

Importance of the Fourth Alpha-Helix within the CAP Homology Domain of Type II Topoisomerase for DNA Cleavage Site Recognition and Quinolone Action

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We report that point mutations causing alteration of the fourth alpha-helix (α 4-helix) of the CAP homology domain of eukaryotic (*Saccharomyces cerevisiae*) type II topoisomerases (Ser⁷⁴⁰Trp, Gln⁷⁴³Pro, and Thr⁷⁴⁴Pro) change the selection of type II topoisomerase-mediated DNA cleavage sites promoted by Ca²⁺ or produced by etoposide, the fluoroquinolone CP-115,953, or mitoxantrone. By contrast, Thr⁷⁴⁴Ala substitution had minimal effect on Ca²⁺- and drug-stimulated DNA cleavage sites, indicating the selectivity of single amino acid substitutions within the α 4-helix on type II topoisomerase-mediated DNA cleavage. The equivalent mutation in the gene for *Escherichia coli* gyrase causing Ser⁸³Trp also changed the DNA cleavage pattern generated by Ca²⁺ or quinolones. Finally, Thr⁷⁴⁴Pro substitution in the yeast type II topoisomerase rendered the enzyme sensitive to antibacterial quinolones. This study shows that the α 4-helix within the conserved CAP homology domain of type II topoisomerases is critical for selecting the sites of DNA cleavage. It also demonstrates that selective amino acid residues in the α 4-helix are important in determining the activity and possibly the binding of quinolones to the topoisomerase II-DNA complexes.

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 (9, 59). Studies in both prokaryotic and eukaryotic cells have demonstrated the importance of topoisomerases in transcription, DNA replication, and chromosome segregation (41, 58). Type II topoisomerases (Top2p) are conserved among eukaryotes. Gyrase and topoisomerase IV are bacterial Top2 enzymes with significant sequence similarity to eukaryotic Top2 proteins (4, 39). Eukaryotic Top2 enzymes are homodimeric and cleave one DNA duplex (the G, or gate, segment) using two tyrosine residues (one from each Top2p monomer) to attack phosphodiester bonds on both DNA strands. In this process, each enzyme monomer becomes covalently attached to the 5' end of the cleaved DNA by a phosphotyrosine linkage. Top2p then opens the DNA, bridging

the gap in the G segment. A second DNA duplex (the T, or transported, segment) passes through the gap. Thereafter, the G segment is resealed, and the T segment is liberated from the enzyme (4, 53, 59).

Under physiological conditions, the covalent Top2p-DNA complexes (referred to as cleavage complexes) are normally short-lived intermediates in the catalytic cycle of the enzyme. Top2p requires the presence of a divalent cation for catalytic activity, which is Mg²⁺ under physiological conditions. However, when magnesium is replaced by Ca²⁺, a higher level of Top2p is trapped in a Ca²⁺-promoted covalent enzyme-DNA complex, and DNA double- and single-stranded DNA cleavage can be detected (44, 45).

Beyond its vital cellular functions, Top2p is the primary cytotoxic target for some of the most active drugs used for the treatment of human cancers (see below) (3, 10, 11, 29, 48). In addition, inhibitors of bacterial type II topoisomerases, such as quinolones, are among the most widely prescribed antibacterial drugs (24, 36). Top2p inhibitors can be divided in two groups: Top2p catalytic inhibitors and Top2p poisons (48). Top2p catalytic inhibitors ("suppressors") are defined as drugs that inhibit enzyme activity but do not stabilize DNA cleavage complexes. Proflavine and 9-aminoacridine belong to this category (2, 54). Top2p poisons inhibit the enzyme by increasing the steady-state levels of DNA cleavage complexes (18, 33, 48). Hence, they convert Top2p into a physiological toxin that creates protein-linked DNA breaks in the genome of treated cells (10, 18, 34, 48). Top2p poisons can, in turn, be subdivided in two groups: DNA intercalators—which include doxorubicin,

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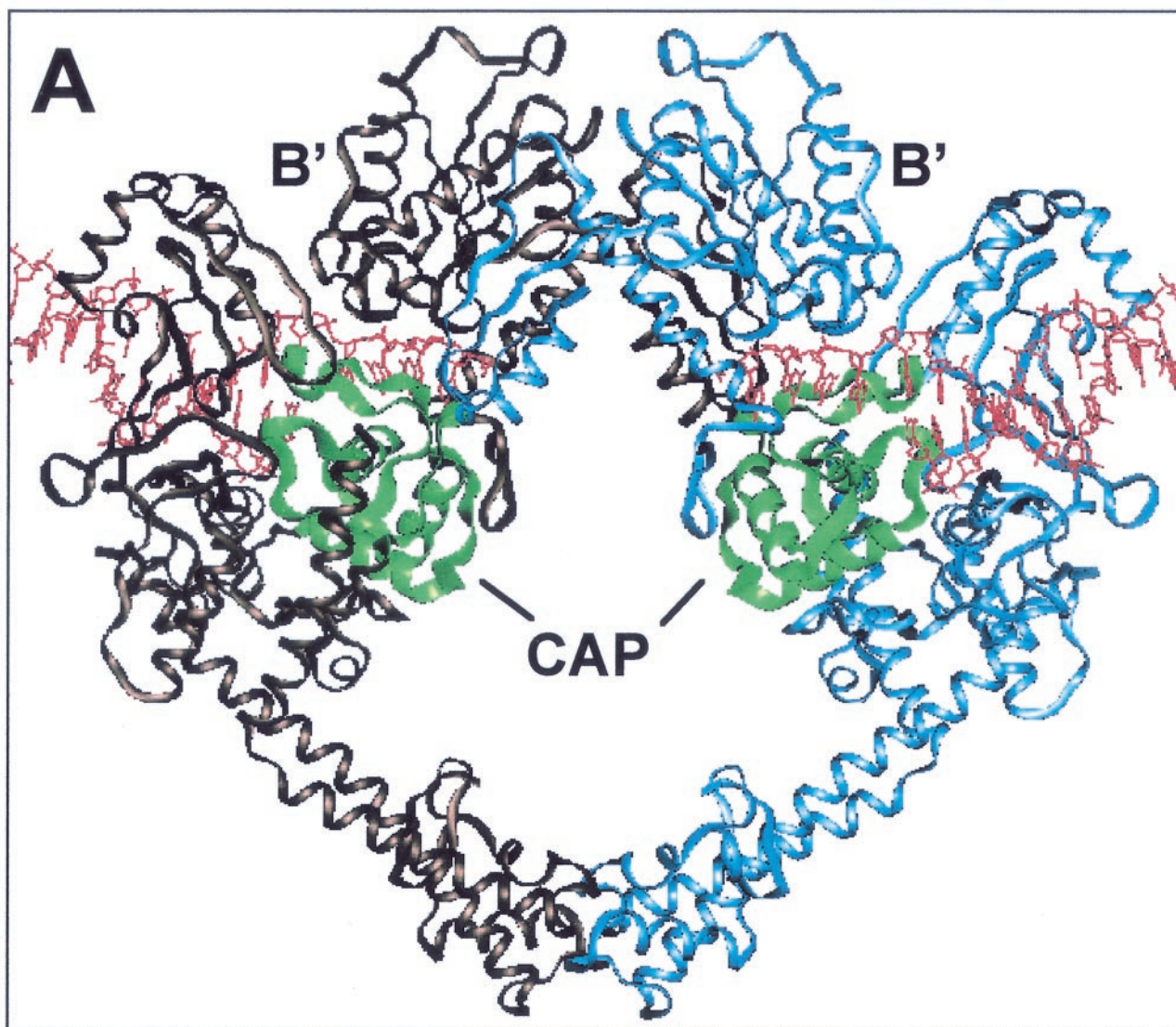


FIG. 1. CAP homology domain and positions of Ser⁷⁴⁰, Gln⁷⁴³, and Thr⁷⁴⁴ in yeast Top2p. (A) Illustration of dimeric *S. cerevisiae* topoisomerase II structure (Protein Data Bank accession number 1bgw). Drawings were generated using the program QUANTA (version 97). The helical ribbon representation shows the 92-kDa fragment of the yeast enzyme with a DNA fragment modeled into each of the putative DNA-binding sites (6). The CAP homology domain of each protomer is highlighted in green. (B) Close view of the putative DNA-binding region, presenting the proposed proximity of the α 4-helix within the CAP homology domain, including Ser⁷⁴⁰, Gln⁷⁴³, and Thr⁷⁴⁴ (in a stick model), to DNA. (C) Alignment of protein sequence for the yeast (Sc Top2p), human Top2p α (Hu Top2p α) and *E. coli* gyrase. The mutated residues studied in the present report are indicated by arrowheads. Shaded residues correspond to the α 4-helix (5). No shading is shown for human Top2p because of lack of structural data. Boxed regions indicate similarity between the amino acid residues. Ser⁷⁶³ in the human Top2p α sequence corresponds to Ser⁷⁴⁰ in yeast Top2p.

mitoxantrone, amsacrine, and ellipticines—and nonintercalators, whose main representatives are the demethylepipodophyllotoxins etoposide (VP-16), teniposide (VM-26), and the quinolones (37, 52), such as ciprofloxacin and norfloxacin, which are specific for bacterial Top2 enzymes (24, 36).

