbers referring to GenBank™ genomic positions using oligonucleotides 5′-GTAATCCAGAACTGGATCGG-3′ for the upper strand and 5′-ATGCGGTCCCTACTCCAAGG-3′ for the lower strand (annealing temperature 56 °C). A 401-base pair DNA fragment from the junction between the first intron and first exon was prepared between positions 2671 and 3072 using oligonucleotides 5′-TGCCGCATCCACGAAACTTT-3′ for the upper strand and 5′-TTGACAAGTCACTTTACCCC-3′ for the lower strand (annealing temperature 60 °C). A 480-base pair fragment from the first exon containing promoters P_1 and P_2 was prepared between positions 2265 and 2745 using the oligonucleotides 5′-GATCCTCTCTCGCTAATCTCCGCCC-3′ for the upper strand and 5′-TCCTTGCTCGGGTGTTGTAAGTTCC-3′ for the lower strand (annealing temperature 70 °C). A 213-base pair fragment from the human c-*JUN* gene was prepared between positions 5′-TGTTGACAGCGGCGGAAAGCAGS-3′ for the upper strand and 5′-CGTCCTTCTTCTCTTGCGTGGCTCT-3′ for the lower strand (annealing temperature 64 °C). Single end labeling of these DNA fragments was obtained by 5′-end labeling of the specific primer oligonucleotide. Ten picomoles of DNA was incubated for 60 min at 37 °C with 10 units of T4 polynucleotide kinase and 10 pM [γ-^{32}P]ATP (100 μCi) in kinase buffer (70 mM Tris-HCl, pH 7.6, 0, 1 M KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin). Reactions were stopped by heat denaturation at 70 °C for 15 min. After purification using Sephadex G-25 columns (Roche Molecular Biochemicals), the labeled oligonucleotides were used for PCR. Approximately 0.1 μg of the c-*MYC* DNA that had been restricted by *Sma*I and *Pvu*II (fragment 2265–2745) and *Xho*I and *Xba*I (fragment 2671–3072 and fragment 3035–3288) was used as template for the PCR. Ten picomoles of each oligonucleotide primer, one of them being 5′-labeled, was used in 22 temperature cycle reactions (each cycle with 94 °C for 1 min, annealing for 1 min, and 72 °C for 2 min). The last extension was for 10 min. DNA was purified using PCR Select-II columns (5 Prime → 3 Prime, Inc., Boulder, CO).

Overexpression and Purification of Yeast and Human Topoisomerase II—Wild-type yeast and human top2, $Ser^{740} \rightarrow Trp$, and $Ser^{763} \rightarrow Trp$ proteins were overexpressed using YEpTOP2-PGAL1 or YEptop2-S*W-PGAL1 using yeast strain JEL1t1$^-$ (25) and purified to homogeneity as described previously (26). The detailed procedure has been described elsewhere (27).

Topoisomerase II-induced DNA Cleavage Reactions—DNA fragments (5–10 × 10^4 dpm/reaction) were equilibrated with or without drug in 1% Me_2SO, 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM Na_2EDTA, 1 mM ATP, and 15 μg/ml bovine serum albumin for 5 min before addition of 8 units (80 ng) of purified top2 in 10-μl final reaction volume. Unless otherwise indicated, reactions were for 30 min at 37 °C. Reactions were stopped by adding 1% SDS (v/v) and further digested with proteinase K (0.4 mg/ml final concentration for 30 min at 55 °C). Calcium-promoted DNA cleavage was performed in the same buffer with 5 mM $CaCl_2$ instead of $MgCl_2$ (24).

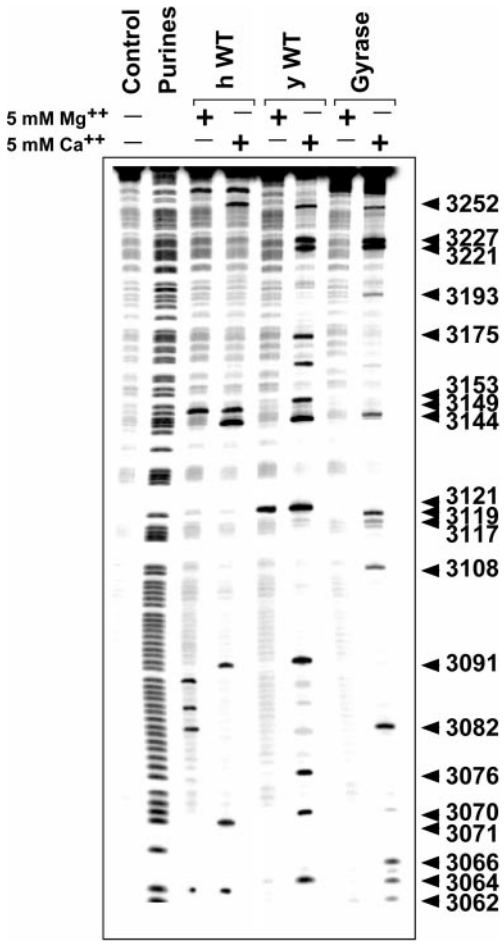

FIG. 1. **Effect of Ca^{2+} on the DNA cleavage patterns of yeast, human, and bacterial top2 enzymes.** A 254-base pair DNA fragment from the human c-*MYC* first intron was prepared by PCR between positions 3035 and 3288. The upper strand was 5′-end-labeled with ^{32}P. Top2 reactions were performed at 37 °C for 30 min in the presence of 5 mM $MgCl_2$ or 5 mM $CaCl_2$ as indicated and stopped by adding EDTA and SDS (25 mM and 1% final concentrations, respectively). Purine ladder was obtained after formic acid reaction. *h WT,* human wild-type top2α; *y WT,* yeast wild-type top2; *Gyrase,* wild-type gyrase from *E. coli*. *Numbers* correspond to genomic positions of the nucleotide covalently linked to top2.

Electrophoresis and Base Preference Analysis—For DNA sequence analysis, samples were precipitated with ethanol and resuspended in 5 μl of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0, 1% xylene cyanol, and 0.1% bromphenol blue). Samples were heated to 95 °C for 5 min and thereafter loaded onto DNA sequencing gels (7% polyacrylamide, 19:1 acrylamide/bisacrylamide) containing 7 M urea in 1× Tris borate/EDTA buffer. Electrophoresis was performed at 2500 V (60 watts) for 2–3 h. The gels were dried on Whatman No. 3MM paper sheets and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software. The determination of preferred bases around top2 cleavage sites was done as described previously (28–30).

