Molecular cloning and expression of the *Candida albicans TOP2* gene allows study of fungal DNA topoisomerase II inhibitors in yeast

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Candida albicans topoisomerase II, encoded by the *TOP2* gene, mediates chromosome segregation by a double-strand DNA break mechanism and is a potential target for anti-fungal therapy. In this paper, we report the characterization of the *C. albicans TOP2* gene and its use to develop a yeast system that allows the identification and study of anti-fungal topoisomerase II inhibitors *in vivo*. The gene, specifying a 1461-residue polypeptide with only 40 % identity with human topoisomerase IIα and β isoforms, was isolated from *C. albicans* on a 6.3 kb *Eco*RI fragment that mapped to chromosome 4. It was used to construct a plasmid in which *TOP*2 expresses a recombinant enzyme (residues 57–1461 of *C. albicans* topoisomerase II fused to the first five residues of *Saccharomyces cerevisiae* topoisomerase II) under the control of a galactose-inducible promoter. The plasmid rescued the lethal phenotype of a temperature-sensitive *S. cerevisiae* DNA topoisomerase II mutant allowing growth at 35 °C. Yeast cells, bearing *ISE2* permeability and *rad52* double-strand-break-repair mutations the growth of which at 35 °C was dependent on *C. albicans* topoisomerase II, were killed by the known topoisomerase II inhibitors amsacrine and doxorubicin. Parallel experiments in yeast expressing human topoisomerase IIα allowed the relative sensitivities of the fungal and host topoisomerases to be examined in the same genetic background. To compare the killing *in vivo* with drug inhibition *in vitro*, the recombinant *C. albicans* topoisomerase II protein was expressed and purified to near-homogeneity from *S. cerevisiae* yielding a 160 kDa polypeptide that displayed the expected ATP-dependent DNA-relaxation and DNA-decatenation activities. The enzyme, whether examined *in vitro* or complementing in *S. cerevisiae*, was comparably sensitive to amsacrine and doxorubicin. Our results suggest that potential topoisomerase II-targeting anti-fungal inhibitors can be identified and studied in *S. cerevisiae*.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that causes life-threatening invasive disease in immunocompromised subjects including AIDS sufferers, those on immunosuppressive therapy for organ transplantation and patients undergoing cancer chemotherapy [1]. The organism is diploid, can exist in yeast and hyphal forms, can undergo switching among different colony morphologies, and is only distantly related to the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* [2]. Unlike these yeasts, the absence of a known sexual cycle in *C. albicans* has slowed the application of genetic approaches. Consequently, compared with *S. cerevisiae*, relatively little is known about its basic biology and the mechanism of action of anti-fungal agents.

Current therapies rely on two main groups of agents: amphotericin B, which disrupts membrane function, and the azoles, such as ketoconazole and fluconazole, which interfere with synthesis of ergosterol, the fungal equivalent of cholesterol needed for membrane biogenesis [3,4]. However, the increasing prevalence of *Candida* infections in immunocompromised patients, dose-limiting toxicity for amphotericin B and emerging resistance to azole agents [3,5,6] have stimulated an interest in exploring and developing other fungal targets including DNA topoisomerases.

Studies in *S. cerevisiae* and *Sch. pombe* have highlighted the essential roles of DNA topoisomerases in DNA replication and chromosome segregation. Topoisomerases alter the topological state of DNA by transiently breaking and rejoining DNA phosphodiester backbone bonds and are classified on the basis of whether they act on one DNA strand at a time (type I) or both strands (type II) [7,8]. *S. cerevisiae* topoisomerase I may act as a swivel during DNA replication but is dispensable for yeast growth [9]. Yeast topoisomerase II is a dimeric protein that mediates the ATP-dependent passage of one DNA duplex through a transient enzyme-bridged double-strand break in another segment [10]. Unlike human topoisomerase II, which exists in two genetically distinct isoforms, α and β [11–14], the *S. cerevisiae* and *Sch. pombe* enzymes are encoded by a single-copy *TOP2* gene [15,16]. Functional topoisomerase II is essential for the growth of yeast: conditional lethal mutations in *TOP2* block chromosome segregation at the non-permissive temperature [17–19]. The *TOP2* genes of *S. cerevisiae* and *Sch. pombe* have been sequenced and their respective 1429- and 1431-residue proteins have been overexpressed and characterized [20–22].

Comparatively little is known about DNA topoisomerases in *C. albicans*. Purification of *C. albicans* topoisomerase I and its inhibition by aminocatechols have been reported and the corresponding *TOP1* gene has been characterized [23–25]. In the case of *C. albicans* topoisomerase II, only one paper has appeared that describes a partial purification procedure [26]. By using this preparation in parallel with mammalian topoisomerase II, the authors identified one compound, a quinolone derivative, that preferentially inhibited the fungal enzyme. Thus selective targeting of topoisomerase II could form a basis for the discovery of fungicidal agents. To gain a better understanding of the genetics and structure of *Candida* topoisomerase II and to study its interactions with inhibitors, we decided to isolate the *C. albicans TOP2* gene and to purify its protein product. In this paper, we describe the molecular cloning of the fungal topoisomerase II and the characterization of a recombinant enzyme overexpressed and purified from *S. cerevisiae*. Moreover, by

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The DNA sequence of the pBK3 insert has been deposited in the EMBL Nucleotide Sequence Database under the accession number Y10377.

complementing with the fungal *TOP2* gene in *S. cerevisiae*, we have developed a yeast system for studying fungal topoisomerase inhibitors *in vivo*.

MATERIALS AND METHODS

Materials

Amsacrine was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda MD, U.S.A. Etoposide was from Sigma or was kindly provided by Bristol Myers Ltd., Syracuse, NY, U.S.A. Doxorubicin was from Farmitalia Carlo Erba, *Eco*RI–*Xho*I adaptor was from Stratagene. Synthetic oligonucleotides were from Oswel, Southampton, Hants, U.K. or were made in our department. The Sequenase version 2.0 sequencing kit, [[α-^{35}S]thio]dATP and [α-^{32}P]dCTP (both 3000 Ci/mmol) were from Amersham International, Little Chalfont, Bucks, U.K. Phosphocellulose P1 was from Whatman, Poole, Dorset, U.K. All other reagents and enzymes were obtained from commercial suppliers.

Strains and plasmids

C. albicans 2402E (Hannington strain) is a clinical isolate obtained from Glaxo Wellcome, Stevenage, Herts., U.K. *S. cerevisiae* strains JCW6 (*top2–4*), JN394t2–4 (*MAT*α *ISE2 ura3-52 top2-4 rad52::LEU2*) and JEL1 (a *leu2 trp1 ura3-52 prb1-1122 pep4-3 Δhis3::PGA10-GAL4*) were kindly provided by Professor James Wang, Harvard University, Cambridge, MA, U.S.A. Plasmids YCpDEDWOB10 and YEpWOB6 were from Dr. Caroline Austin, University of Newcastle, Newcastle, U.K. Plasmid pCR was from Invitrogen, YCp50 was from New England Biolabs and pBluescript was from our laboratory collection. Supercoiled pBR322 was purified by CsCl-density ultracentrifugation as described previously [27].