Previous studies showed that substitution of Gly⁷⁴⁷ to Glu of *Saccharomyces cerevisiae* Top2p (Fig. 1C) conferred resistance to doxorubicin, suggesting that the fourth alpha-helix (α 4-helix) is implicated in drug action (46). Furthermore, we recently reported that substitution of a conserved serine residue (Ser⁷⁴⁰Trp) in yeast Top2p confers resistance to quino-

lones and hypersensitivity to etoposide (26) and is associated with changes in DNA sequence preference at sites of cleavage (56). The first aim of the present study was to further investigate the role of the fourth helix (α 4) within the CAP homology domain for DNA cleavage site recognition. CAP homology domains, i.e., regions that are similar to the DNA-binding domain of the *Escherichia coli* catabolite activator protein (23, 40), have been determined in both the crystal structure of a 92-kDa fragment of yeast Top2p (6) and a 59-kDa fragment of the gyrase A subunit (GyrA) of *E. coli* gyrase (39). We examined the effect of different single-amino-acid substitutions

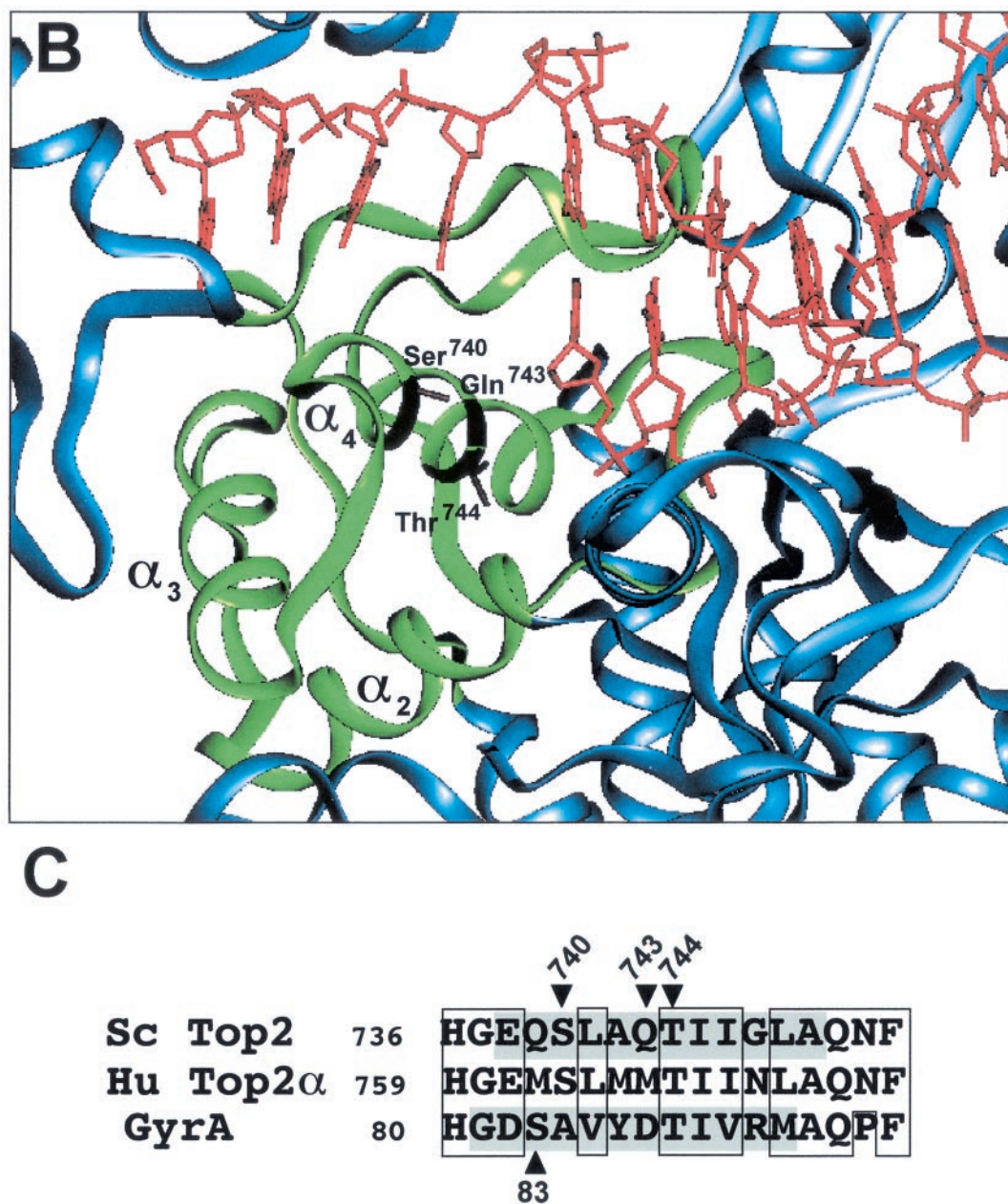


FIG. 1—Continued.

within the α_4 -helix (Fig. 1) on DNA cleavage patterns generated in the presence of Ca^{2+} or in the presence of nonintercalative and intercalative Top2p inhibitors. Four mutant yeast Top2p (Ser⁷⁴⁰Trp, Gln⁷⁴³Pro, Thr⁷⁴⁴Pro, and Thr⁷⁴⁴Ala) were compared to the wild-type enzyme. The Ser⁸³Trp substitution in the GyrA subunit, which is equivalent to the Ser⁷⁴⁰Trp substitution in yeast Top2p (Fig. 1C), was also compared to wild-type gyrase. The second aim of this study was to examine the role of the α_4 -helix for drug activity to gain further insight in the interaction between Top2p inhibitors and the Top2p-DNA complexes. Together, our data demonstrate that specific amino acid residues within the α_4 -helix are critical for DNA sequence recognition with regard to cleavage of the G segment

and for drug interaction within the enzyme-DNA complex. We also found that Thr⁷⁴⁴Pro substitution in yeast Top2p confers hypersensitivity to antibacterial quinolones.

MATERIALS AND METHODS

Materials, chemicals, and enzymes. Etoposide (VP16) was obtained from the Bristol-Myers Squibb Co., Wallingford, Conn. Mitoxantrone was from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, Md.). Azatoxin was provided by T. Macdonald, Department of Chemistry, University of Virginia, Charlottesville. CP-115,953 was a gift from P. R. McGuirk and T. D. Gootz of Pfizer Laboratories. Drug stock solutions were made in dimethyl sulfoxide 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 Labs (Gaithersburg, Md); Life Technologies, Inc. (Gaithersburg, Md.); New England Biolabs (Beverly, Mass.), or Qiagen Inc. (Valencia, Calif.). [γ - 32 P]ATP (6,000 Ci/mmol) was purchased from DuPont NEN (Boston, Mass.). PCR oligonucleotide primers were obtained from GIBCO BRL (Gaithersburg, Md.).