RESULTS

Calcium-promoted DNA Cleavages Differ between Human, Yeast, and Bacteria Top2 Enzymes—The calcium-promoted, drug-independent DNA cleavage sites induced by yeast and human wild-type top2 and by *E. coli* gyrase (*i.e.* in bacterial type II topoisomerase) (31, 32) were mapped on the upper strand of the c-*MYC* first intron fragment (Fig. 1). Even in the presence of magnesium, differences in the cleavage sites could

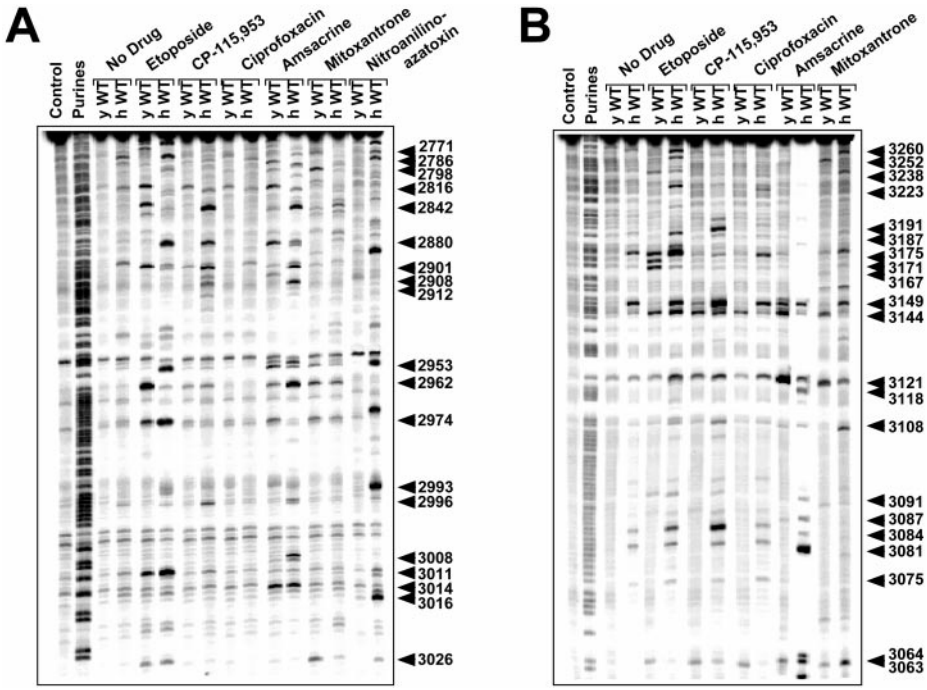

FIG. 2. **DNA cleavage patterns induced in yeast and human top2 enzymes by non-intercalating and intercalating drugs.** DNA fragments from the junction between the c-*MYC* first intron and first exon between positions 2671 and 3072 (*panel A*) and from the c-*MYC* first intron (*panel B*) were prepared by PCR using one primer labeled with ^{32}P at the 5′ terminus. *Panel A,* labeling of the lower DNA strand at position 3072. *Panel B*, labeling of the upper DNA strand at position 3035. Drugs are indicated *above each lane.* Concentrations used were as follows: etoposide, 100 μM; CP-115,953, 100 μM; ciprofloxacin, 100 μM; amsacrine, 200 μM; mitoxantrone, 1 μM; and 11β(4″-nitroanilino)azatoxin, 100 μM. Purine ladder was obtained after formic acid reaction. *Control,* no top2, no drug treatment. *Numbers* correspond to genomic positions of the nucleotide covalently linked to top2. *y WT,* yeast wild-type enzyme; *h WT,* human wild-type enzyme.

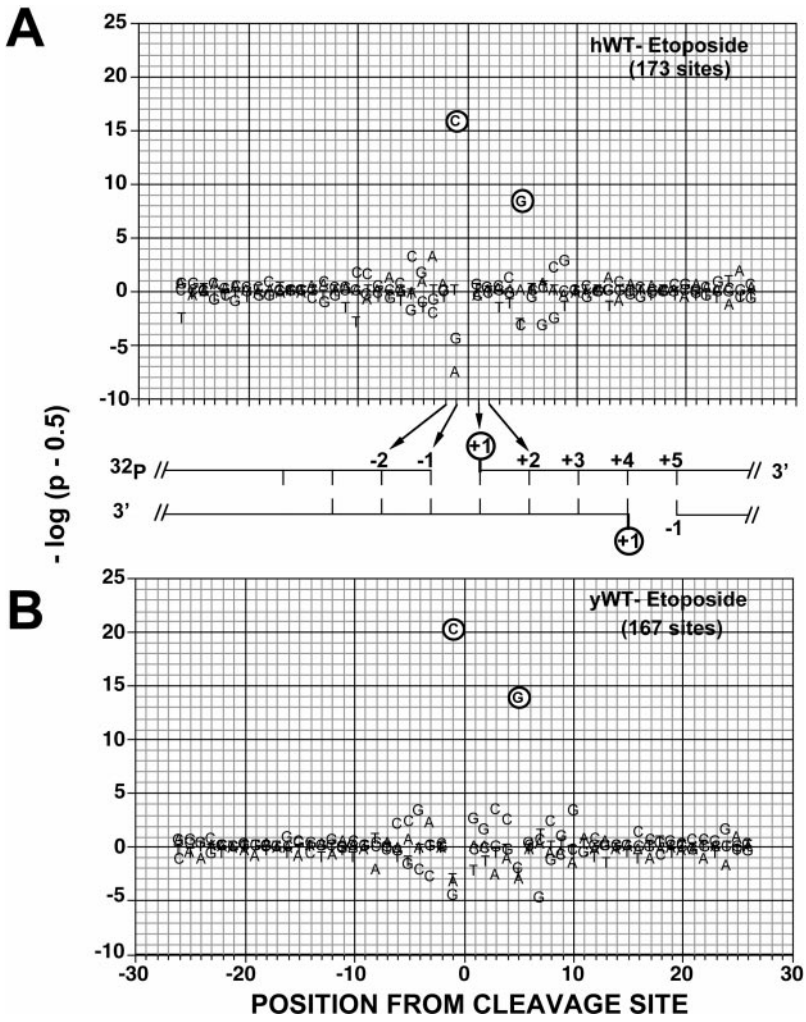

FIG. 3. **Probability of the observed base frequency deviations at top2 cleavage sites for the human and yeast wild-type enzymes in the presence of etoposide.** Drug concentration was 100 μM. Position 0 corresponds to the cleavage site. The panels present the probability of the observed base frequency deviations from expectation for the indicated enzyme. In the *y* axis, *P* is the probability of observing that deviation or more, either as excess (*above base line*) or deficiency (negative values *below base line*) relative to the expected frequency of each individual base (29). Cleavage sites for the human (*panel A*) and the yeast (*panel B*) wild-type enzymes were analyzed. Drug concentration was 100 μM. A schematic representation of a top2 cleavage complex is shown between *panels A* and *B*. The top2 covalent linkage to the 5′-DNA termini is shown as a *circle* at the +1 position.

be observed. When magnesium was replaced by calcium, higher levels of DNA cleavage were seen in the yeast protein. DNA cleavage sites common to both proteins were seen in the presence of Ca^{2+}. However, there were also major differences in the intensity of cleavage at other sites. Although a number of DNA cleavage sites in yeast were also found in gyrase, *e.g.* at posi-

TABLE I

Base distribution at each position of etoposide-, amsacrine-, and mitoxantrone-induced DNA cleavage sites

Underlined numbers represent base frequencies significantly ($p < 0.001$) greater or lower than expected.