Cloning of *TOP2*

Genomic DNA from spheroplasts of *C. albicans* 2402E was isolated by standard procedures [28]. Two primers corresponding to *S. cerevisiae TOP2* sequence were designed to allow amplification of a *C. albicans TOP2* gene fragment: forward primer 5′-TGTTGTTGGTAGAGATTATTATGG (nucleotide positions 1829–1852) and reverse primer 5′-CCATCATGATCTTGATC-GGTCATG (complementary to nucleotides 2012–2035). PCR was carried out in a 100 μl reaction sample containing 0.1 μg of genomic DNA from *C. albicans* 2402E (or control DNA from *S. cerevisiae*) as template, 50 pmol of each primer, 200 μM each dNTP, 2 units of *Taq* polymerase and 1.5 mM $MgCl_2$ in buffer supplied by Bioline. The following temperature profile was used: 94 °C for 5 min; then 30 cycles of 94 °C for 1 min, 48 °C for 1 min, 73 °C for 1 min; finally, 73 °C for 5 min. *S. cerevisiae* and *C. albicans* templates generated 205 and 213 bp PCR products respectively. The PCR product amplified from *C. albicans* was recovered by electrophoresis in 2% low-melting-point agarose, cloned into plasmid vector pCRII and verified as *TOP2* by DNA sequence analysis (see below) in comparison with that of the *S. cerevisiae* PCR product. To identify *TOP2* gene fragments by Southern-blot hybridization [29], *C. albicans* 2404E DNA was digested with restriction enzymes, transferred to Hybond-N nylon membranes and cross-linked by UV irradiation. Filters were prehybridized and hybridized at 65 °C to the 213 bp *C. albicans TOP2* PCR product that had been radiolabelled by random priming using [α-^{32}P]dCTP and the Multiprime kit (Amersham International). A single 6.3 kb *C. albicans* fragment hybridized to the probe; there was no hybridization to *S. cerevisiae* DNA. *C. albicans*. *Eco*RI fragments in the 5.5–6.5 kb size range were isolated by agarose-gel electrophoresis before ligation into pBluescript SK + and transformation of *Escherichia coli* DH5α. Ampicillin-resistant colonies were streaked on to Hybond-N nylon membranes placed on Luria broth–ampicillin agar plates and then grown overnight. Replica membranes were made and grown on Luria broth–agar plates for several hours until 0.5–1.0 mm-diameter colonies were obtained. The membranes were placed sequentially on Whatman 3MM paper soaked in 10% SDS for 10 min, in 1.5 M NaCl/0.5 M NaOH for 5 min, twice for 5 min on paper soaked with 1.5 M NaCl/0.5 M Tris-HCl, pH 8.0, and finally on 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 5 min. Filters were air-dried and DNA was fixed to the membranes by UV cross-linking. The filters were probed using the same conditions as for Southern-blotting except that hybridization was at 60 °C. A positive clone, pBK1, was identified that contained multiple 6 kb inserts. These *Eco*RI inserts were cloned individually into pBluescript SK +, and colony hybridization and DNA sequencing identified a plasmid, pBK3, that carried a 6.3 kb full-length *TOP2* insert.

DNA sequence analysis

DNA was sequenced on both strands by the dideoxy-chain termination method using the Sequenase version 2.0 kit [30]. PCR products were cloned into pCRII and sequenced from T3 and T7 primers. The pBK3 insert was sequenced from both ends by using a series of primers made to accumulated sequence. Some regions of the pBK3 insert were subcloned into M13mp19 to allow single-strand sequencing of regions exhibiting compressions in the double-strand sequencing approach.

Yeast transformation and complementation

Plasmids were transformed into yeast strains using the modified lithium acetate procedure of Schiestl and Gietz [31]. For complementation studies, yeast transformants were streaked on to URA$^-$ plates and incubated for 5 days at 25 and 35 °C.

Construction of YEpWCa10

The yeast vector YEpWCa10 allowing expression of *C. albicans* topoisomerase II was constructed by a single-step ligation reaction involving four DNA fragments (see Figure 4). First, the vector portion of YEpWOB6 [32], carrying the *GAL1* promoter, *amp* and *URA* genes and the replication origins for the 2 μm plasmid and pBR322, was isolated as a *Bam*HI–*Xho*I fragment. A second fragment carrying *Bam*HI and *Age*I ends was made by annealing two synthetic self-complementary oligonucleotides, 5′-GATCCCGTAACCATGTCAACTGAA and 5′-CCGGTTCA-GTTGACATGGTTACGG. This fragment specifies sequence from the *GAL1* promoter and the first five codons of *S. cerevisiae* topoisomerase II. A third DNA fragment bearing sequence from codon 57 of *C. albicans TOP2* and finishing at a *Hpa*I site internal to *TOP2* was obtained by PCR. The forward primer was 5′-CCTAAGCCTAC<u>ACCGGT</u>A[AAT GCT TCA GAG ACA TAT] in which the *Age*I site is underlined and the sequence in brackets encodes *C. albicans* topoisomerase II beginning at codon 57; the reverse primer was 5′-CCCATTTCTACCACC-AGT corresponding to nucleotide positions 944–961, i.e. downstream of the *Hpa*I site in the *C. albicans TOP2* gene. Using pBK3 as template, and 2 units of Vent DNA polymerase at 1.5 mM $MgCl_2$, PCR conditions were: 94 °C, 5 min; then 30 cycles of 94 °C, 30 s; 48 °C, 1 min; 73 °C, 1 min; and finally 73 °C, 5 min. The amplified PCR product was digested with *Age*I

and *Hpa*I to yield a 358 bp fragment which was gel-purified. Ligation of fragments 2 and 3 through the *Age*I site was expected to join the *C. albicans TOP2* gene sequence to the beginning of the *S. cerevisiae TOP2* coding sequence. Finally, a *Hpa*I–*Xho*I fragment bearing the 3′ region of *C. albicans TOP2* and its natural stop codon was obtained by ligating *Eco*RI–*Xho*I adaptors to the ends of the 6.3 kb *Eco*RI *TOP2* insert isolated from pBK3 (Figure 1), digesting with *Hpa*I and *Xho*I and isolating the 5390 bp *Hpa*I-*Xho*I *TOP2* fragment. In addition to *TOP2* sequence (nucleotides 551–4765), the fragment contains a partial *SDHA* gene (Figure 1). The four fragments were combined in equimolar ratios, ligated overnight at 16 °C using T4 DNA ligase and used to transform *E. coli* DH5α to ampicillin-resistance. Plasmids were isolated and characterized by restriction enzyme digestion. YEpWCa10 had the expected restriction map and was confirmed to be the required construct by DNA sequence analysis across the critical *Bam*HI, *Age*I and *Hpa*I restriction sites.

Cytotoxicity assays

The drug-sensitivity of JN394 top2-4 carrying YEpWCa10 or YEpWOB6 was determined as previously described [33]. Briefly, cells were grown in minimal medium lacking uracil and supplemented with 2% (w/v) glucose at 35 °C to late exponential phase, diluted to an A_{600} of 0.4 and grown in the absence or presence of different concentrations (0–100 μg/ml) of amsacrine or doxorubicin or solvent. After 5, 10 and 24 h, cell suspensions were diluted appropriately, plated out on uracil-minus medium and grown for 5 days at 35 °C, after which the number of colonies was counted. Drug-sensitivity was plotted as percentage relative survival (cell number at 5, 10 or 24 h relative to that at time zero).

C. ablicans topoisomerase II

The enzyme was purified by a modification of the method of Worland and Wang [22] that has been developed for *S. cerevisiae* topoisomerase II. *S. cerevisiae* strain JEL1 transformed with YEpWCa10 was grown to late exponential phase in minimal medium lacking uracil and supplemented with 2% (w/v) glucose, 3% (v/v) glycerol and 2% (w/v) lactic acid. Cells were diluted 1:50 in glucose-supplemented medium and grown to late exponential phase, diluted 1:50 into 1 litre of glucose-free medium and grown to an A_{600} of 0.7. A 10 ml sample was removed and used as an uninduced control. To the remainder of the culture, 110 ml of 20% (w/v) galactose was added, and growth was continued for another 12 h. Cells were harvested, washed with water, resuspended in buffer I (50 mM Tris/HCl, pH 7.7, 1 mM EDTA and 1 mM EDTA) containing inhibitors (1 mM PMSF, 1 mM dithiothreitol, 1 mM benzamidine, 10 μg/ml leupeptin and 10 μg/ml pepstatin A) (1 ml of buffer per g of wet packed cells), flash-frozen in liquid nitrogen and stored at −70 °C.