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-bp DNA fragment from the first intron was prepared between positions 3035 and 3288 (GenBank accession X00364) using the following oligonucleotides: 5'-GT AATCCAGAAGTGGATCGG-3' for the upper strand and 5'-ATGCGGTCCC TACTCCAAGG-3' for the lower strand (annealing temperature, 56°C). A 401-bp DNA fragment from the junction between the first intron and the first exon was prepared between positions 2671 and 3072 using the following oligonucleotides: 5'-TGCCGCATCCACGAAACTTT-3' for the upper strand and 5'-TTGACAAGTCACTTTACCCC-3' for the lower strand (annealing temperature, 60°C). A 480-bp fragment from the first exon containing promoters P₁ and P₂ was prepared between positions 2265 and 2745 using the following oligonucleotides: 5'-GATCCTCTCTCGCTAATCTCCGCC-3' for the upper strand and 5'-TCCTTGCTCGGGTGTGTAAGTTC-3' for the lower strand (annealing temperature, 70°C). A 213-bp fragment from the human *c-jun* gene was prepared between positions 5'-TGTTGACAGCGGGCGAAAGCAGS-3' for the upper strand and 5'-CGTCCTTCTTCTTGCGTGGCTCT-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 U of T4 polynucleotide kinase and 10 pM [γ - 32 P]ATP (100 μ Ci) in kinase buffer (70 mM Tris-HCl, pH 7.6; 0.1 M KCl; 10 mM MgCl₂; 5 mM dithiothreitol; bovine serum albumin [0.5 mg/ml]). Reactions were stopped by heat denaturation at 70°C for 15 min. After purification using Sephadex G-25 columns (Boehringer Mannheim), the labeled oligonucleotides were used for PCR. Approximately 0.1 μ g of the *c-myc* DNA that had been restricted by *Sma*I and *Pvu*II (fragment spanning positions 2265 to 2745) and *Xho*I and *Xba*I (fragment spanning positions 2671 to 3072 and fragment 3035 to 3288) was used as a 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, and DNA was purified using PCR Select-II columns (5Prime-3Prime Inc., Boulder, Colo.).

Overexpression and purification of yeast topoisomerase II and *E. coli* DNA gyrase. Wild-type yeast Top2p, Ser⁷⁴⁰Trp, Gln⁷⁴³Pro, Thr⁷⁴⁴Pro, and Thr⁷⁴⁴Ala proteins were overexpressed using YEptOP2-PGAL1 or YEptop2-S*W-PGAL1 and yeast strain JEL111⁻ (32) and purified to homogeneity as previously described (59). The detailed procedure has been described elsewhere (15). GyrA (gift of N. A. Gormley), GyrA^{S83W} (gift of C. J. R. Willmott), and GyrB were purified as described previously (38). GyrA^{S83W} was shown to have the expected level of quinolone-resistant supercoiling activity when complexed with wild-type gyrB.

Topoisomerase II-generated DNA cleavage reactions. For the yeast Top2p experiments, DNA fragments (5×10^4 to 10×10^4 dpm/reaction) were equilibrated with drug or with the corresponding 1% dimethyl sulfoxide concentration in 10 mM Tris-HCl (pH 7.5)–50 mM KCl–5 mM MgCl₂–2 mM dithiothreitol–0.1 mM Na₂EDTA–1 mM ATP–bovine serum albumin (15 μ g/ml) for 5 min before the addition of 8 U (80 ng) of purified Top2p in a 10- μ l final reaction volume. Unless otherwise indicated, reactions were for 30 min at 37°C. Reactions were stopped by adding EDTA and sodium dodecyl sulfate (25 mM and 1% final concentrations, respectively) and were further digested with proteinase K (0.4-mg/ml final concentration for 30 min at 55°C). For the DNA gyrase experiments, similar conditions were used except that the DNA cleavage reactions were performed with 10 to 15 U of purified *E. coli* gyrase for 120 min at 25°C (60).

Electrophoresis and base sequence analysis. For DNA sequence analysis, samples were precipitated with ethanol and resuspended in 5 μ l of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol 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 \times Tris-borate-EDTA buffer. Electrophoresis was performed at 2,500 V (60 W) for 2 to 3 h. The gels were dried on Whatman no. 3MM paper sheets and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and ImageQuant software. The determination of preferred bases around Top2p cleavage sites was done as described previously (8, 49, 57).

Molecular dynamics simulation. Modeling of the Thr⁷⁴⁴Pro substitution into published crystal structure data of a 92-kDa fragment of yeast Top2p (6) (PDB identification code 1bgw) was performed with the molecular modeling package QUANTA (version 96) run on both an SGI Crimson and an SGI Octane workstation. Energy calculations and related operations, such as energy minimizations

and the molecular dynamics simulations, were carried out with QUANTA's associated molecular mechanics program CHARMM, version 23.1, run on a separate computation host, a Digital 2100 AlphaServer 4/275, using the MSI parameter set version 22.0.

In order to generate a low-energy starting geometry for the subsequent molecular dynamics simulation, each of the wild-type and mutant structures was energy minimized in 5,000 steps of Adopted-Basis Newton-Raphson minimization. Each of these minimized structures was subjected to a total of 300 ps of molecular dynamics simulation using the Verlet algorithm for integration of the equations of motion. Using a step size of 1 fs, the structures were heated from 0 to 310 K in approximately 10 ps (9,982 steps), then equilibrated at 310 K for 40 ps (40,000 steps) with an equilibration frequency of 200 steps, and finally subjected to a simulation at 310 K for 250 ps (250,000 steps). Snapshot frames containing the current coordinates were written out every 50 steps.

RESULTS

Alteration of Ca²⁺-promoted DNA cleavage by Ser⁷⁴⁰Trp, Gln⁷⁴³Pro, Thr⁷⁴⁴Pro, and Thr⁷⁴⁴Ala substitutions in yeast Top2p and by the Ser⁸³Trp substitution in bacterial GyrA. It is well known that DNA cleavage by type II DNA topoisomerases can be stimulated by a variety of drugs (2, 18, 33, 34, 36, 37, 52), but cleavage can also be stimulated when Ca²⁺ is substituted for Mg²⁺ in the enzyme reaction (45, 50). In this case it is likely that the cleavage specificity reflects the natural sequence specificity of the enzyme. The Ca²⁺-promoted, drug-independent DNA cleavage sites generated by yeast wild-type Top2p (Top2p^{WT}) and four yeast mutant proteins (Top2p^{S740W}, Top2p^{O743P}, Top2p^{T744P}, and Top2p^{T744A}) were mapped in two different *c-myc* DNA fragments. Figure 2A and B show enzyme-specific differences. Cleavage was reduced at several sites for Top2p^{S740W} and Top2p^{O743P} compared to Top2p^{WT}. For example, cleavage at positions 3124, 3147, 3156, 3226, 2706 or 2707, 2735, 2741, 2745, and 2754 was reduced for Top2p^{S740W} and at that at positions 3094, 3170, 2754, and 2735 was reduced for Top2p^{O743P}. Most reduction in Ca²⁺-promoted cleavage was seen for Top2p^{T744P} (Fig. 2B), which is in contrast to the intensity of the drug-induced cleavage sites generated by Top2p^{T744P} (Fig. 3). Cleavage was enhanced for the Top2p^{S740W} enzyme at several positions (e.g., 3170, 3174, 3241, 2898, and 2704) compared to Top2p^{WT}. By contrast, the DNA cleavage pattern of the Top2p^{T744A} enzyme was consistently similar to Top2p^{WT} (Fig. 3A and B and data not shown in other *c-myc* fragments). These results indicate that yeast Top2p^{WT} and the mutant enzymes Top2p^{S740W}, Top2p^{O743P}, and Top2p^{T744P} present different Ca²⁺-promoted DNA cleavage patterns, whereas Top2p^{T744A} is comparable to Top2p^{WT}.

The GyrA^{S83W} substitution in *E. coli* gyrase is equivalent to Top2p^{S740W} in yeast (Fig. 1C) (26). Figure 2C shows a comparison of the Ca²⁺-promoted DNA cleavage patterns for wild-type gyrase (gyrase^{WT}) and GyrA^{S83W}. The number of Ca²⁺-promoted DNA cleavage sites was changed for GyrA^{S83W}, which is consistent with the corresponding results for Top2p^{S740W} (Fig. 2A and B). For GyrA^{S83W}, cleavage at positions 3110, 3120, 3205, 3226, and 3230 was very low or not detectable. Conversely, cleavage was enhanced at positions 3150 and 3198. Differences in Ca²⁺-promoted DNA cleavage patterns between GyrA^{S83W} and gyrase^{WT} were also observed in other *c-myc* fragments (data not shown). Together, these results demonstrate that single-amino-acid substitutions within the α 4-helix of the CAP homology domain affect Ca²⁺-promoted DNA cleavage for both Top2p and gyrase.