hWT-Etoposide (173 sites)																				
A	40	34	45	51	38	40	51	61	41	12	33	39	45	46	42	43	46	40	34	35
C	63	61	44	47	56	67	37	30	48	99	45	43	53	54	28	48	54	61	45	52
G	49	41	50	41	49	33	57	42	51	23	47	53	49	46	82	43	27	30	64	50
T	21	37	34	34	30	33	28	40	33	39	48	38	26	27	21	39	46	42	30	36
yWT-Etoposide (167 sites)																				
A	39	33	24	40	37	49	41	53	32	21	38	35	21	29	20	31	40	32	31	26
C	51	49	46	44	63	61	28	27	52	103	45	45	70	61	32	52	57	61	36	41
G	47	47	50	47	40	31	64	51	50	23	59	62	46	42	92	52	21	31	54	67
T	30	38	47	36	27	26	34	36	33	20	25	25	30	35	23	32	49	43	46	33
hWT-AMSA (64 sites)																				
A	21	14	12	16	16	14	21	21	18	14	47	18	4	17	15	18	17	15	20	20
C	15	14	24	17	14	20	12	8	6	26	0	21	27	13	15	25	23	20	12	21
G	20	23	15	18	21	19	17	20	24	10	9	24	15	6	24	8	9	11	18	13
T	8	13	13	13	13	11	14	15	16	14	8	1	18	28	10	13	15	18	14	10
yWT-AMSA (61 sites)																				
A	13	16	11	10	16	23	25	22	21	12	29	15	14	9	28	11	22	10	14	17
C	18	12	13	17	21	16	6	9	6	14	6	15	23	22	7	20	14	26	10	10
G	17	18	21	18	12	10	24	16	20	3	20	18	15	8	16	13	6	8	17	22
T	13	15	16	16	12	12	6	14	14	32	6	13	9	22	10	17	19	17	20	12
hWT-Mitoxantrone (106 sites)																				
A	25	32	25	35	28	23	33	44	35	12	38	19	30	26	33	22	31	31	26	21
C	33	38	34	33	35	28	27	8	29	62	20	38	38	31	20	27	35	48	36	37
G	32	16	31	14	25	25	25	30	24	10	23	35	24	31	39	24	12	14	32	29
T	16	20	16	24	18	30	21	24	18	22	25	14	15	18	14	33	28	13	12	19
yWT-Mitoxantrone (110 sites)																				
A	25	12	25	29	36	41	39	32	18	6	38	27	26	20	34	19	38	25	22	17
C	36	48	33	34	36	28	17	17	33	60	41	41	29	26	16	52	37	40	24	27
G	29	24	37	29	25	23	36	25	36	4	21	27	35	37	46	16	11	14	27	34
T	20	26	15	18	13	18	18	36	23	40	10	15	20	27	14	23	24	31	37	32
Position from cleavage site																				
	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10

tions 3227, 3221, 3064, the majority of cleavage sites were specific for either protein. These results suggest that human, yeast, and bacterial top2 are different regarding Ca^{2+}-promoted DNA cleavage in the absence of a top2 drug.

Cleavage Sites Induced by Yeast and Human Top2 in the Presence of Drugs—To investigate further possible differences between yeast and human top2 DNA cleavage patterns, we compared the drug-induced cleavage sites (Fig. 2). Several cleavage sites induced by etoposide were much stronger with the yeast enzyme, for example in Fig. 2, *panel A,* at positions 2816, 2842, 2901, and 2962, and in Fig. 2, *panel B,* at positions 3171 and 3167. Conversely, some etoposide-induced sites were stronger with the human than with the yeast enzyme, *e.g.* in Fig. 2, *panel A,* at position 2880 and in Fig. 2, *panel B,* at positions 3260, 3252, 3223, 3187, 3149, and 3084.

In the case of CP-115,953, human top2α caused greater cleavage than the yeast enzyme at several sites (in Fig. 2, *panel A,* at positions 2842, 2880, 2901, and 2996, and in Fig. 2, *panel B,* at positions 3187, 3149, and 3084). In the case of the gyrase-specific quinolone ciprofloxacin, yeast top2 showed minimal cleavage induction at position 3144 (Fig. 2, *panel B*).

With amsacrine, human top2α cleaved more extensively than yeast top2 (in Fig. 2, *panel A,* at positions 2842, 2912, 2962, and 3008 and in Fig. 2, *panel B,* at positions 3064, 3081, 3084, 3091, and 3118). At some sites (*e.g.* positions 3144, 3121, and 2974), however, cleavage was stronger with the yeast enzyme. Similarly, several changes in cleavage sites induced by mitoxantrone were seen. The azatoxin-derivative 11β(4″-nitroanilino)azatoxin (15) (Fig. 2, *panel A*) was markedly more active against the human top2α than the yeast top2 in the DNA fragment examined. Taken together, these results show different DNA cleavage patterns for yeast and human top2 in the presence of both intercalating and non-intercalating drugs.

Different Base Preferences of Amsacrine- and Mitoxantrone-stabilized Cleavage Complexes for the Yeast and Human Top2—Because the yeast and human top2 enzymes presented different cleavage activity in the presence of drugs, we compared the DNA base preferences for both proteins in the presence of etoposide, amsacrine, mitoxantrone, and CP-115,953 (Figs. 3-6 and Tables I and II). Cleavage sites for the three c-*MYC* DNA fragments and the c-*JUN* fragment (see "Experimental Procedures") were analyzed for both DNA strands.

For yeast and human proteins, etoposide preferentially stabilized sites with C at position −1 (C^{-1}) (Fig. 3 and Table I). This result agrees well with previous analyses (18, 29). Preference on the opposite strand showed a complementary (although slightly weaker) preference for G at position +5. Thus, the different cleavage patterns for yeast and human top2 in the presence of etoposide were not associated with detectably altered base preferences.

In the presence of amsacrine (Fig. 4 and Table I), the human enzyme showed a clear preference for A^{+1} (47 of 64 sites) and a complementary (although weaker) preference for T^{+4} (28 of 64 sites), which conforms with earlier studies (29, 33, 34). The yeast protein also demonstrated a strong preference for A^{+1} (29 of 61 sites) but an additional preference for T at position −1 (32 of 61 sites) as well as the complementary A at position +5 (28 of 61 sites), which was not seen in the human enzyme.