For enzyme preparation, all steps were carried out at 4 °C except where mentioned. Approx. 30 ml of cell suspension (from 6 litres of induced culture) were thawed on ice and distributed equally to three 30 ml round-bottomed plastic tubes (Sarstedt). An equal volume of glass beads was added and each tube was vortexed for 20 s and cooled on ice for 40 s. This procedure was repeated 15 times. Cell debris was removed by centrifugation for 15 min at 12000 rev./min in a Sorvall SS34 rotor. The supernatant (fraction I) was diluted to a protein concentration of 5 mg/ml with buffer I containing 25 mM KCl before the dropwise addition of 10% (v/v) poly(ethyleneimine) (Polymin P) (pH adjusted to 7.7) to a final concentration of 0.1%. After stirring on ice for 30 min, Celite was added (8 g per 100 ml of fraction I). The slurry of Celite and Polymin P precipitate was poured into a column on to 4 g of prewetted Celite and allowed to settle. The column was washed with 1 column volume of buffer I containing 25 mM KCl, 3 vol. of buffer I containing 500 mM KCl, and finally 3 column volumes of buffer I containing 1 M KCl to elute the topoisomerase II activity. An equal volume of saturated $(NH_4)_2SO_4$ was added to the eluate with stirring, and additional solid $(NH_4)_2SO_4$ was added with stirring to 65% saturation. After 40 min, the precipitate was pelleted by centrifugation for 25 min at 11000 ***g*** at (12000 rev./min) in a Sorvall SS34 rotor. The precipitate was dissolved in buffer I (fraction II) and applied to a phosphocellulose column (1 ml volume per 5 mg of protein) equilibrated with buffer I plus 250 mM KCl. The column was washed with 1 column volume of buffer I plus 250 mM KCl and eluted with a gradient of 250 mM to 1 M KCl in buffer I. Topoisomerase II activity was eluted at about 0.5 M salt.

Recombinant *S. cerevisiae* DNA topoisomerase II and human topoisomerase IIα were purified from *S. cerevisiae* JEL1 strains transformed with plasmids bearing the appropriate *TOP2* genes under *GAL1* control as described previously [32]. The purification protocol was essentially identical with that outlined above for the *C. albicans* enzyme and resulted in proteins that were more than 95% pure by SDS/PAGE analysis.

Assays of topoisomerase II

Enzyme activity was determined by the ATP-dependent relaxation of supercoiled plasmid pBR322 DNA. The standard reaction mixture contained 50 mM Tris/HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 30 μg/ml BSA and 0.4 μg of supercoiled pBR322 DNA (total volume 20 μl). One unit is the amount of enzyme required to relax 50% of the DNA in 30 min at 37 °C. Reactions were stopped by the addition of loading buffer containing 25% glycerol, 5% SDS and 0.25 mg/ml Bromophenol Blue and examined by electrophoresis in 0.8% agarose gels run in TBE buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA). Gels were stained with ethidium bromide and photographed under UV transillumination. Decatenation assays were carried out similarly at pH 7.5, using 150 mM KCl, 50 μg/ml BSA and substituting 0.25 μg of kinetoplast DNA (from *Crithidia fasciculata*).

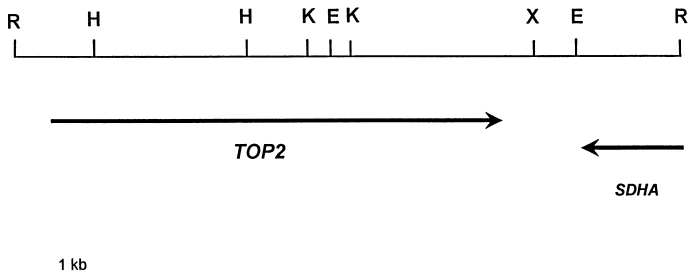
DNA cleavage

Reaction mixtures contained 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 5% DMSO, 0.4 μg of supercoiled pBR322 DNA human topoisomerase IIα or recombinant *C. albicans* topoisomerase II, and reactions were carried out in the presence or absence of amsacrine. Samples were incubated for 30 min at 37 °C, and then further incubated with 1 μl of 10% (w/v) SDS and 1 μl of 0.5 mg/ml proteinase K for 30 min at 37 °C. Samples were analysed by electrophoresis in 0.8% agarose.

RESULTS

Topoisomerase II encoded by *C. albicans TOP2* shares closest homology with the *S. cerevisiae* enzyme

A PCR-based approach was used to isolate a full-length *C. albicans TOP2* gene. First, a 213 bp *C. albicans TOP2* PCR product was amplified from *C. albicans* strain 2402E genomic DNA using two oligonucleotide primers made to the *S. cerevisiae TOP2* sequence corresponding to VVGRDYYG and MTDQDHDG motifs (residues 464–471 and 525–532) (see the