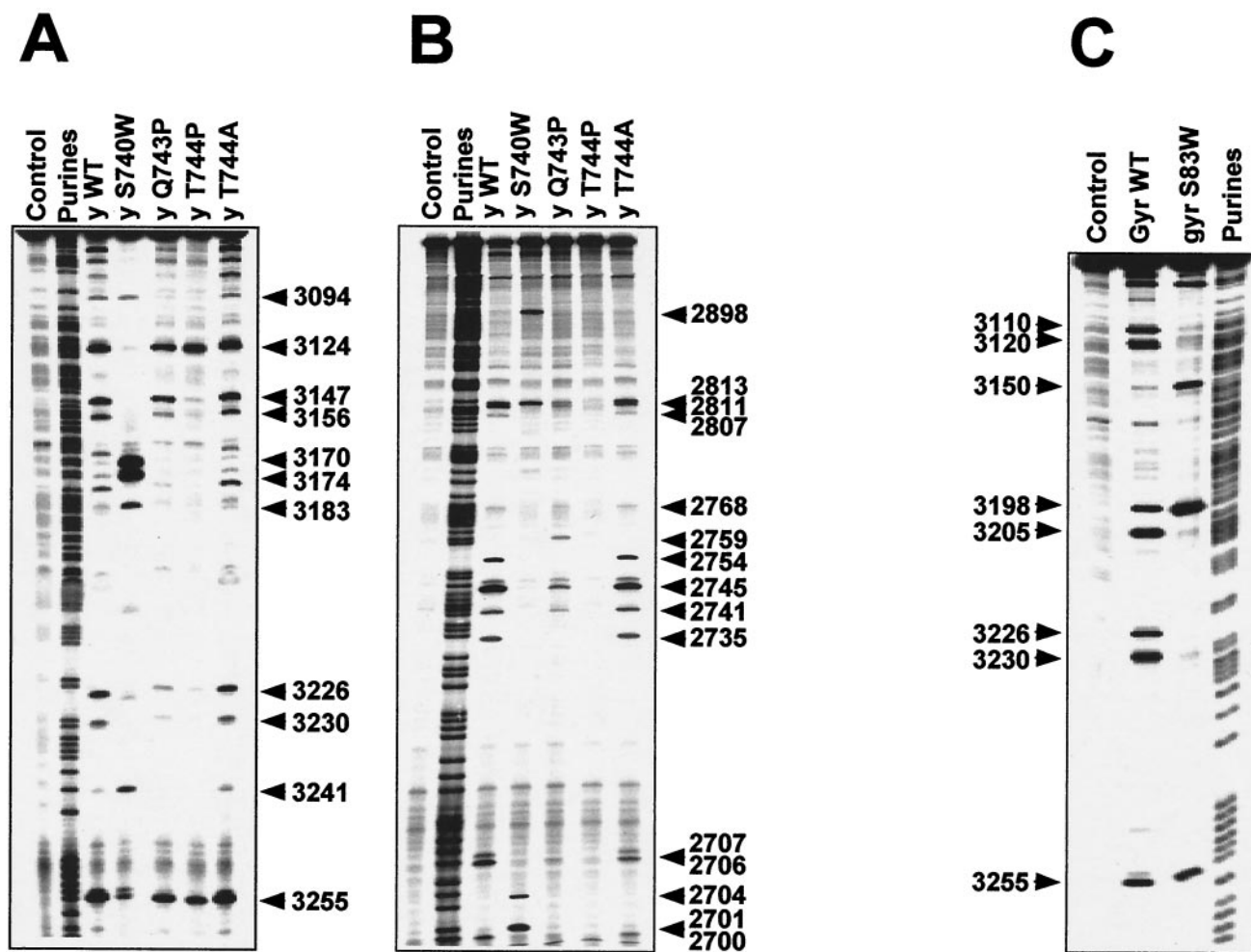


FIG. 2. Comparison of Ca^{2+} -promoted DNA cleavage patterns of yeast Top2p^{WT}, Top2p^{S740W}, Top2p^{Q743P}, Top2p^{T744P}, and Top2p^{T744A} as well as gyrase^{WT} and GyrA^{S83W}. DNA fragments from the human *c-myc* first intron (A) and from the junction between the *c-myc* first intron and first exon (B) were prepared by PCR. For each fragment, 5'-end-labeling was performed with ^{32}P . Yeast Top2p reactions were performed at 37°C for 30 min (A and B), and gyrase reactions were performed at 25°C for 2 h (C). In all reactions, the CaCl_2 concentration was 5 mM. Lanes: Control, without Top2 enzyme; Purine, ladder obtained after formic acid reaction; y WT, yeast wild-type Top2p; y S740W, yeast Top2p^{S740W}; y Q743P, yeast Top2p^{Q743P}; y T744P, yeast Top2p^{T744P}; y T744A, yeast Top2p^{T744A}; gyr^{WT}, gyrase^{WT} from *E. coli*; GyrA^{S83W}, mutant Ser⁸³Trp gyrase. Numbers correspond to genomic positions of the nucleotide covalently linked to the enzyme via the 5' phosphate.

Differences in DNA cleavage patterns generated by yeast Top2p^{S740W}, Top2p^{Q743P}, Top2p^{T744P}, Top2p^{T744A}, and Top2p^{WT} in the presence of Top2p poisons. To further investigate differences between Top2p^{S740W}, Top2p^{Q743P}, Top2p^{T744P}, Top2p^{T744A}, and Top2p^{WT}, we compared drug-stimulated DNA cleavage patterns. Etosiposide results are shown in Fig. 3A. Results for the fluoroquinolone CP-115,953 in are shown in Fig. 3B, and those for mitoxantrone are shown in Fig. 3C.

Top2p^{S740W} (as well as the corresponding yeast mutant) has been previously characterized as being resistant to the fluoroquinolone CP-115,953 and hypersensitive to etosiposide (26, 56). Compared with Top2p^{WT} and the other mutant proteins, several cleavage sites stimulated by etosiposide were markedly enhanced with Top2p^{S740W}, e.g., at positions 2711 to 2712, 2781, 2983, 3011, 3019, and 3026 (Fig. 3A).

Proline substitutions at positions 744 and 743 also changed the distribution of Top2p-mediated DNA cleavages. These

effects were more pronounced for Top2p^{T744P} than for Top2p^{Q743P} (Fig. 3). By contrast, Top2p^{T744A} behaved very similarly to Top2p^{WT} (Fig. 3).

Together, the data shown in Fig. 3 demonstrate that single mutations in the α 4-helix of Top2p (Ser⁷⁴⁰Trp, Gln⁷⁴³Pro, and Thr⁷⁴⁴Pro) changed enzyme-specific DNA cleavage patterns produced by nonintercalating and intercalating drugs.