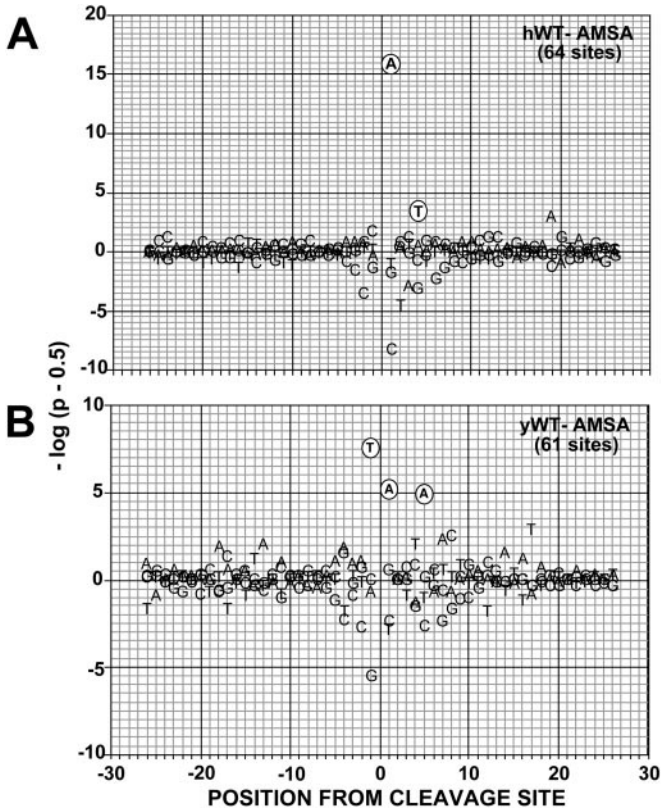

FIG. 4. **Probability of the observed base frequency deviations at top2 cleavage sites for the human and yeast wild-type enzymes in the presence of amsacrine (*AMSA*).** Position 0 corresponds to the cleavage site. The *panels* present the probability of the observed base frequency deviations from expectation for the indicated enzyme. Cleavage sites for the human (*panel A*) and the yeast (*panel B*) wild-type enzymes were analyzed. Drug concentration was 200 μM. *yWT,* yeast wild-type enzyme; *hWT,* human wild-type enzyme.

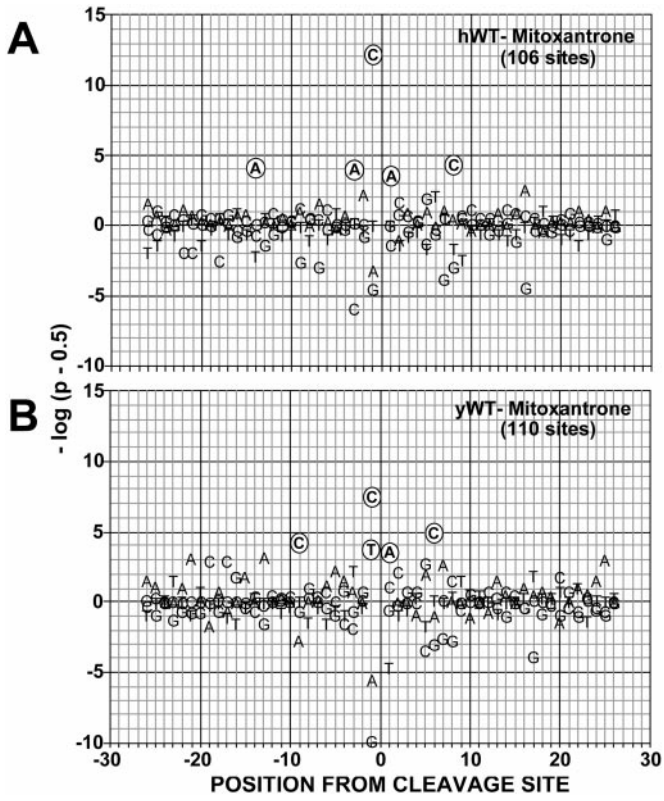

FIG. 5. **Probability of the observed base frequency deviations at top2 cleavage sites for the human and yeast wild-type enzymes in the presence of mitoxantrone.** Position 0 corresponds to the cleavage site. The *panels* present the probability of the observed base frequency deviations from expectation for the indicated enzyme. Cleavage sites for the human (*panel A*) and the yeast (*panel B*) wild-type enzymes were analyzed. Drug concentration was 1 μM. *yWT,* yeast wild-type enzyme; *hWT,* human wild-type enzyme.

In case of mitoxantrone (Fig. 5 and Table I), the consensus sequence for the preferred mitoxantrone intercalation site (5′-AC(A/G)) (35) was reflected by a preference for A^{+1} in yeast top2 (38 of 110 sites) and human top2α (38 of 106 sites). The preference of C at position +2 did not reach significance for either protein, and position +3 did not show any preference. The human enzyme showed a strong preference for C^{-1} (62 of 106 sites). This preference for C^{-1} was also seen in the yeast protein, although less strong (60 of 110 sites). Besides the yeast protein revealed an additional preference of T at position −1 (40 of 110 sites) that was not apparent in the human top2α. In addition to differences in the base preferences for the positions flanking the cleavage sites, the proteins also showed individual preferences at positions −3 and +8 for the human top2α and at positions −9 and +6 for the yeast top2. Thus, our data show significant differences in base sequence preferences between human and yeast top2 enzymes in the presence of amsacrine and mitoxantrone. In the case of etoposide, no significant difference in base preference was observed despite clear differences in observed DNA cleavage patterns.

Different Base Preferences for Yeast, Human, and E. coli Top2 in the Presence of CP-115,953—In the presence of the fluoroquinolone CP-115,953 (Fig. 6 and Table II), the human protein preferred cleavage sites with C^{-1} (44 of 79 sites), A^{+1} (38 of 79 sites), and (more weakly) A^{-2} (29 of 79 sites). These results are consistent with other reports (33, 36). Complementary preferences for T^{+4} and G^{+5} were also observed. The yeast top2 showed additional preferences for T^{-1} (C^{-1} and T^{-1}, 49 of 65 sites) and for G^{+1} (A^{+1} and G^{+1}, 54 of 65 sites). There was no clear preference at position −2 for the yeast protein. Interestingly, gyrase showed the T^{-1} and G^{+1} preferences observed for yeast top2 (41 and 50 of 107 sites, respectively). These preferences are in agreement with previous reports obtained with a different fluoroquinolone (37). Thus, it appears that the base preferences for the CP-115,953-induced sites in gyrase are more similar to the yeast than to the human top2α.

Homologous Mutations of Conserved Serine Residues Alter the Enzyme-DNA and Drug Interactions for Both Yeast and Human Top2—We recently reported that mutation of $Ser^{740} \rightarrow$ Trp in yeast top2 affects both DNA and drug interactions (24). To analyze the effect of the homologous mutation in human top2α ($Ser^{763} \rightarrow$ Trp), we compared the calcium-promoted DNA cleavages for both mutant proteins (Fig. 7). Even in the absence of drug (in the presence of Mg^{2+}), both mutants presented different cleavage patterns compared with the corresponding wild-type proteins. When magnesium was replaced by calcium, higher levels of DNA cleavage were only seen in the yeast proteins, *i.e.* in the wild-type enzyme and in $top2^{S740W}$. New DNA cleavage sites common to both of the mutant proteins were seen in the presence of Mg^{2+} and Ca^{2+}, although there were considerable differences in cleavage intensity. Most of the DNA cleavage sites in the upper and lower strands were staggered by 4 base pairs with a 5′-overhang, as expected for concerted top2-induced double-strand cleavage (see Fig. 3) (2, 8, 11).