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1       GAATTCAAAATGAAATAATGAAAAAGAAGAAATAACTCACTACTGAAAAGAAAGGGTTATATAGATGTTTAAGATTTATAATATATGTATAGGAGTGTTTAATGGTAACAAAAATATATT
121     GACGCGAAGCATAATTAGGATTAAGCGTGTCTTGAGAGACAAAAGGAACACTAACAATTAAAATTCTCGCATTTACAACAATACTCACACAGATCATCATCTAAAATCAAAACAAAAAAA
241     ATTGTACTGTTGATGATTTTTTTCCTCTGTGCGTTGCCAAATTTGATTTGATTTGAGATTGATACTTCTATTATTTTCTTTTCCTTTACAACCAAATAATAATCTATTCTTAATTATTGT
                                M  S  E  S  E  S  D  Y  F  T  D  G  S  E  D  D  F  V  P  T  S  K  K  S  T  K  K  N  A  S  S  K  S
361      TTGATTGAATCACTCATCTACAATGTCTGAATCAGAATCTGATTATTTTACTGATGGGTCAGAAGATGATTTTGTTCCAACTTCTAAAAAATCCACCAAGAAAAACGCGTCTTCAAAACT
          K  Q  P  L  G  D  A  T  N  S  T  V  S  S  S  R  S  S  T  P  K  P  T  N  A  S  E  T  Y  Q  K  L  S  Q  L  E  H  I  L  K
481      GAAACAACCTCTTGGTGATGCAACCAATAGCACTGTCAGTTCATCGCGCTCTTCCACACCTAAGCCTACCAATGCTTCAGAGACATATCAAAAGCTTTCCCAATTAGAGCATATTTTAAA
          R  P  D  T  Y  I  G  S  V  E  K  T  K  T  E  M  W  C  F  D  A  E  T  E  S  M  V  F  K  E  V  T  I  V  P  G  L  Y  K  I
601      AAGACCCGATACTTATATCGGATCTGTTGAGAAAACCAAAACAGAAATGTGGTGTTTTGACGCAGAAACTGAATCTATGGTGTTCAAAGAAGTCACTATTGTTCCTGGGTTGTATAAAAT
          F  D  E  I  L  V  N  A  A  D  N  K  I  R  D  P  S  M  K  N  I  R  V  K  I  D  A  E  N  N  I  I  E  V  M  N  D  G  K  G
721      CTTTGATGAAATTCTAGTTAATGCTGCTGATAACAAAATCAGAGATCCCAGTATGAAGAATATCAGAGTCAAAATCGATGCTGAAAACAACATAATAGAAGTAATGAATGATGGGAAAGG
          I  P  I  E  M  H  T  K  E  N  M  Y  I  P  E  L  I  F  G  N  L  L  T  S  S  N  Y  D  D  D  Q  K  K  V  T  G  G  R  N  G
841      TATACCAATTGAAATGCACACCAAAGAAAATATGTACATTCCTGAATTGATTTTTGGTAACTTGTTAACTTCTTCCAACTATGATGACGACCAGAAAAAAGTTACTGGTGGTAGAAATGG
          F  G  A  K  L  C  N  I  F  S  T  Q  F  E  V  E  T  A  D  L  N  M  G  K  L  Y  K  Q  S  W  T  N  N  M  S  N  V  S  K  P
961      GTTTGGTGCTAAGTTGTGTAATATTTTCTCTACCCAATTTGAAGTGGAAACTGCTGATTTGAATATGGGGAAATTGTATAAACAATCTTGGACAAATAATATGTCAAATGTGTCAAAACC
          K  I  T  T  L  R  T  K  K  E  Y  T  K  I  T  F  R  P  D  L  S  K  F  D  M  D  C  L  D  N  D  L  L  S  V  L  R  R  R  V
1081     AAAAATCACCACTTTAAGAACCAAAAAGGAATATACCAAGATTACTTTCAGACCCGACTTGAGTAAATTCGATATGGACTGTTTGGATAATGATCTTTTATCTGTTTTAAGAAGAAGAGT
          Y  D  L  C  G  T  V  K  N  C  N  I  Y  L  N  D  K  R  L  N  I  S  S  F  K  G  Y  V  E  M  Y  V  K  A  I  K  E  R  S  P
1201     TTATGATTTATGTGGTACTGTGAAAAACTGTAACATTTATTTAAATGATAAGAGGTTGAACATCTCCAGTTTCAAAGGTTACGTGGAAATGTATGTCAAAGCTATCAAAGAAAGGTCACC
          E  P  E  P  Q  D  G  T  I  K  N  F  T  T  I  V  H  E  V  F  N  D  R  W  E  V  A  F  A  V  S  D  G  S  F  N  Q  V  S  F
1321     TGAACCTGAACCACAAGATGGAACCATTAAAAATTTCACCACCATTGTACATGAAGTGTTTAATGATAGATGGGAAGTTGCATTTGCTGTTAGTGATGGTTCATTTAATCAAGTCAGTTT
          V  N  S  I  A  T  T  S  G  G  T  H  V  K  Y  V  S  D  Q  I  I  N  K  L  V  E  T  L  S  K  K  E  K  G  K  K  K  L  M  I
1441     TGTCAACTCCATTGCCACAACATCTGGAGGTACACATGTCAAATACGTTTCCGATCAAATAATCAATAAGTTAGTAGAGACCTTATCCAAGAAGGAAAAAGGTAAGAAGAAACTTATGAT
          K  P  Q  E  V  R  D  N  M  F  L  F  I  N  C  L  I  E  N  P  A  F  T  S  Q  T  K  E  Q  L  T  T  K  V  S  Q  F  G  G  K
1561     TAAGCCTCAAGAAGTGAGGGATAATATGTTTTTATTTATCAACTGTCTTATTGAAAACCCTGCTTTCACTTCCCAGACTAAAGAACAATTAACAACAAAAGTGTCTCAATTTGGTGGTAA
          D  K  F  V  A  N  D  N  L  I  N  R  I  L  K  T  S  I  V  D  K  I  R  A  I  A  N  A  N  E  D  K  A  L  Q  K  A  D  G  S
1681     AGATAAGTTTGTTGCTAATGATAACTTAATCAACCGTATTTTGAAAACCAGTATAGTAGATAAAATCAGAGCTATTGCCAACGCCAACGAAGACAAGGCACTTCAAAAAGCCGATGGTAG
          R  K  S  R  I  K  G  Q  V  N  L  V  D  A  N  K  A  G  T  K  D  G  H  N  C  T  L  I  L  T  E  G  L  S  A  M  N  L  A  V
1801     TAGAAAACTGCGTATTAAGGGTCAAGTTAATTTGGTTGATGCAAACAAAGCTGGTACAAAAGATGGACACAATTGTACTTTGATCTTAACAGAAGGGCTTTCTGCTATGAATTTAGCTGT
          A  G  L  S  V  V  G  R  D  Y  Y  G  C  F  P  L  R  G  K  L  L  N  V  R  E  A  S  A  D  Q  I  S  K  N  A  E  I  N  S  L
1921     TGCTGGGCTTTCCGTTGTTGGGAGAGATTATTATGGATGTTTCCCGTTACGTGGTAAATTATTGAATGTTCGTGAAGCTTCGGCAGATCAAATTTCCAAAAATGCAGAAATTAACTCATT
          K  Q  I  I  G  L  Q  H  K  K  V  Y  T  A  E  N  I  K  S  L  R  Y  G  H  I  M  I  M  T  D  Q  D  Q  D  G  S  H  I  K  G
2041     GAAACAAATTATTGGGTTGCAACATAAAAAAGTTTACACAGCTGAAAATATCAAATCATTGAGATATGGTCATATTATGATCATGACAGATCAAGATCAGGATGGGTCGCATATCAAGGG
          L  I  I  N  F  L  E  T  S  F  P  G  L  L  D  I  P  G  F  L  L  E  F  I  T  P  I  V  K  V  T  V  K  A  R  G  A  G  G  K
2161     GTTAATTATTAACTTTTAGAAACTTCCTTCCCTGGATTGTTAGACATTCCCGGATTCTTACTTGAGTTTATTACCCCTATTGTTAAAGTCACTGTTAAAGCAAGAGGTGCTGGTGGTAA
          R  V  I  P  F  Y  T  M  P  E  F  E  H  W  R  D  T  E  G  K  Q  C  R  W  T  Q  K  Y  Y  K  G  L  G  T  S  T  P  M  E  A
2281     ACGTGTTATACCATTCTACACAATGCCGGAATTTGAGCATTGGAGAGATACTGAAGGGAAACAATGTCGTTGGACTCAGAAGTATTACAAAGGTTTGGGTACTTCTACTCCTATGGAAGC
          R  E  Y  F  T  A  L  D  R  H  L  K  R  F  H  A  L  Q  G  E  D  K  D  Y  I  D  L  A  F  S  K  K  K  A  D  E  R  K  E  W
2401     ACGTGAATATTTTACTGCCTTGGATCGTCATTTAAAGAGATTTCATGCATTGCAAGGTGAAGATAAAGATTACATTGATTTGGCATTTCTGAAAAAGAAAGCTGATGAAAGAAAGGAATG
          L  Q  G  F  L  P  G  T  H  L  D  P  E  I  T  E  I  P  I  S  D  F  I  N  K  E  F  I  L  F  S  M  S  D  N  V  R  S  I  P
2521     GTTACAAGGGTTTTTACCAGGTACCCATCTTGATCCTGAGATTACTGAAATTCCAATAAGTGATTTTATCAACAAAGAGTTTATTTTGTTTTCAATGTCTGATAATGTAAGATCGATTCC
          S  V  L  D  G  F  K  P  G  Q  R  K  V  L  Y  G  C  F  K  K  L  R  S  E  I  K  V  A  Q  L  A  G  Y  V  S  E  N  T  G
2641     GTCGGTTTTAGATGGGTTCAAACCAGGTCAAAGAAAAGTCTTGTATGGTTGTTTCAAGAAAAAATTGAGAAGTGAAATTAAAGTTGCACAATTAGCAGGGTATGTGAGTGAAAACACGGG
          Y  H  H  G  E  Q  S  L  V  Q  T  I  I  G  L  A  Q  N  F  V  G  S  N  N  I  N  V  L  K  P  N  G  S  F  G  S  R  A  A  G
2761     ATATCATCATGGTGAACAGTCATTAGTGCAAACTATAATTGGTCTTGCCCAAAATTTCGTTGGATCAAACAACATTAACGTATTAAAACCAAATGGTTCTTTTGGTTCGCGTGCTGCTGG
          G  K  D  F  S  A  A  R  Y  I  F  T  E  L  S  E  I  T  R  K  I  F  N  P  L  D  D  P  L  Y  T  Y  V  Q  D  D  E  Q  T  V
2881     TGGTAAAGATTTCTCTGCTGCGAGATATATTTTCACAGAATTGAGTGAGATTACTCGTAAAATATTCAATCCATTGGATGATCCCTTGTACACTTATGTTCAAGATGATGAGCAAACTGT
          E  P  E  W  Y  L  P  V  L  P  M  I  L  V  N  G  A  E  G  I  G  T  G  W  S  T  N  I  P  S  Y  N  P  K  D  L  V  T  N  I
3001     TGAGCCTGAATGGTACTTGCCAGTTTTGCCTATGATTTTGGTAAATGGTGCTGAAGGTATTGGTACCGGTTGGTCTACAAATATTCCTTCATACAATCCAAAAGATCTTGTTACAAATAT
          R  R  L  M  N  G  E  E  L  Q  E  M  T  P  W  Y  K  G  W  G  G  D  L  E  P  M  G  P  Q  K  F  K  V  S  G  R  I  E  Q  I
3121     CAGAAGATTAATGAATGGTGAAGAATTGCAGGAGATGACACCATGGTATAAAGGATGGGGTGGTGACCTTGAACCAATGGGACCGCAAAAGTTTAAAGTTTCTGGACGAATCGAACAAAT
          D  S  N  T  V  E  I  T  E  I  P  V  K  T  W  T  N  N  V  K  E  F  L  L  S  G  F  G  N  E  K  T  Q  P  W  I  K  D  N  E
3241     TGATCTGAATACAGTTGAAATTACTGAAATCCCAGTCAAAACTTGGACTAACAACGTCAAAGAATTTTTGCTCAGTGGGTTTGGGAATGAAAAGACCCAGCCATGGATTAAAGATATGGA
          E  H  H  T  T  S  I  R  F  V  V  K  L  T  D  A  E  M  Q  K  S  L  R  I  G  L  L  E  R  F  K  L  V  S  S  L  S  L  A  N
3361     AGAACACCATACTACATCGATCAGGTTTGTTGTCAAATTGACTGATGCGGAAATGCAAAAGTCGTTGCGTATTGGTCTTTTGGAGAGGTTCAAGTTGGTTTCTTCATTGAGTTTGGCCAA
          M  V  A  F  D  P  M  G  R  I  K  K  Y  N  D  V  L  E  I  I  K  D  F  Y  Y  V  R  L  E  Y  Y  Q  K  R  K  D  Y  M  T  D
3481     TATGGTTGCCTTTGATCCTATGGGTAGAATCAAAAAATATAACGATGTTTTAGAAATAATTAAAGATTTCTATTATGTTCGATTAGAATACTATCAAAAGAGAAAAGATTATATGACTGA
          N  L  Q  N  Q  L  L  M  L  S  E  Q  A  R  F  I  K  M  I  I  E  K  Q  L  S  V  A  N  K  K  K  K  Q  L  V  A  L  L  E  E
3601     CAATTTACAAAATCAATTGTTGATGTTGAGTGAACAGGCGAGATTTATCAAGATGATAATTGAGAAACAATTGAGTGTTGCTAACAAGAAAAAGAAACAATTGGTTGCATTGCTAGAAGA
          H  N  F  T  K  F  S  K  D  G  K  P  I  K  S  S  E  E  L  L  T  G  D  D  A  D  E  E  E  E  E  T  Q  E  Q  E  G  D  E  D  V
3721     ACATAATTTTACAAAATTCAGTAAAGATGGGAAACCAATCAAATCCAGTGAAGAATTATTGACTGGTGACGATGCCGATGAGGAAGAGGAAACACAAGAACAAGAAGGTGACGAAGACGT
          G  N  T  S  V  A  N  I  Q  E  G  E  P  E  Q  A  A  H  V  P  E  T  I  Y  S  S  Y  D  Y  L  L  G  M  A  I  W  S  L  T  Y
3841     CGGTAATACCTCTGTAGCTAATATTCAAGAAGGAGAACCTGAGCAAGCAGCCCATGTTCCAGAAACAATCTATTCATCCTATGATTATTTGTTGGGTATGGCAATTTGGTCTTTAACTTA
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Figure 1 For legend see facing page.