Differences in quinolone-generated DNA cleavage patterns by the mutant GyrA^{S83W} and wild-type DNA gyrase. We next tested the role of the corresponding α 4-helix in the bacterial topoisomerase II, DNA gyrase. Figure 4 shows a comparison of the DNA cleavage patterns observed in the presence of two different quinolones, CP-115,953 and ciprofloxacin. Because the GyrA^{S83W} mutant is quinolone resistant (13, 42, 58, 60) and therefore bound to give less cleavage than gyrase^{WT}, we chose drug concentrations (100 μM) that are approximately three times the 50% inhibitory concentration for supercoiling (data not shown). As for yeast Top2p, the S83W substitution

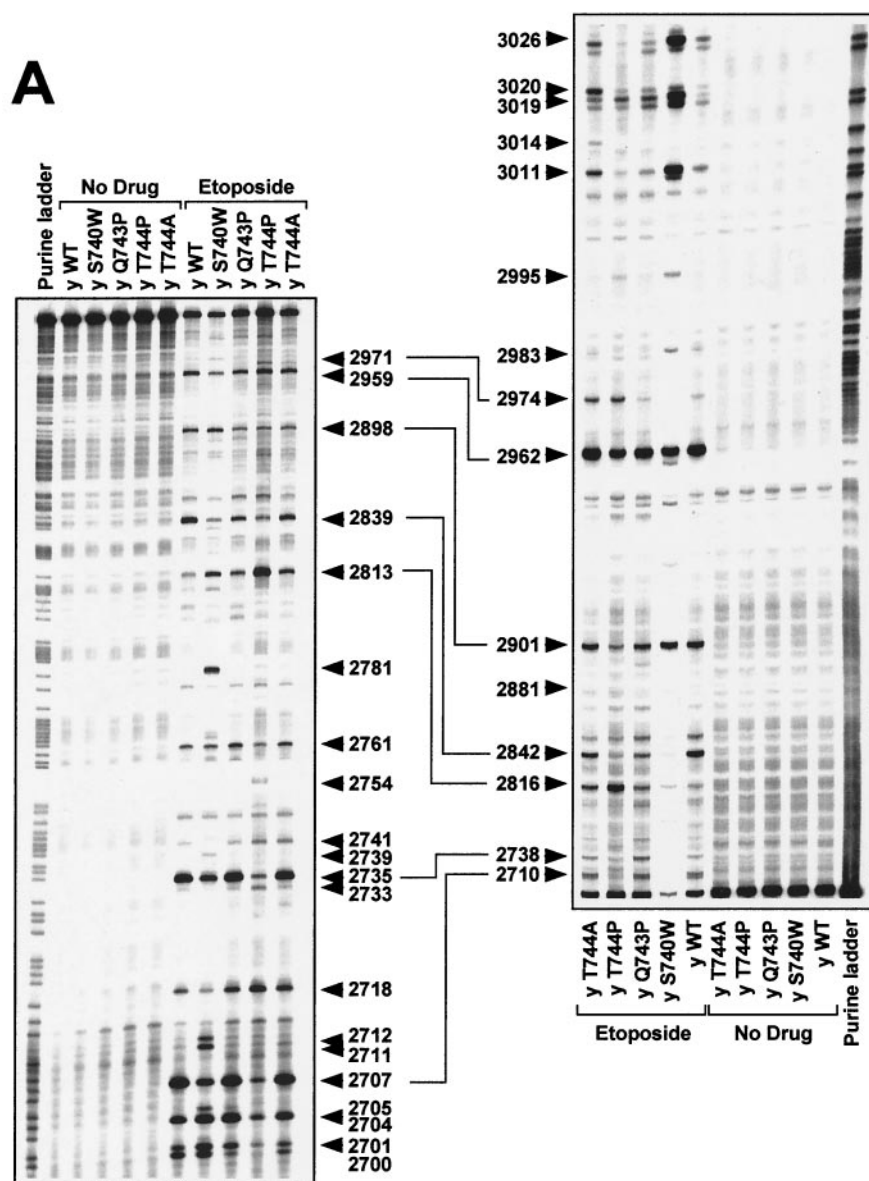


FIG. 3. DNA cleavage patterns generated by yeast Top2p^{WT}, Top2p^{S740W}, Top2p^{Q743P}, Top2p^{T744P}, and Top2p^{T744A} enzymes in the presence of etoposide, the fluoroquinolone CP-115,953, and the intercalator mitoxantrone. DNA fragments from the junction between the *c-myc* first intron and first exon between positions 2671 and 3072 were prepared by PCR using one primer labeled with ³²P at the 5' terminus. The left-hand panels show labeling of the upper DNA strand at position 2671. The right-hand panels show that labeling of the lower DNA strand was at position 3072. Top2 enzymes are indicated above each lane. Lanes: y WT, yeast wild-type Top2p; y S740W, yeast Top2p^{S740W}; y Q743P, yeast Top2p^{Q743P}; y T744P, yeast Top2p^{T744P}; y T744A, yeast Top2p^{T744A}. Concentrations were 100 μ M for etoposide (A) and CP-115,953 (B) and 1 μ M for mitoxantrone (C). Reactions were performed at 37°C for 30 min in the presence of 5 mM MgCl₂. Purine ladders were obtained after formic acid reaction. Numbers correspond to genomic positions of the nucleotide covalently linked to Top2p via the 5' phosphate. Double-headed arrows correspond to DNA cleavage sites with a 4-bp stagger that represent potential DNA double-strand breaks.

changed the enzyme cleavage pattern. Several cleavage sites were reduced in intensity for GyrA^{S83W} in the presence of CP-115,953 and ciprofloxacin (e.g., 2747, 2744, 2772, 2770, 2991, 3020, 3017, and 3020), whereas other sites were enhanced (e.g., 2722, 2893, 2768, 2794, 2805, and 2962). These results demonstrate that the GyrA^{S83W} mutation markedly affects the DNA sequence specificity of DNA gyrase.

Comparison of yeast Top2p^{T744P} and *E. coli* gyrase DNA

cleavage sites generated in the presence of quinolones. In contrast to the mutation causing Ser⁷⁴⁰Trp, which confers quinolone resistance, the Thr⁷⁴⁴Pro substitution causes hypersensitivity to CP-115,953 (14) (Fig. 3). Figure 5 compares DNA cleavage produced by CP-115,953, ciprofloxacin, and norfloxacin for GyrA^{WT}, Top2p^{WT}, and Top2p^{T744P}. Remarkably, prominent cleavage was observed for Top2p^{T744P} in the presence of ciprofloxacin and norfloxacin, whose antibacterial ac-