Since the $Ser^{740} \rightarrow$ Trp mutation in yeast affects the DNA cleavage patterns induced by both intercalating and non-intercalating drugs (24) and confers partial resistance to fluoroquinolones and collateral hypersensitivity to etoposide (38), we compared the drug-induced DNA cleavage sites for the human

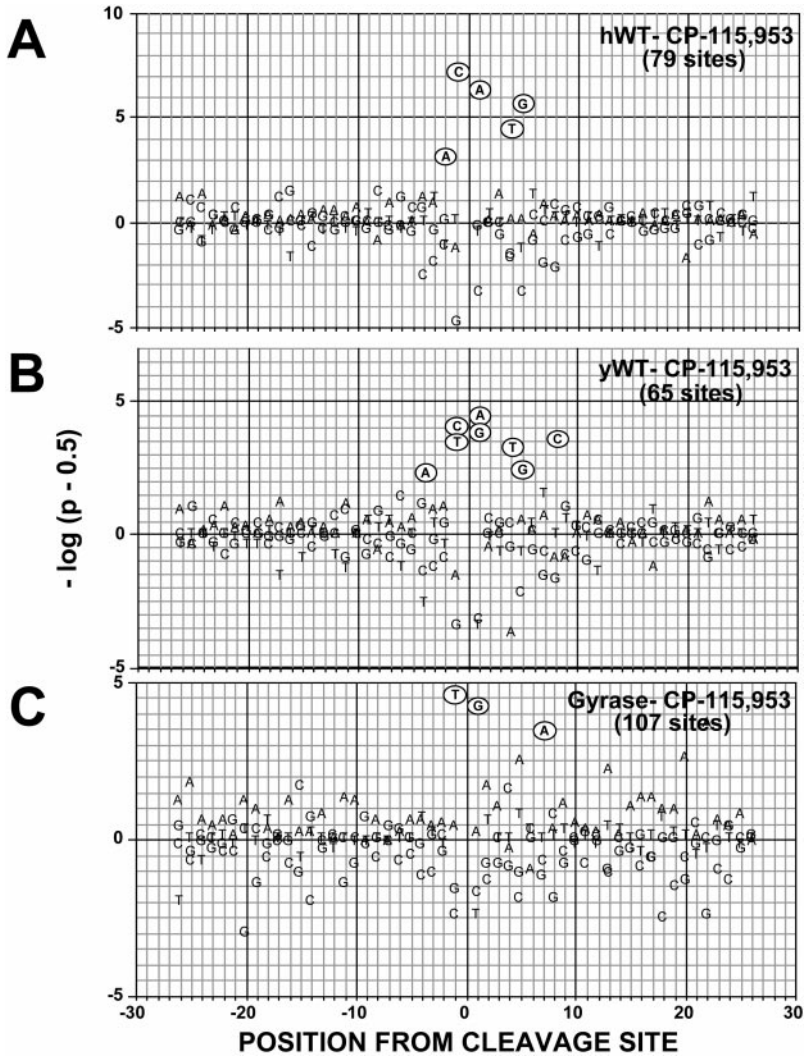

FIG. 6. **Analysis of base preferences at top2 cleavage sites for the human, yeast, and bacterial wild-type enzymes in the presence of CP-115,953. (100 μM).** Position 0 corresponds to the cleavage site. *Panels A—C,* probability of the observed base frequency deviations from expectation. Cleavage sites for the human (*panel A*), the yeast (*panel B*), and the *E. coli* (*panel C*) wild-type enzymes were analyzed. *yWT,* yeast wild-type enzyme; *hWT,* human wild-type enzyme.

TABLE II

Base distribution at each position of CP-115,953-induced DNA cleavage sites

Underlined numbers represent base frequencies significantly ($p < 0.001$) greater or lower than expected.

hWT-CP115,953 (79 sites)																				
A	22	16	12	24	16	19	27	26	29	12	38	19	25	21	17	12	22	20	17	19
C	23	23	31	19	18	27	10	10	15	44	9	19	22	10	9	27	24	27	16	28
G	21	19	19	18	31	18	25	20	25	7	17	22	15	14	42	16	10	11	26	16
T	13	21	17	18	14	15	17	23	10	16	15	19	17	34	11	24	23	21	20	16
yWT-CP115,953 (65 sites)																				
A	15	19	11	18	18	22	27	23	20	9	25	12	14	4	17	15	18	9	9	14
C	17	14	13	12	25	16	9	9	12	26	6	21	20	20	9	19	14	30	13	13
G	18	12	23	17	14	13	24	17	21	7	29	21	20	14	27	13	9	9	22	20
T	15	20	18	18	8	14	5	16	12	23	5	11	11	27	12	18	24	17	21	18
Gyrase-CP115,953 (107 sites)																				
A	33	23	29	22	25	29	30	28	29	30	27	35	30	21	35	17	39	29	32	29
C	29	26	24	28	24	25	20	21	31	15	18	21	30	41	20	32	24	36	25	28
G	24	37	33	37	35	30	28	33	26	21	50	26	24	22	21	31	21	18	23	30
T	21	21	21	20	23	23	29	25	21	41	12	25	23	23	31	27	23	24	27	20
Position from cleavage site																				
	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10

top2α^{S763W} protein to the corresponding wild-type human top2α (Fig. 8). Several cleavage sites induced in the presence of CP-115,953 were markedly reduced in the top2α^{S763W} (at positions 2842, 2883, 2901, 2908, 2912, and 2959). On the other hand, the human top2α^{S763W} caused increased cleavage at specific sites in the presence of etoposide (at positions 2771, 2784, 2816, 2901, and 2996), compared with the human wild-type top2α. Reduced cleavage in the presence of etoposide was detected at other sites (for instance at 2974). Multiple changes were also observed in cleavage sites induced in the presence of

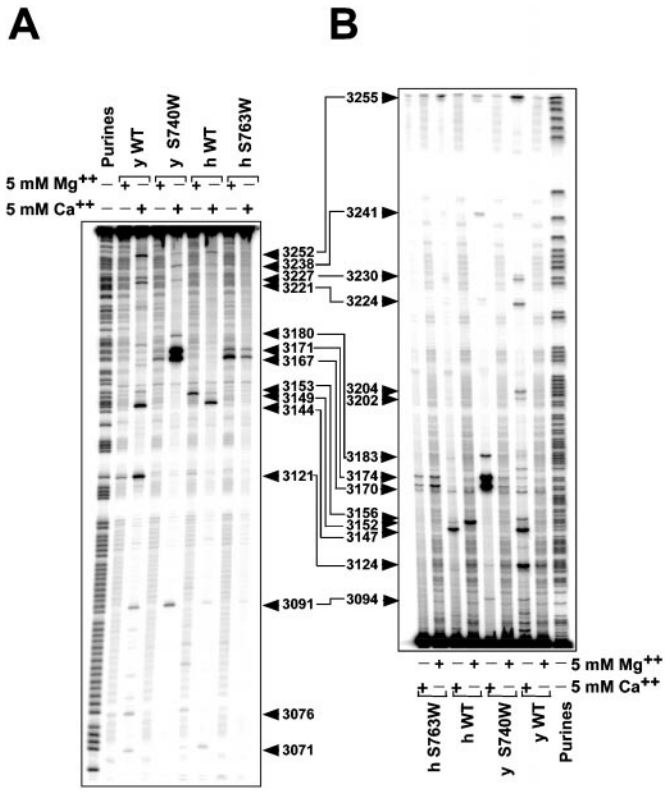

FIG. 7. **Homologous mutations in yeast top2 and human top2α alter the calcium-promoted DNA cleavages.** A 254-base pair DNA fragment from the first c-*MYC* intron was prepared. *Panel A,* labeling of the upper DNA strand at position 3035. *Panel B,* labeling of the lower DNA strand at position 3288. Top2 reactions were performed at 37 °C for 30 min in the presence of 5 mM $MgCl_2$ or 5 mM $CaCl_2$ as indicated and stopped by adding EDTA and SDS (25 mM and 1% final concentrations, respectively). Purine ladders were obtained after formic acid reaction. *y WT*, yeast wild-type top2; *h WT,* human wild-type top2α; *y S740W,* yeast top2^{S740W}; *h S763W,* human top2α^{S763W}. *Double-headed arrows* correspond to DNA cleavage sites with a 4-base pair stagger that represent potential DNA double-strand breaks.