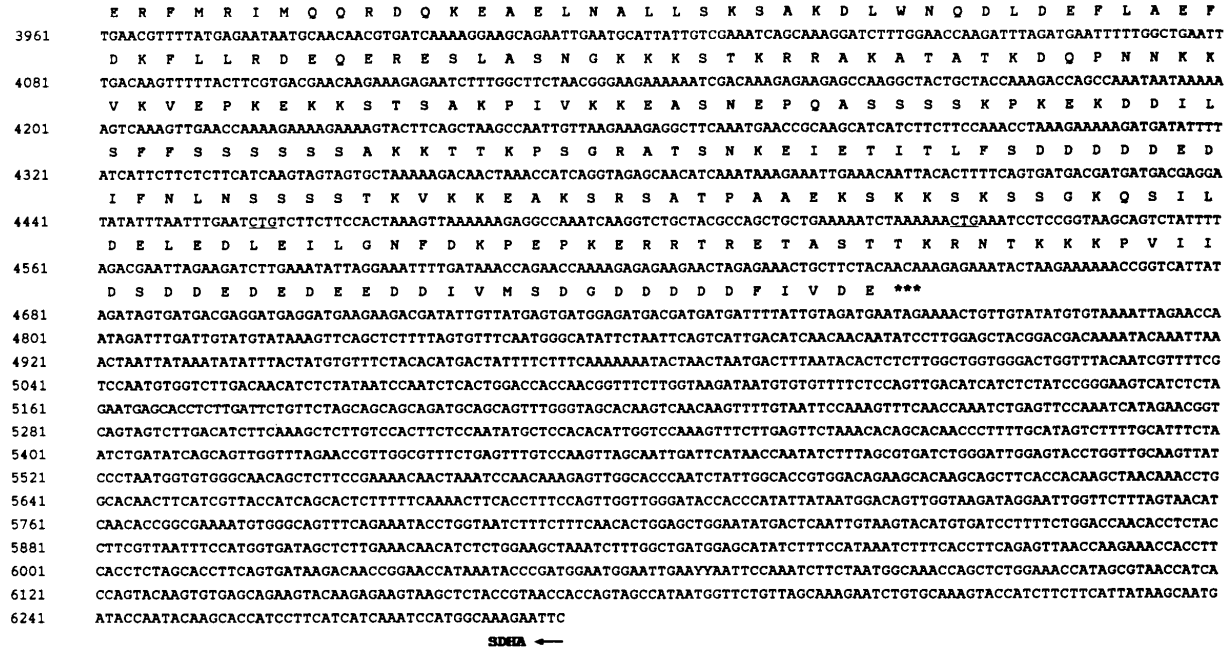

Figure 1 Restriction map (top) and nucleotide sequence (bottom) of a 6290 bp *Eco*RI fragment containing the *TOP2* gene of *C. albicans*

E, H, K, R and X denote restriction sites for *Eco*RV, *Hin*dIII, *Kpn*I, *Eco*RI and *Xba*I respectively. The deduced amino acid sequence of topoisomerase II (one-letter code) is shown above the nucleotide sequence. The initiation codon is underlined, as are six CTG codons translated as serine in *C. albicans* (see the text); *** Termination codon. *SDHA* and the arrow denote an open reading frame on the complementary strand specifying a protein homologous with the flavoprotein subunit of succinate dehydrogenase of *S. cerevisiae*.

Materials and methods section) that are also conserved in *Sch. pombe* topoisomerase II. When used to probe a Southern blot of 2404E DNA, the PCR product hybridized to a single ~ 6 kb *Eco*RI fragment (not shown). Plasmid clone pBK3 containing the 6.3 kb *Eco*RI fragment was obtained by colony hybridization using the radiolabelled PCR product to probe a size-selected library of 2402E *Eco*RI fragments in plasmid pBluescript (Figure 1 top). The nucleotide sequence of the 6290 bp pBK3 insert was determined in both strands and is presented in Figure 1 bottom. Two convergent non-overlapping open reading frames were identified. An incomplete open reading frame that we designated *SDHA* specified a protein homologous to the C-terminal region of the flavoprotein subunit of *S. cerevisiae* succinate dehydrogenase (Figure 1) [34–36]. The second open reading frame encoded a 1461-residue protein displaying close homology with the topoisomerase II proteins of *S. cerevisiae* and *Sch. pombe* (Figure 2). Homology with the known yeast topoisomerases II was greatest in the N-terminal 1–1200 residues. By analogy with the *S. cerevisiae* enzyme [22], the putative catalytic tyrosine of *S. albicans* topoisomerase II was identified as Tyr-842 (Figure 2). The C-terminal tail of *C. albicans* topoisomerase II (residues 1200–1461) was not particularly conserved and contained a number of highly acidic and highly basic tracts as well as polyserine sequences. Table 1 compares the relative sequence identities of the three yeast topoisomerases with the human topoisomerase IIα and IIβ isoforms. *C. albicans* topoisomerase II was most closely related to its counterpart in *S. cerevisiae*, i.e. 55% identity compared with 42–44% identity with the other proteins.