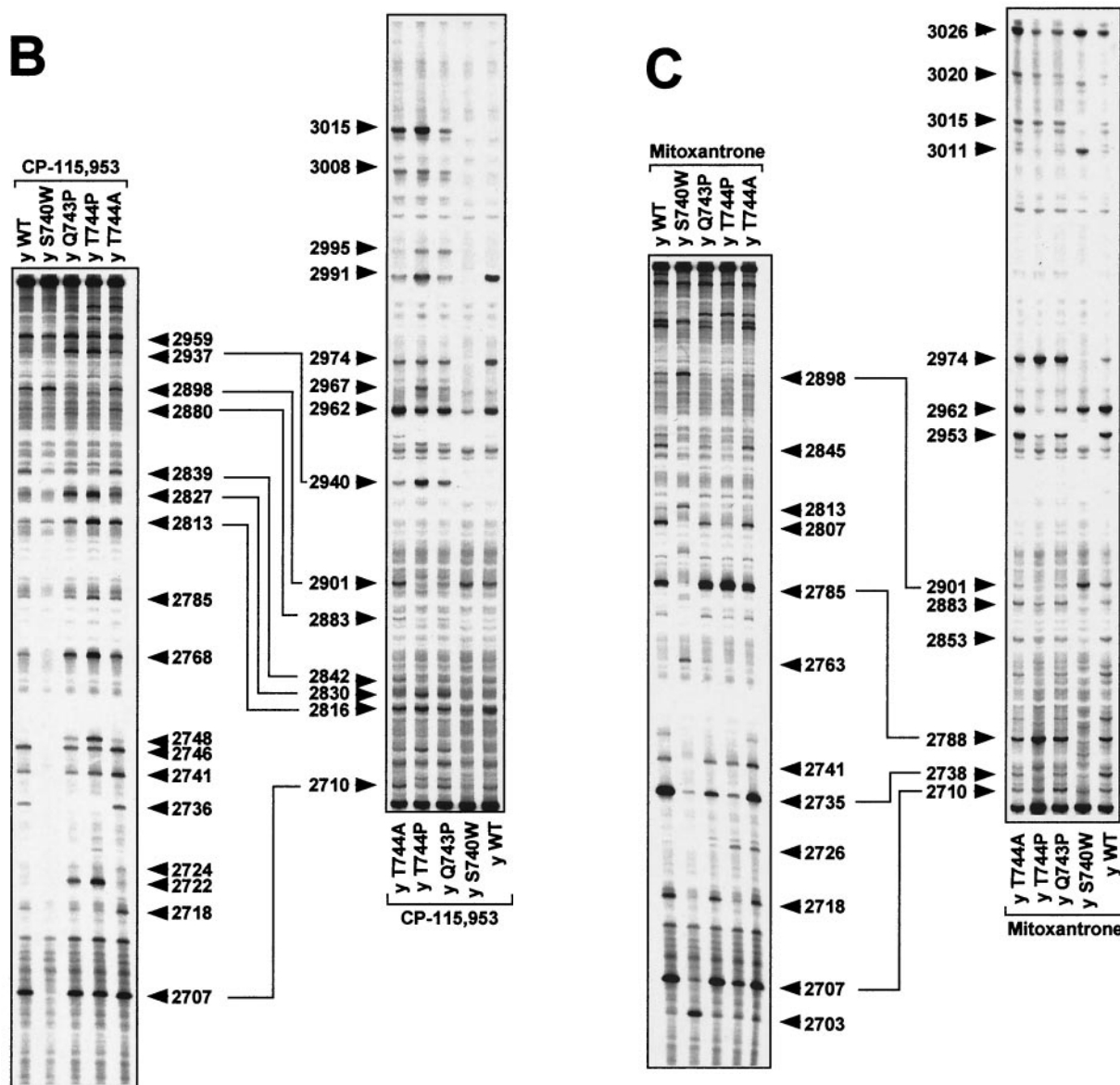


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tivities are due to specific inhibition of bacterial Top2p and have no effect on mammalian Top2p.

A number of cleavage sites stimulated by Top2p^{T744P} in the presence of quinolones matched the *E. coli* DNA gyrase sites (e.g., at positions 3202, 3179, 3145, 3121, 3108, 3066, and 3111). However, other sites were different for DNA gyrase. Enhanced sensitivity to antibacterial quinolones by the Thr⁷⁴⁴Pro substitution in yeast Top2p was also detected in other DNA fragments (data not shown).

Base preference of the CP-115,953-stabilized cleavage complexes for mutant Top2^{T744P} enzyme. Because we observed that the Thr⁷⁴⁴Pro substitution enhanced sensitivity to quinolones (including the fluoroquinolone CP-115,953) and produced common DNA cleavage sites with *E. coli* DNA gyrase, we examined whether this particular amino acid substitution affected the DNA base preference of yeast Top2p in the presence of CP-115,953. Cleavage sites for the three c-myc DNA

fragments and one c-jun fragment (see Materials and Methods) (Fig. 6) were analyzed for both DNA strands. For the wild-type yeast Top2p, CP-115,953 preferentially stabilized sites with C or T at position -1 (C⁻¹ + T⁻¹: 49 out of 65 sites) and A or G at position +1 (A⁺¹ + G⁺¹: 54 out of 65 sites). Complementary preferences (although slightly weaker) were seen for T⁺⁴ and G⁺⁵.

Top2p^{T744P} also demonstrated a preference for T at position -1, which was higher than that for the wild-type enzyme (41 out of 89 sites), in combination with preference for G at position +1 (44 out of 89 sites). However, preferences for C at position -1 and A at position +1 were not detectable in the mutant protein (Thr⁷⁴⁴Pro). Moreover, complementary preference also changed to A⁺⁵. Because bacterial Top2 enzymes are also sensitive to CP-115,953 (Fig. 5), we analyzed the base preferences for DNA gyrase. The observed preferences for T at position -1 (41 out of 107 sites) and G at position +1 (50

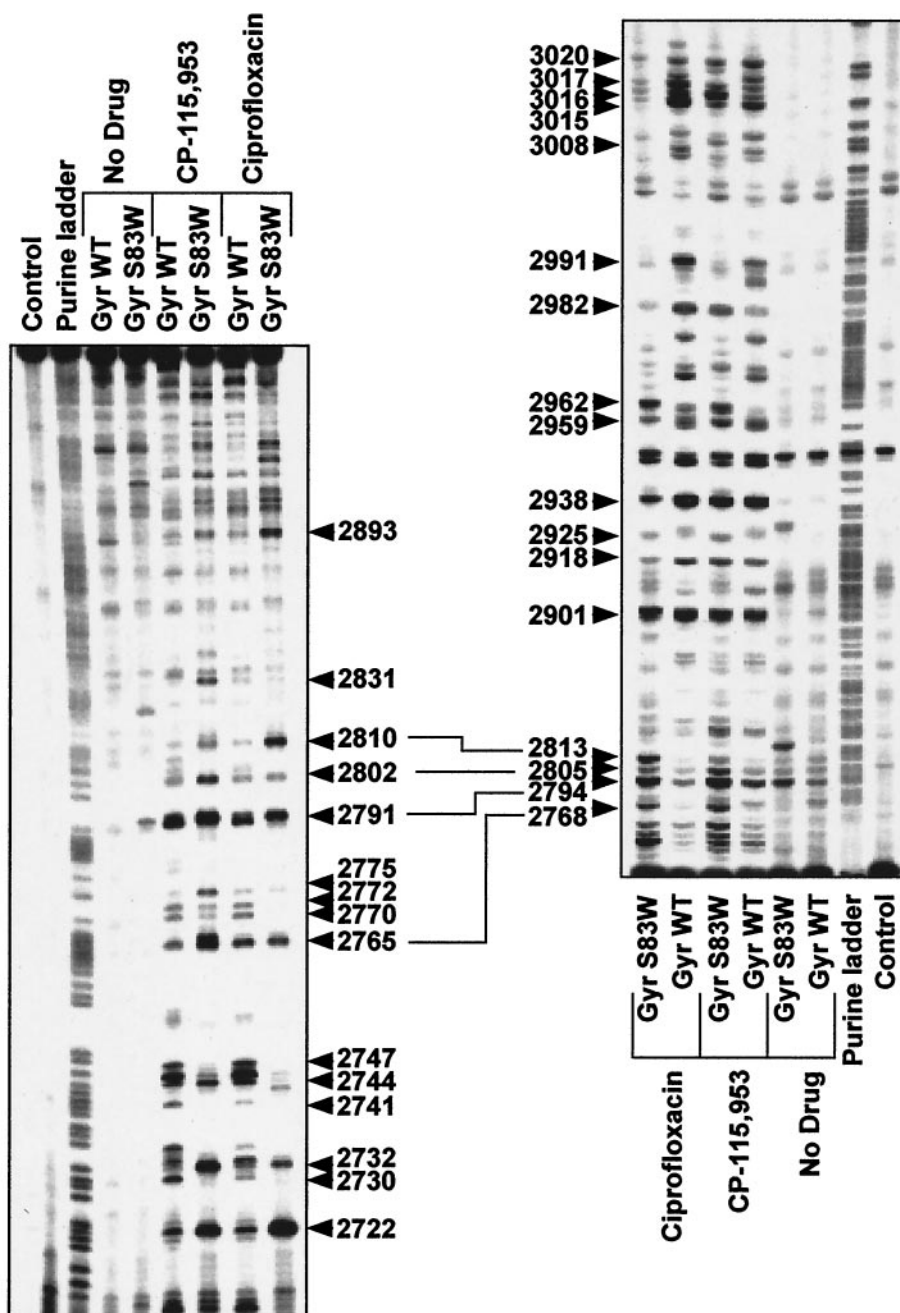


FIG. 4. DNA cleavage patterns generated by gyrase^{WT} and mutant GyrA^{S83W} in the presence of the quinolones CP-115,953 and ciprofloxacin. The same DNA fragments presented in Fig. 3 were used. The left-hand panel shows labeling of the upper DNA strand; the right-hand panel shows labeling of the lower DNA strand. Drugs and enzymes are indicated above each lane. Concentrations were 100 μ M for CP-115,953 and ciprofloxacin, respectively. Cleavage reactions were performed at 25°C for 2 h in the presence of 5 mM MgCl₂. Purine ladders were obtained after formic acid reaction. Lanes: Control, no Top2p, no drug treatment; Gyr WT: gyrase^{WT} from *E. coli*; GyrA^{S83W}, mutant Ser⁸³Trp gyrase. Numbers correspond to genomic positions of the nucleotide covalently linked to Top2p. Double-headed arrows correspond to DNA cleavage sites with a 4-bp stagger that represent potential DNA double-strand breaks.

out of 107 sites) are in agreement with previous reports obtained with the antibacterial fluoroquinolones (12, 17, 21). These data indicate that enhanced quinolone sensitivity of the mutation causing Thr⁷⁴⁴Pro is associated with a shift in base sequence preferences, resembling that of DNA gyrase.