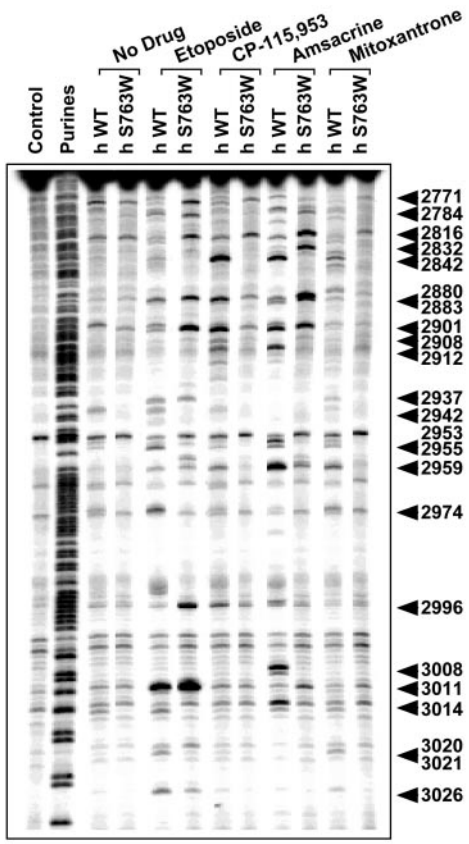

FIG. 8. **Ser^{763} → Trp mutation in human top2α alters DNA cleavage patterns induced by various top2 inhibitors in the human c-*MYC* gene.** A DNA fragment from the junction between the c-*MYC* first intron and first exon between positions 2671 and 3072 was labeled at the lower DNA strand at position 3072 with ^{32}P. Drugs are indicated *above each lane*. Concentrations used were as follows: etoposide, 100 μM; CP-115,953, 100 μM; amsacrine, 200 μM; and mitoxantrone, 1 μM. Purine ladder was obtained after formic acid reaction. *Control,* no top2, no drug treatment. *Numbers* correspond to genomic positions of the nucleotide covalently linked to top2. *H WT,* human wild-type top2α; *h S763W,* human top2α^{S763W}.

amsacrine and mitoxantrone (Fig. 8). Taken together, these results show that both mutations, yeast top2^{S740W} and human top2α^{S763W}, alter DNA cleavage in the absence or presence of top2 poisons.

Same Base Preference Alterations of the Etoposide-stabilized Cleavage Sites for the Yeast Mutant Top2^{S740W} and the Human Mutant Top2α^{S763W}—As described above, the yeast top2^{S740W} and the human top2α^{S763W} are hypersensitive to etoposide. Since the Ser^{740} → Trp mutation affects the DNA base preference of yeast top2 in the presence of this drug (24), it was therefore of interest to examine the effect of homologous mutation in the human protein. Cleavage sites for three c-*MYC* DNA fragments and one c-*JUN* fragment (see "Experimental Procedures") were analyzed for both DNA strands (Fig. 9 and Table III). As already shown for the human and yeast wild-type top2 enzymes (see above), both yeast top2^{S740W} and human top2α^{S763W} demonstrated a strong preference for C^{-1} in combination with the complementary (although slightly weaker) preference for G^{+5}. Human top2α^{S763W} and yeast top2^{S740W} extended the cleavage site preferences to include the C^{-2} and G^{+6} positions. It is remarkable that this relaxation of recognition position occurred in the same way in both the human and the yeast enzymes (Fig. 9) (24). A χ^2 test indicated that the combination of the C^{-1} and C^{-2} preference in yeast top2^{S740W} as well as in human top2α^{S763W} was not significantly more frequent than having C^{-1} or C^{-2} alone (data not shown). Thus, the novel C^{-2} base preference in both mutant proteins is independent of the C^{-1} preference. These data suggest a change in the protein-DNA interaction resulting from the homologous mutations Ser^{740} → Trp in yeast and Ser^{763} → Trp in human top2α, leading to an extension of the base preference to C^{-2} in the presence of etoposide.

Since we demonstrated that the Ser^{740} → Trp in yeast and the Ser^{763} → Trp mutation in human top2α increased sensitivity to etoposide and changed the base preferences in the same way, we tested whether human top2α^{S763W} and yeast top2^{S740W} enhanced DNA cleavage by etoposide at the same positions. Fig. 10 shows that a number of cleavage sites were enhanced for both mutant proteins (at positions 3252, 3091, 2996, 2959 and to lesser extent at positions 3141 and 3073). In addition, reduced cleavage for both mutants was observed at positions 2974 and 3121. Several sites, however, showed differences between human top2α^{S763W} and yeast top2^{S740W}, *e.g.* at positions 3026, 3020, 2901, and 2816. In particular, cleavage at position 3175 was enhanced for yeast top2^{S740W} but markedly reduced for human top2α^{S763W}. Thus, human top2α^{S763W} and yeast top2^{S740W} preserve, at least partially, the differences described above between human and yeast protein-DNA interactions.

Base Preference of Etoposide-induced, Heat-stable Cleavage Complexes Induced by Human Top2α^{S763W}—We recently reported that cleavage complexes mediated by yeast top2^{S740W} in the presence of etoposide have enhanced stability (24, 38). The

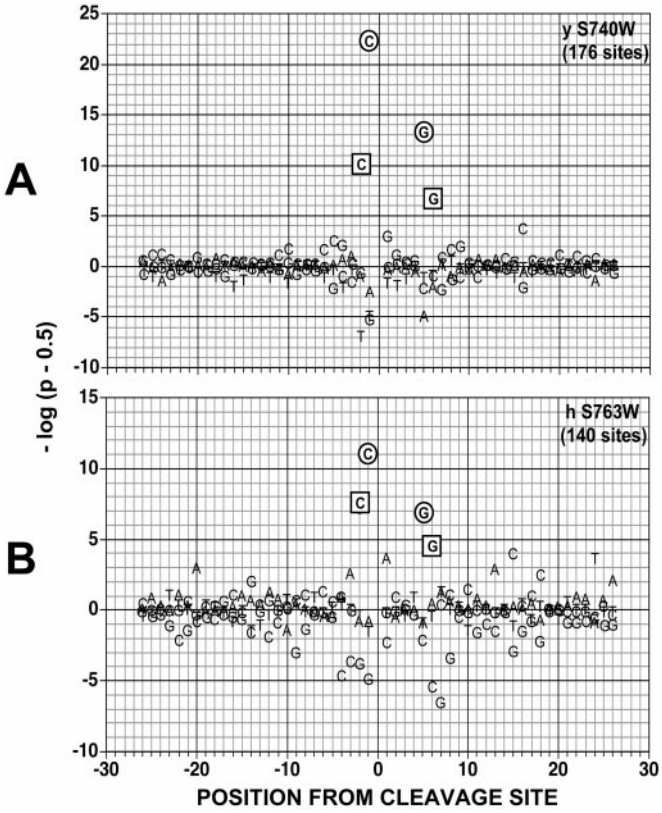

FIG. 9. **Probability of the observed base frequency deviations at top2 cleavage sites for yeast top2^{S740W} and human top2α^{S763W} in the presence of etoposide.** Reactions were performed in the presence of etoposide (100 μM). *Panels A* and *B,* position 0 corresponds to the cleavage site. Probability of the observed base frequency deviations from expectation. *y,* yeast; *h,* human.