Chromosome mapping of the *C. albicans TOP*2 and *SDHA* genes was carried out independently using gene-specific PCR products as probes. Both genes mapped to chromosome 4 of *C. albicans*. Syngeneity does not extend to *S. cerevisiae*, the corresponding *TOP2* and *SDH1* genes of which map to chromosomes XIV and XI respectively [37,38].

C. albicans TOP2 gene rescues growth of temperature-sensitive *top2 S. cerevisiae* mutants: a yeast system for studying fungal topoisomerase II inhibitors *in vivo*

Previous studies have elegantly shown that *S. cerevisiae* topoisomerase II is the target in yeast for a variety of anti-cancer agents including amsacrine and etoposide [33,39–41]. Moreover, plasmids expressing either the human *TOP2*α or *TOP2*β gene can complement a temperature-sensitive topoisomerase II mutation in *S. cerevisiae*, allowing the effects of inhibitors to be studied against the individual α or β isoform in the same yeast background [32,42,43]. We noted that the inferred sequence of the fungal topoisomerase is more similar to that of its yeast counterpart than to either the human α or β isoform (Table 1). This observation suggested that the fungal enzyme might also complement, opening the possibility of studying anti-fungal inhibitors in the well-defined yeast system. Therefore we initially tested the putative *C. albicans TOP2* gene for its ability to rescue the temperature-sensitive growth of the *ura-S. cerevisiae* strain JCW6 conferred by the *top2–4* mutation. The pBK3 insert was ligated into the shuttle vector YCp50 carrying selectable *amp* and *URA3* genes yielding construct pBK50. Strain JCW6 was separately transformed with YCp50, pBK50 or plasmid YCpDEDWOB10 bearing the *S. cerevisiae TOP2* gene under the control of the constitutive *S. cerevisiae DED1* gene promoter [32], and transformants were selected on uracil-minus plates. Transformants on YPDA plates were incubated for 3 days at 25

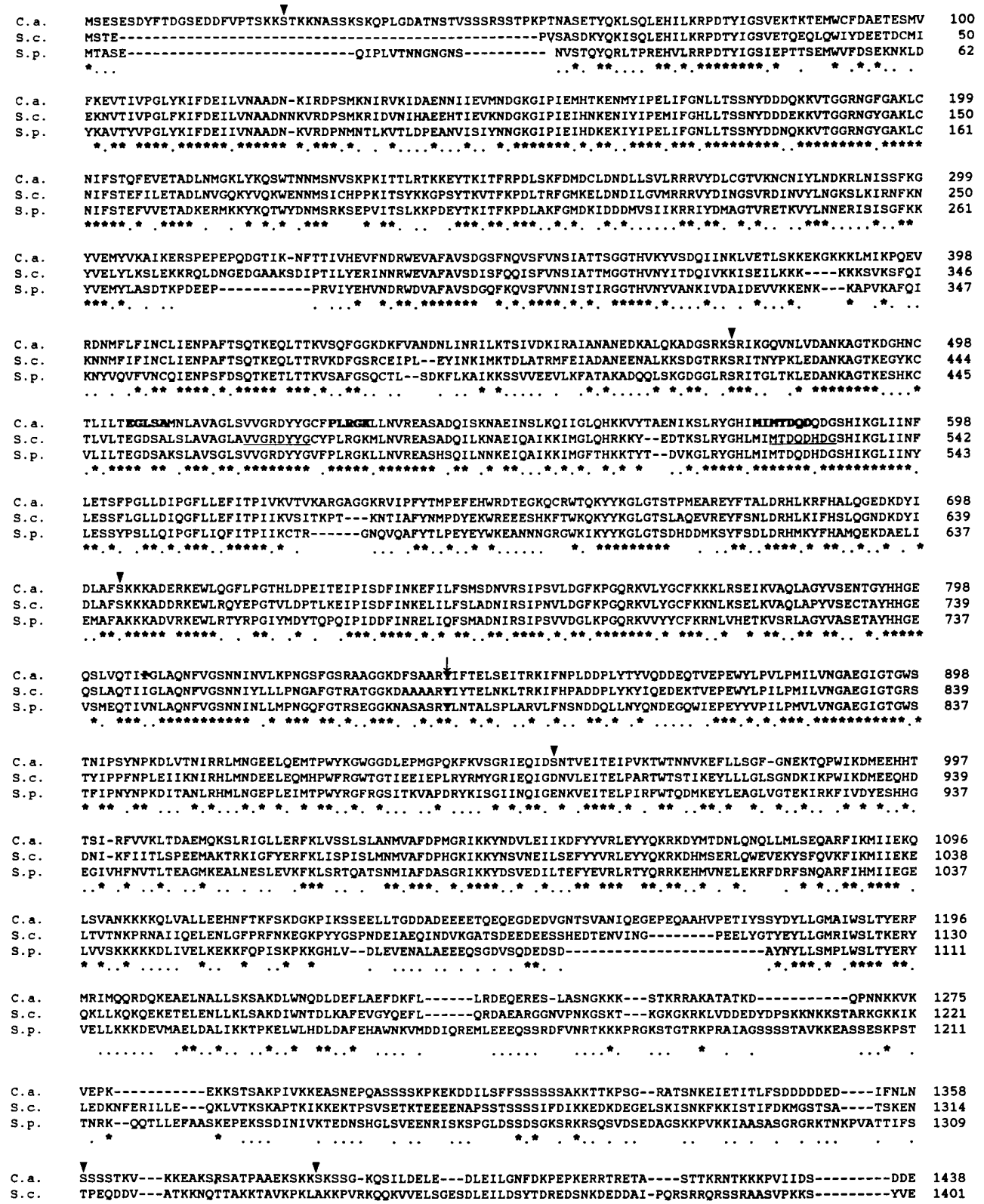

Figure 2 For legend see facing page.

Table 1 Pairwise sequence identities between topoisomerases II of *C. albicans*, *S. cerevisiae*, *Sch. pombe* and *Homo sapiens*

Values are percentage amino acid identities.

	S. cerevisiae	*Sch. pombe*	Human α	Human β
C. albicans	55.4	47.6	41.6	43.1
S. cerevisiae	—	47.5	42	43
Sch. pombe	—	—	43.1	44

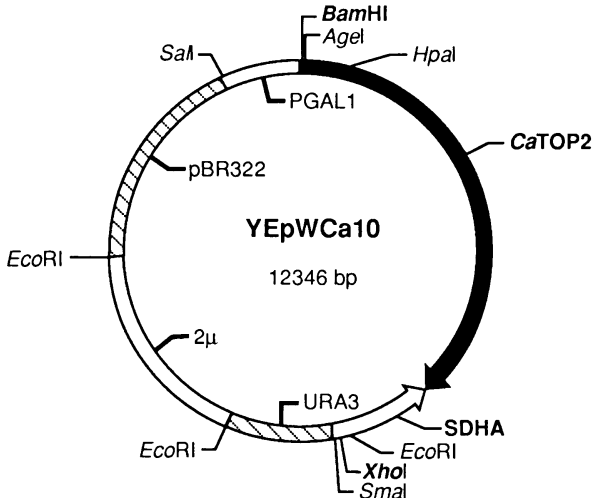

Figure 4 Structure and restriction map of YEpWCa10, a plasmid designed to allow overexpression of *C. albicans* topoisomerase II in *S. cerevisiae*

*Sal*I and *Xho*I sites are unique in the plasmid.

and 35 °C, the permissive and non-permissive temperatures for growth. The results are shown in Figure 3. JCW6 and its plasmid transformants all grew at 25 °C as expected. At 35 °C, neither JCW6 nor its YCp50 transformant was able to grow, consistent with the *top2–4* phenotype [32]. Plasmid YCpDEDWOB10 bearing the *S. cerevisiae TOP2* gene rescued growth at 35 °C as described previously [32]. Significantly, pBK50 also rescued growth at 35 °C showing that the *C. albicans TOP2* gene is expressed from its own promoter and can functionally substitute for *TOP2* in *S. cerevisiae* (Figure 3). However, JCW6 transformed with pBK50 grew less well than the YCpDEDWOB10 transformant, perhaps indicating that the promoter of the *C. albicans TOP2* gene (or its topoisomerase II product) exhibits suboptimal activity in this yeast background.