DISCUSSION

The A' domains of eukaryotic Top2 enzymes (homologous to the amino terminus of the A subunit of gyrase) comprise the primary DNA binding sites and include the catalytic tyrosine

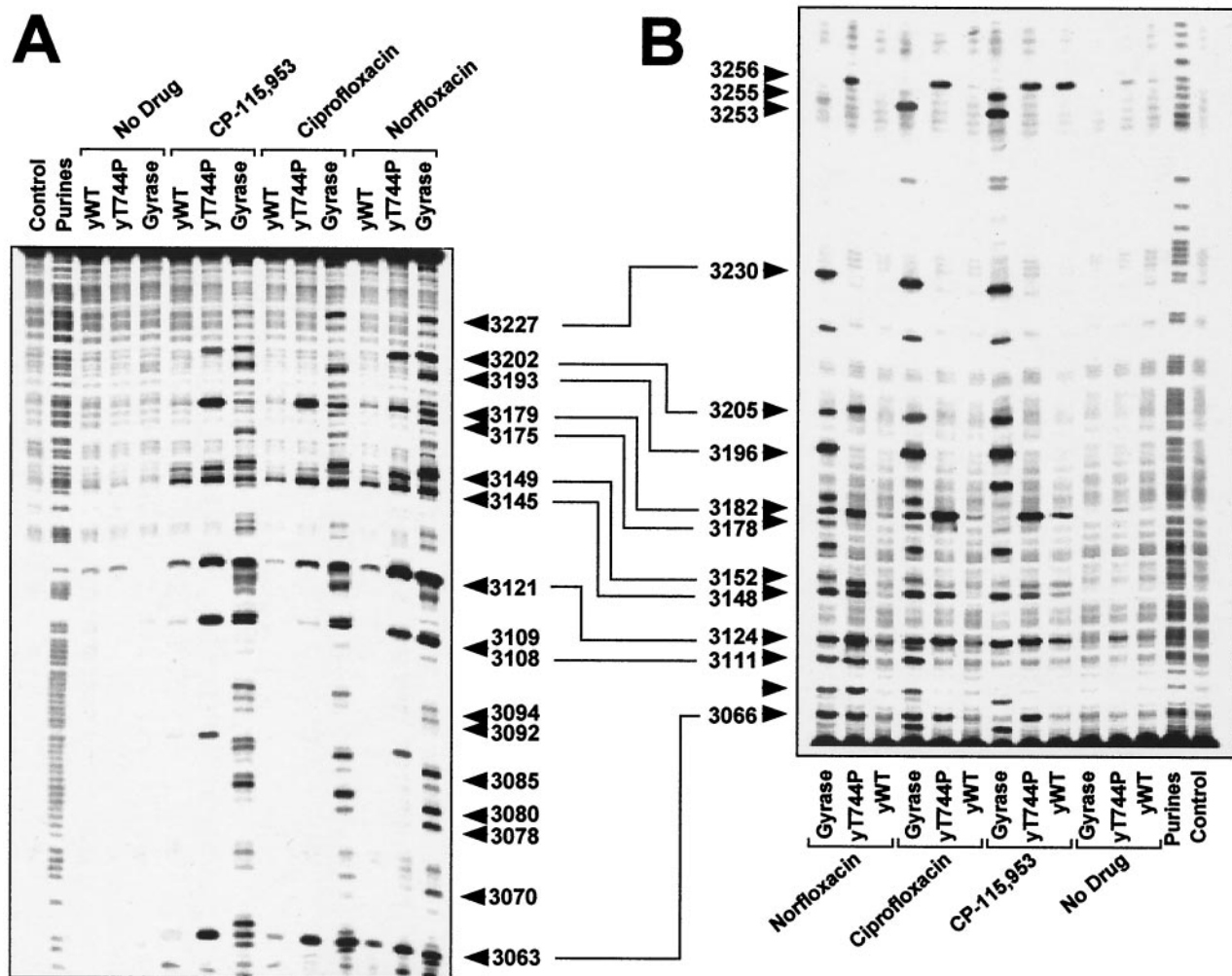


FIG. 5. Generation of DNA cleavage by the yeast Top2p^{T744P} mutant in the presence of antibacterial quinolones. A 254-bp DNA fragment from the *c-myc* first intron was prepared. (A) Labeling of the upper DNA strand at position 3035. (B) Labeling of the lower DNA strand at position 3288. Drugs (100 μ M each) and enzymes are indicated above the lanes. Lanes: yWT, yeast wild-type Top2p; Y T744P, Top2p^{T744P}. Numbers correspond to genomic positions of the nucleotide covalently linked to Top 2p. Double-headed arrows correspond to DNA cleavage sites with a 4-bp stagger that represent potential DNA double-strand breaks.

Y⁷⁸² for the yeast *S. cerevisiae* (59), which is homologous to Y¹²² for *E. coli* gyrase (6, 25, 39). DNA footprinting analyses indicate that for eukaryotic Top2p and bacterial topoisomerase IV, ~15 to 35 bp of DNA is tightly bound to the enzyme (30, 47). In the case of gyrase, a more extensive stretch of DNA (~130 bp) is protected by the enzyme (43). However, the central section of this region around the DNA cleavage site is very similar in size to that bound by Top2p and topoisomerase IV (12, 21, 39). The length of this DNA segment is consistent with the combined length of the A' grooves present in the Top2p and GyrA structures (6, 39). Within the A' domain of Top2p, a 29-kDa fragment containing the active-site tyrosine and the helix-turn-helix motif from the CAP homology domain can be cross-linked to DNA (27), and protein footprinting suggest that the binding of DNA protects lysines from chemical modification within the same region (31). CAP homology domains contain typically a three-alpha-helix bundle backed by β -sheets. Two adjacent alpha-helices, usually the second helix

(referred to as α 3-helix in yeast Top2p according to reference 6) and the third helix (referred to as α 4-helix of the bundle in yeast Top2p according to reference 6), are connected by a short turn. This motif known as the helix-turn-helix is responsible for many of the critical contacts between CAP-like proteins and DNA. The second helix of this motif generally inserts into the major groove of DNA with the turn contacting the phosphodiester backbone (5, 23, 40) (Fig. 1).

Our observations suggest that specific substitutions in the α 4-helix (Thr⁷⁴⁴Pro, Gln⁷⁴³Pro, and Ser⁷⁴⁰Trp of eukaryotic Top2p), which corresponds to the third helix of the CAP homology domain, can change the enzyme-DNA interactions, as reflected by differences in DNA cleavage patterns induced in the presence of Ca²⁺ or in the presence of intercalating and nonintercalating drugs. We also observed differences in base sequence preferences. We interpret these results as an indication that the α 4-helix of the Top2p CAP homology domain is critical for DNA sequence recognition prior to cleavage of the