effect of the Ser^{763} → Trp mutation on the stability of human top2α-DNA cleavage complexes was determined by examining the heat reversibility of the ternary complexes formed with drug, protein, and DNA. Cleavage reactions were carried out with the human top2α or top2α^{S763W} for 30 min at 37 °C, after which reaction mixtures were heated to 65 °C for various times prior to the addition of SDS. Fig. 11 shows that most of the etoposide-stabilized cleavage sites were readily reversible for the wild-type protein. In contrast, a number of cleavage sites induced by the human top2α^{S763W} showed slow reversal (positions 3091, 3207, 3223, 3238, 3124, 3183, etc.) or no detectable reversal after incubation at 65 °C for 20 min (positions 3167, 3171, 3252, 3170, 3174, 3210, etc.). Enhanced heat stability of the DNA cleavage sites induced by human top2α^{S763W} was also observed in other c-*MYC* DNA fragments (data not shown). Enhanced heat stability was also observed with the human wild-type top2α at certain sites (positions 3252, 3175, 3194, 3178, etc.). However, the stability was considerably less than for the human top2α^{S763W} protein. As already shown for yeast top2^{S740W} (24), cleavage sites with slow reversibility exhibited highly significant preferences for C^{-1} in combination with less strong C^{-2} preference in human top2α^{S763W}, whereas rapidly reversible cleavage sites did not show any preferences at positions −1 and −2 (data not shown). Hence, both mutations Ser^{740} → Trp in yeast and Ser^{763} → Trp in human top2α similarly alter the DNA recognition of the corresponding enzyme, markedly affect the interaction with inhibitors, and enhance the stability of the top2 cleavage complexes in the presence of etoposide.

DISCUSSION

The DNA sequence preference of drugs that target DNA top2 has been widely investigated (34). Early studies showed that topoisomerase-targeting drugs influence the sequence specificity of DNA cleavage by top2 compared with sites of DNA cleavage in the absence of drugs (28, 29, 39). Not surprisingly, drugs that bind DNA in the absence of enzyme most commonly resulted in cleavage specificities that differed from that seen with the enzyme in the absence of drug. Nonetheless, the cleavage specificity induced by intercalating drugs frequently differed from that expected, based on the binding of drugs to DNA in the absence of enzyme.

A key issue in understanding the mechanism of action of top2-targeting drugs is the determination of where drugs bind in the covalent complex. Important clues can be obtained from the DNA sequence of cleavage sites induced by intercalating drugs. For example, the intercalator amsacrine with human top2α exhibited the strongest preference at the +1 base (29). Recent biochemical studies by Kreuzer and colleagues (17) using a photoreactive amsacrine analog demonstrated reactivity only with the −1 and +1 bases, in agreement with the results suggested from the DNA cleavage pattern.

Only recently have investigators begun to compare the effects of different enzymes on DNA cleavage specificities with the same top2 poison. This problem is of particular interest because mammalian cells express two different top2 isoforms, α and β (2). A recent study compared recombinant forms of human α and β and found similar cleavage specificities for teniposide and the anthracycline 4-demethoxy-3′-deamino-3′-hydroxy-4′-epidoxorubicin (40). The cleavage specificity was also found to be the same for mouse top2.

Yeast has been commonly used to analyze topoisomerase functions and to study the biochemistry and molecular biology of topoisomerase inhibitors (2, 3, 22). Of particular importance is the determination of two different structures of the breakage/rejoining domains of the enzyme by x-ray crystallography (41, 42). A model for the binding of top2 to DNA has been proposed (43). Although details of specific protein:nucleic acid contacts will require a solution of the structure of the protein bound to DNA, the model is consistent with the notion that residues in the helix-turn-helix domain play key roles in interacting with DNA near the cleavage site and that this domain is also close to sites where top2-targeting drugs interact with DNA (24).

Results reported here showed strong similarities between yeast top2 and recombinant human top2α in the cleavage site preferences for several agents. However, several intriguing differences were noted. Interestingly, the non-intercalating agent etoposide showed clear similarities. Both human and yeast top2 have a strong preference for a C at the −1 position, along with a complementary preference for G at the +5 position. In addition, yeast and human enzymes with homologous mutations in the helix-turn-helix domain (Ser^{740} → Trp and Ser^{763} → Trp for yeast and human, respectively) showed the same change in cleavage specificity, a preference for a C at −2 (and G at +6) that is independent of the base at the −1 position. This result is consistent with an etoposide-binding site that is well conserved between the two enzymes.

Clerocidin is a top2 poison that has an action that is analogous to the Ser^{740} → Trp mutant of yeast top2 and the Ser^{763} → Trp mutant of human top2α. Clerocidin generated heat- and salt-stable covalent complexes with human top2α (44) and also heat-stable complexes with yeast top2.[2] The sequence preference for clerocidin with human top2α was G at position −1 (45),

[2] J. L. Nitiss, unpublished results.

TABLE III

Base distribution of DNA cleavage sites for top2^{S740W} or top2^{S763W} in the presence of etoposide

Underlined numbers represent base frequencies significantly ($p < 0.001$) greater or lower than expected.

yS740W (176 sites)																				
A	32	44	40	41	37	46	51	47	26	25	36	38	39	36	17	23	40	32	38	37
C	65	54	43	52	64	65	37	36	94	114	49	52	55	50	29	41	62	61	37	43
G	51	38	50	43	45	31	62	61	45	20	62	61	54	51	100	84	32	34	61	54
T	28	40	43	40	30	34	26	32	11	17	29	25	28	39	30	28	42	49	40	42
hS763W (140 sites)																				
A	21	34	34	30	27	30	41	46	22	27	52	28	32	33	26	36	42	41	36	34
C	40	49	48	36	50	43	16	20	70	80	25	44	39	30	20	18	44	47	32	51
G	40	20	25	32	34	30	43	42	22	12	30	38	41	36	70	62	14	18	35	34
T	37	38	34	41	30	36	38	31	26	22	32	31	28	41	24	24	41	36	36	22
Position from cleavage site																				
	−10	−9	−8	−7	−6	−5	−4	−3	−2	−1	1	2	3	4	5	6	7	8	9	10