To study the effects of topoisomerase II inhibitors *in vivo*, it was necessary to utilize *S. cerevisiae* strain JN394t2–4. In addition to the *top2–4* mutation that blocks growth at 35 °C, the strain also has an *ISE2* mutation allowing drug uptake and a *rad52* repair defect that sensitizes the yeast to damage through double-strand DNA breaks [33,40,41]. To examine the drug susceptibilities of JN394t2–4 growing at 35 °C by virtue of the *C. albicans* topoisomerase II *in vivo*, we transformed the strain with plasmid YEpWCa10 in which the fungal *TOP2* gene is under the control of the *GAL*1 promoter. YEpWCa10 allows expression of recombinant *C. albicans* topoisomerase II constituting residues 57–1461 fused to the first five residues of the *S. cerevisiae* topoisomerase II (Figure 4). The sequence alignments in Figure

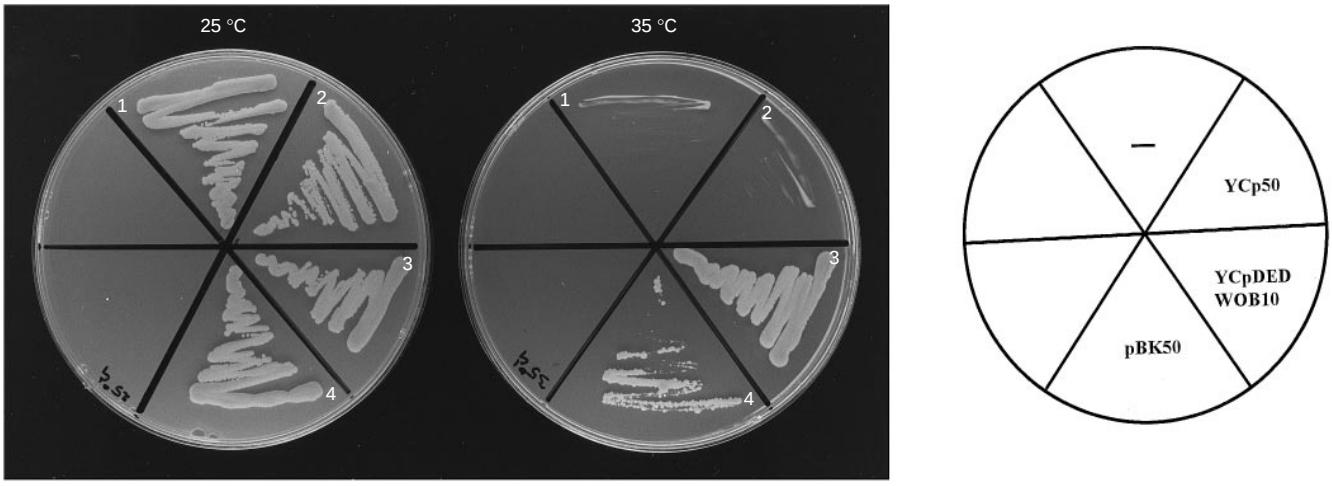

Figure 3 Growth of *S. cerevisiae top2-4ts* strain JCW6 and its *TOP2* plasmid transformants at 25 °C (left) and 35 °C (middle)

Yeast strains were streaked on YPDA medium and grown for 5 days at the indicated temperatures: 1, JCW6; 2, JCW6 (YCp50); 3, JCW6 transformed with YCpDEDWOB10 carrying *S. cerevisiae TOP2* gene; 4, JCW6 transformed with pBK50, i.e. *C. albicans TOP2* in YCp50.

Figure 2 Alignment of the topoisomerase II protein sequences from yeasts *C. albicans* (C.a.), *S. cerevisiae* (S.c.) and *Sch. pombe* (S.p.)

Asterisks and dots denote identical and conserved residues respectively. Amino acid residues are numbered on the right. Underlining indicates conserved sequences used in the design of PCR primers for isolation of the *C. albicans TOP2* gene (see the text). Letters in bold denote three motifs that are highly conserved in topoisomerase II proteins. Arrowheads identify residues specified by the CUG codon, which is decoded as Ser in *C. albicans* and not as Leu as would be expected in the 'universal' genetic code. The arrow indicates the catalytic tyrosine involved in enzymic DNA breakage-reunion. *S. cerevisiae* and *Sch. pombe* sequences were from refs. [20,21].

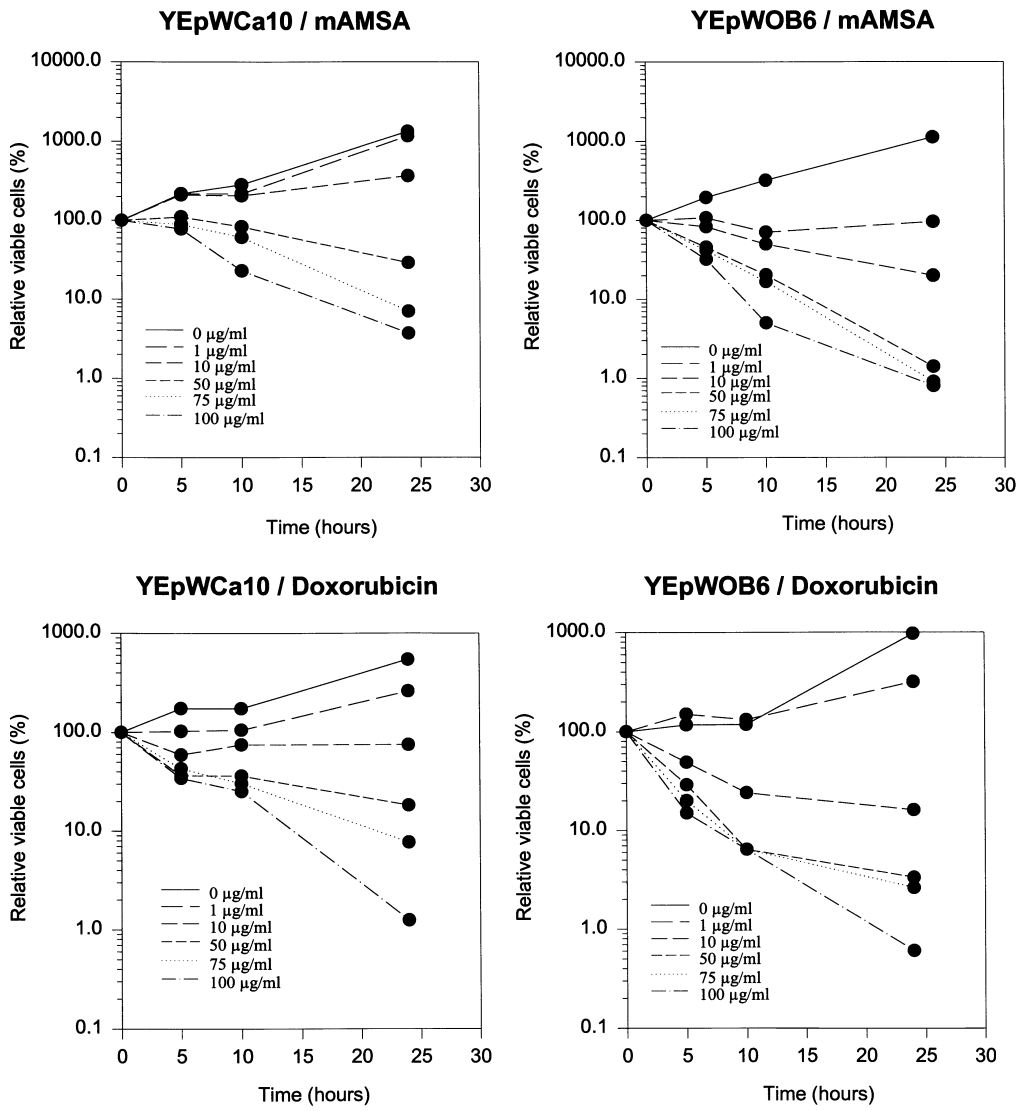

Figure 5 Effects of topoisomerase II-targeting agents on the viability of *S. cerevisiae* JN394 *top2-4ts* transformants growing at 35 °C by virtue of human topoisomerase IIα or *C. albicans* topoisomerase II expressed from plasmids YEpWOB6 (right hand panels) or YEpWCa10 (left hand panels) respectively

Viable counts were determined after growth of JN394t2–4 transformants at 35 °C in the absence or presence of amsacrine (m-AMSA) (top) or doxorubicin (bottom) and are expressed as a percentage of viable counts present at time zero.