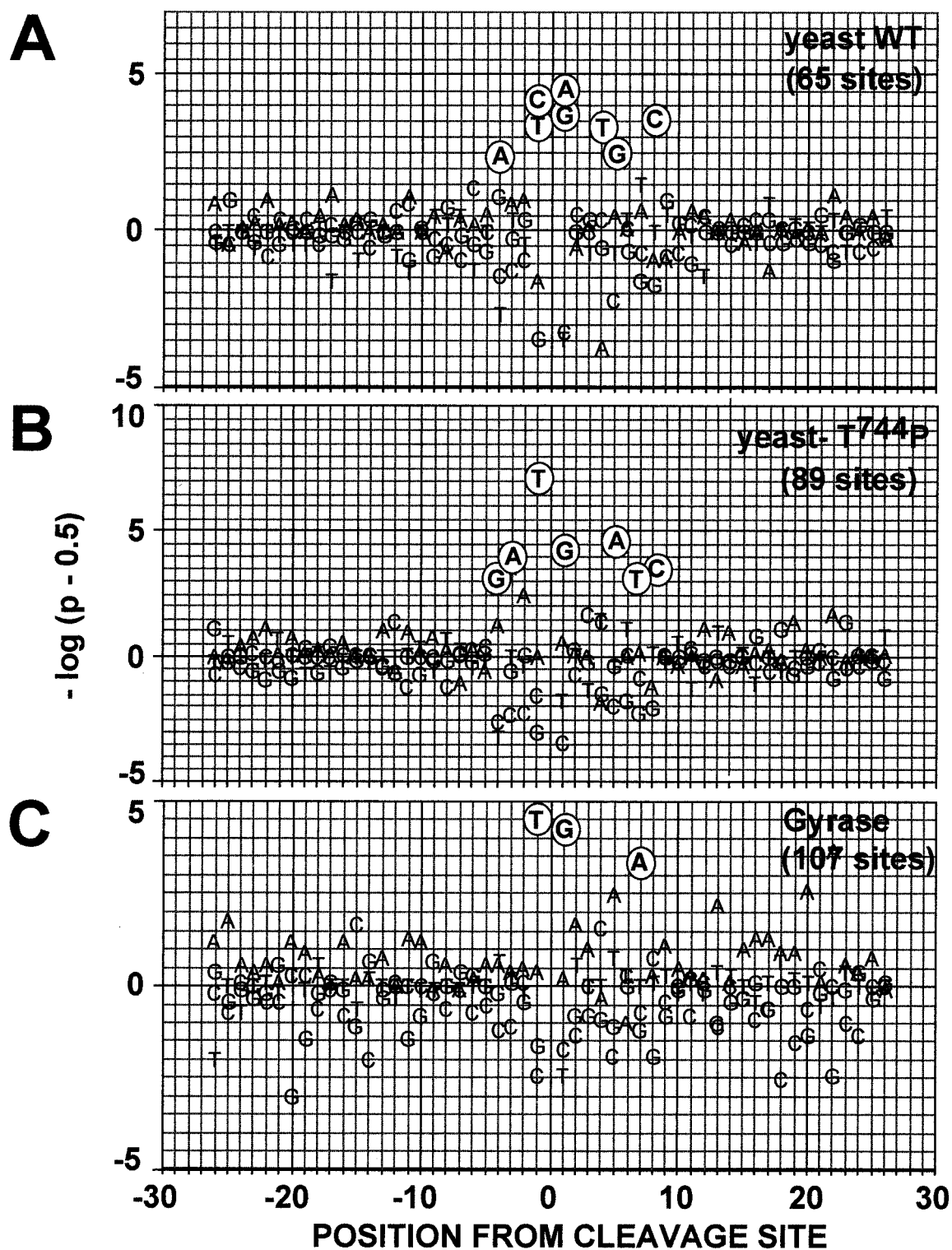


FIG. 6. Probability of the observed base frequency deviations at Top2p cleavage sites for the yeast wild-type enzyme, for Top2p^{T744P} and for *E. coli* gyrase in the presence of CP-115,953. Position 0 corresponds to the cleavage site, and positions -1 and +1 to the bases immediately 3' and 5' from the cleavage site, respectively. The panels present the probability of the observed base frequency deviations from expectation for the indicated enzyme. On the y axis, P is the probability of observing that deviation or more, either as excess (above baseline) or deficiency (below baseline), relative to the expected frequency of each individual base (48). Cleavage sites for the yeast wild-type Top2p (A), the yeast Top2p^{T744P} (B), and *E. coli* gyrase (C) were analyzed after treatment with 100 μM CP-115,953.

G strand, which is consistent with the proposed binding of DNA to yeast Top2p with the α 4-helix close to the catalytic tyrosine and to the ends of the cleaved DNA (6). Figure 1 presents the positions of Ser⁷⁴⁰, Gln⁷⁴³, and Thr⁷⁴⁴ in the yeast Top2 protein (4, 6). These conserved residues (Fig. 1C) tend to be buried inside the protein, while the nonconserved residues Ser⁷⁴⁰ and Gln⁷⁴³ are more solvent accessible (22) (Fig. 1B). The nonconserved amino acid residues might contribute to the observed differences in DNA sequence selectivity and drug sensitivity between the bacterial, the yeast, and the human Top2 enzymes (22, 55).

Other regions of Top2p must interact with different DNA segments, such as the T segment for the strand passage reaction. The B' domain (homologous to B subunit of gyrase) is also essential for DNA binding and cleavage (7, 19, 20, 31, 35). Recently it was proposed that DNA breakage and rejoining by Top2p involve the coordinated action of the CAP homology domain in the A' fragment containing the active-site tyrosine together with the Rossmann fold in the B' fragment (5, 16, 35; J. G. Heddle, and A. Maxwell, submitted for publication). The Rossmann fold, which is important for DNA binding and cleavage reactions, contains a number of highly conserved acidic residues that probably bind divalent metals (5, 16, 35). Structural and biochemical studies suggest that the active-site tyrosine in the DNA-binding domain of one protomer cooperates with several residues in the Rossmann fold of the other protomer (16, 35). Our data suggest that not only the active-site tyrosine and the Rossmann fold domain but also the α 4-helix of the CAP domain are involved in the concerted molecular actions, including DNA interactions leading to sequence-specific cleavage of the G-strand duplex DNA.

We have shown that the Ca²⁺- and drug-generated DNA cleavage patterns of Top2p^{T744P} and Top2p^{Q743P} are different from those of Top2p^{T744A} and Top2p^{WT} and that Top2p^{T744A} is comparable to Top2p^{WT}. Since the Top2p^{Q743P} substitution does not lead to enhanced drug sensitivity (14), the change in the observed pattern of Top2p cleavage sites cannot arise solely from global quantitative alterations. Also, since Top2p^{T744A} does not have a pattern of cleavage that differs from the wild-type enzyme, Thr⁷⁴⁴ probably does not play a crucial role in determining cleavage site selection by the enzyme. Since detailed structural data for any of the mutant proteins are not yet available, we modeled the specific molecular changes that might result from the Thr⁷⁴⁴Ala/Pro and Gln⁷⁴³Pro substitutions in the yeast Top2p structure (6). Molecular dynamics simulations of the Thr⁷⁴⁴Pro mutant Top2p predicted structural changes within the CAP homology domain (data not shown; see also reference 14). Compared to wild-type Top2p and Top2p^{T744A}, the Thr⁷⁴⁴Pro substitution was associated with a kink in the α 4-helix and a bending angle of approximately 37°. The helical structure was preserved on both sides of the kink (data not shown). These simulations suggest that an alteration in the secondary structure of the α 4-helix can alter protein-DNA interactions. It is important to note, however, that changes in other parts of the protein also occurred. Therefore, it is premature to conclude that all of the biochemical alterations we observed in the mutant proteins arise solely from changes in the α 4-helix.

A key issue in understanding the mechanism of action of Top2p targeting drugs is the determination of where the drugs

bind in the Top2p/DNA complex. Our present findings indicate that the same Top2p domain that presumably interacts with DNA is also critical for drug interaction in the Top2p/DNA/drug ternary complex. In particular, the solvent-exposed residues Ser⁷⁴⁰, Gln⁷⁴³, and to a lesser extent Thr⁷⁴⁴, which affect drug activity, are important, directly or indirectly, for drug binding to the Top2p/DNA complex (22).

An intriguing finding arising from this work is that a quinolone-hypersensitive yeast Top2p mutant, resulting in Thr⁷⁴⁴Pro substitution, changed the DNA cleavage pattern in the presence of quinolones so that it more closely resembled the DNA cleavage pattern of a quinolone-sensitive prokaryotic topoisomerase II. Quinolones act against eukaryotic topoisomerase II by accelerating the rate of DNA cleavage (51). Recent studies have shown that along with gyrase and topoisomerase IV of *E. coli*, quinolones can also inhibit religation (1, 28). A plausible model is that the quinolone-hypersensitive mutant allows quinolones to inhibit religation as well. By this hypothesis, the action of quinolones against Top2p^{Thr744Pro} more closely resembles the action of quinolones against prokaryotic topoisomerase II, demonstrating conservation of mechanisms of drug action between eukaryotic and prokaryotic type II topoisomerases. Thus, the quinolone-protein interaction domains between the prokaryotic and eukaryotic enzymes may be comparable, with only limited differences preventing antibacterial quinolones from acting against eukaryotic Top2p.

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