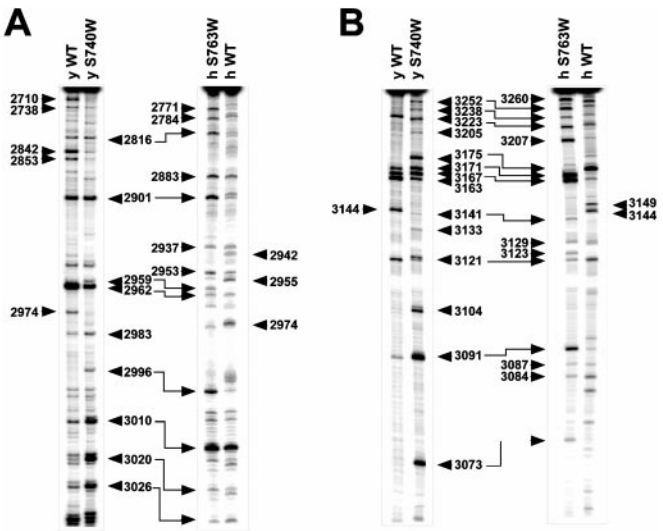

FIG. 10. **Comparison of yeast top2^{S740W}- and human top2α^{S763W}-induced DNA cleavage sites in the presence of etoposide.** DNA fragments were the same as described for Fig. 2. Reactions were performed in the presence of etoposide (100 μM). *y S740W,* yeast top2^{S740W}; *h S763W,* human top2α^{S763W}; *WT,* wild type.

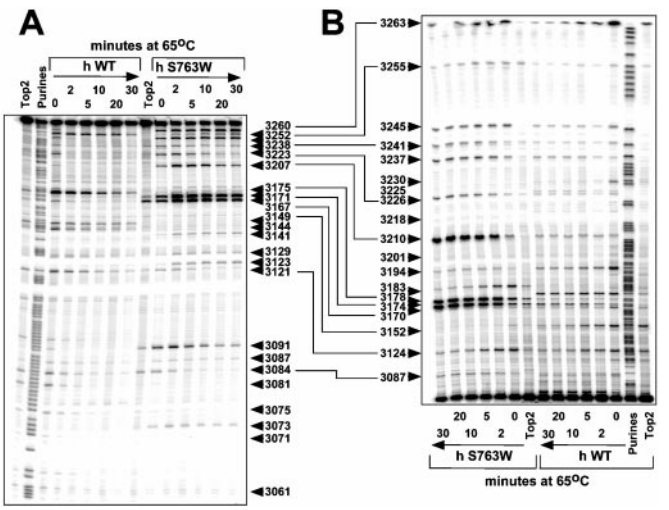

FIG. 11. **Cleavage complexes stabilized by etoposide with human top2α^{S763W} exhibit enhanced heat stability.** A 254-base pair DNA fragment from the first c-*MYC* intron was prepared. *Panel A,* labeling of the upper DNA strand at position 3035. *Panel B,* labeling of the lower DNA strand at position 3288. Top2 reactions were performed at 37 °C for 30 min in the presence of etoposide (100 μM). The reactions were then incubated at 65 °C for the indicated times prior to the addition of SDS and proteinase K. *Top2,* no drug treatment; *Control,* no top2, no drug treatment. *Numbers* correspond to genomic positions of the nucleotide covalently linked to top2. *h WT,* human wild-type top2α; *h S763W,* human top2α^{S763W}. *Connected arrowheads* correspond to DNA cleavage sites with a 4-base pair stagger that represent potential DNA double-strand breaks.

suggesting that interactions between the −1 base and drug may be an important determinant of the stability of covalent complexes.

The helix-turn-helix domain is also important in drug action with the non-intercalating fluoroquinolones. It is well established that amino acids around Ser^{83} of *gyrA* are the principal site of resistance mutations to fluoroquinolones in *E. coli* (32, 46). Biochemical results also suggested the presence of a quinolone-binding site in the vicinity of Ser^{83} (47). Resistance to quinolones has also been observed in yeast mutants with changes in this region (38, 48). Interestingly, we detected differences between yeast top2 and human top2α in DNA cleavage specificity induced by the fluoroquinolone CP-115,953. For all three topoisomerases examined, there were clear sequence preferences at both the −1 and +1 bases. The specificities for all three enzymes were somewhat different, but in each case the specificities at the −1 and +1 positions were pyrimidine and purine, respectively. As was the case for etoposide, complementary preferences, in this case at positions +4 and +5, were also seen.

The most extensive differences between yeast and human top2α in cleavage pattern specificities were observed with drugs that intercalate in DNA. For human top2α, a preference for A at the +1 position was observed, along with a complementary T^{+4} preference as previously reported (18, 29). A statistically significant preference at the −1 position was not observed. By contrast, yeast showed a clear preference for T at the −1 position, along with A at +1. Thus, unlike the human enzyme, there is a clear preference with the yeast enzyme at both positions −1 and +1. This result demonstrates that different drugs cannot be categorized just on the DNA sequence preference around the cleavage site.

The differences between human top2α and yeast top2 seen with the intercalating drug mitoxantrone are more complicated. Both the human and yeast enzymes exhibited preferences at both −1 and +1 positions, but other preferences were also seen, such as A at position −3 with the human enzyme and C at position +6 with the yeast enzyme. One factor that may contribute to this more complicated pattern is the strong inhibition of cleavage seen at high mitoxantrone concentrations (49–51). Perhaps the complex pattern that arises for both enzymes may be due in part to the ability of mitoxantrone to inhibit cleavage in a sequence-dependent manner.

A recent model has attempted to explain the similar sequence preferences of different top2 poisons by suggesting that

drugs that have a common sequence preference share a common pharmacophore (52). In this model, a top2 poison is modeled as consisting of two "modules," one that mediates DNA binding, *e.g.* which intercalates in DNA, and a second module that interacts with the enzyme. By this model, sequence specificity would be determined mainly by the DNA binding module, whereas the potency of the drug would also depend on the second module. The results presented here demonstrate that the same topoisomerase poison with the same DNA substrate exhibits different sequence specificities with different top2 enzymes. Thus, our results require a modification of the model. For example, enzyme binding, DNA cleavage, and strand separation of the 4-base overlap between sites of cleavage may lead to a reorientation of the drug interacting with DNA (or enzyme), and the reorientation may affect the ability of the drug to prevent religation. Such reorientation seems particularly plausible for a drug molecule like amsacrine that intercalates between the −1 and +1 bases. The reorientation may involve specific contacts between the drug and the enzyme, and these contacts may be different for the yeast and human top2 enzymes.

Recent results from Osheroff and colleagues (19, 53) have stressed the importance of the enzyme in determining cleavage site specificity with non-intercalating top2 poisons. The results described here indicate that the enzyme plays a very important role in the cleavage specificity of intercalating top2 poisons as well. Since intercalators bind DNA with a distinct sequence preference, part of the base sequence specificity of top2 poisoning by these agents depends on drug-DNA interactions. The results presented here highlight the importance of interactions of all three components of the trapped covalent complex with protein, DNA, and drug.

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