2 show that the N-terminal 56 residues of *C. albicans* topoisomerase II are not present in the protein from either *S. cerevisiae* or *Sch. pombe* and are thus unlikely to be necessary for catalysis. YEpWCa10 is otherwise identical with plasmid YEpWOB6 which allows expression of a recombinant human α isoform in which the first 28 of the 1531 residues are replaced by the first five residues of *S. cerevisiae* topoisomerase II. Transformation of JN394t2–4 with YEpWCa10 and YEpWOB6 facilitated direct comparison of fungal and human enzymes *in vivo*.

Constitutive high level expression of topoisomerases II in *S. cerevisiae* is known to inhibit growth, and so glucose was used as the carbon source in our experiments. At present, there are no commercially available inhibitors known to be selective for *C. albicans* topoisomerase II. Therefore to test the yeast system it was decided to use amsacrine and doxorubicin, two anti-cancer drugs known to inhibit mammalian topoisomerase II. JN394t2–4 transformants were grown in minimal medium lacking uracil at 35 °C for 5, 10 and 24 h in the absence or presence of inhibitors, and then diluted and plated on to uracil-minus plates to determine the number of viable colonies (Figure 5). In the absence of drug, JN394t2–4 (YEpWCa10) grew at 35 °C giving a 10-fold increase in viable counts after 24 h (Figure 5 left hand panels). Inclusion of either amsacrine or doxorubicin led to time-dependent and dose-dependent reductions in viable counts. At higher drug doses, some killing was apparent after 5 h but was most marked after 24 h. For amsacrine at 10 μg/ml, there was notable reduction in viable counts and nearly three logs of killing at 100 μg/ml. Similar results were seen with doxorubicin. These experiments establish that topoisomerase II inhibitors do kill yeast cells growing by virtue of the *C. albicans* topoisomerase II. The right hand panels of Figure 5 show parallel experiments using JN394t2–4 (YEpWOB6) in which the drug target was human topoisomerase IIα. Dose-dependent killing of the transformants

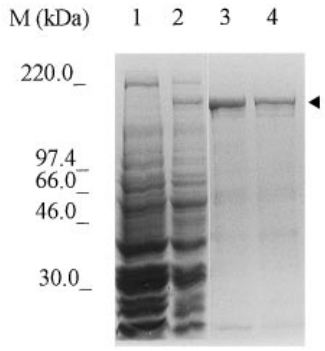

Figure 6 Purification of *C. albicans* topoisomerase II overexpressed in *S. cerevisiae* strain JEL1 bearing plasmid YEpWCa10

The SDS/5%-polyacrylamide gel shows the cleared lysate from yeast cells uninduced (lane 1) or induced with galactose (lane 2) and the two peak fractions from the phosphocellulose column (lanes 3 and 4). The arrowhead indicates topoisomerase II protein. The positions of protein size markers are shown on the left.

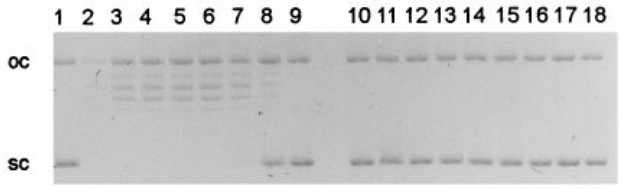

Figure 7 ATP-dependent DNA-relaxation activity of recombinant *C. albicans* topoisomerase II

Supercoiled plasmid pBR322 DNA was incubated with 0, 600, 300, 120, 60, 30, 12, 6 and 3 ng of recombinant protein in the presence (lanes 1–9) or absence (lanes 10–18) of 1 mM ATP. oc and sc denote open circular and supercoiled pBR322 respectively. DNA was analysed by 0.8% agarose-gel electrophoresis.

was again seen for both amsacrine and doxorubicin, and the kill curves suggest that YEpWOB6 conferred slightly greater drug sensitivity than YEpWCa10. The results in Figure 5 indicate that amsacrine and doxorubicin are inhibitors of the fungal enzyme and that such inhibitors can be studied in the yeast system.

Overexpression and purification of the recombinant fungal enzyme from *S. cerevisiae*: role of non-universal codons present in *C. albicans TOP2*

To confirm that amsacrine and doxorubicin do inhibit *C. albicans* topoisomerase II, it was necessary to purify the fungal protein to homogeneity to permit studies of enzyme inhibition *in vitro*. To achieve this aim, we exploited the fact that, in YEpWCa10, the *TOP2* gene is under the control of the *S. cerevisiae GAL1* promoter, thus allowing inducible expression in *S. cerevisiae* on addition of galactose. The protease-deficient *ura3 S. cerevisiae* strain JEL1 transformed with YEpWCa10 was grown in minimal medium lacking uracil and containing 3% glycerol and 2% lactic acid. Galactose was added to 2% and growth was continued for 12 h. Recombinant protein was purified essentially by the method of Worland and Wang [22] using a decatenation assay to follow activity. In short, the protocol involves cell lysis, Polymin P and $(NH_4)_2SO_4$ fractionation followed by chromatography on phosphocellulose. Figure 6 shows an SDS/PAGE analysis of protein fractions from a typical purification. The Coomassie-stained gel revealed a 160 kDa protein present in extracts of induced JEL1 cells containing YEpWCa10 (lane 2) that was absent from uninduced extracts (lane 1). The 160 kDa protein in peak fractions from the phosphocellulose column was more than 90% homogeneous (lanes 3 and 4). Approx. 300 ng of highly purified topoisomerase II could be produced per litre of yeast culture, a yield similar to that obtained for human topoisomerase IIα or β isoforms expressed using a similar approach [32,42].

Purified fungal topoisomerase II catalysed the relaxation of supercoiled pBR322 (Figure 7). It was found that 6 ng of protein was sufficient to cause 50% relaxation under standard conditions (lane 8) yielding a specific activity of 1.7×10^5 units/mg, a value similar to that of the purified *S. cerevisiae* enzyme and human α and β isoforms [22,42]. Characteristic of a eukaryotic type-II activity, relaxation was fully dependent on the inclusion of ATP (lanes 1–9); omission of this cofactor led to no detectable relaxation even using 600 ng of enzyme (lanes 10–18). The results indicate that the preparation was highly active in ATP-dependent relaxation and was substantially free ($< 1\%$) of topoisomerase I activity.

Interestingly, when a kDNA decatenation assay was used, the specific activity of the *Candida* enzyme was 3×10^3 units/mg, i.e. some 500-fold lower than that measured for either purified *S. cerevisiae* topoisomerase II or the human α isoform (results not shown). One explanation for this difference could be that the native *Candida* enzyme is inherently less efficient in decatenation. It is also possible that the standard conditions that we used to assay and compare the decatenation activities, i.e. 1 mM ATP and 10 mM Mg^{2+}, may not be optimal for the *C. albicans* protein. Alternatively, it could be a property of the recombinant enzyme expressed in yeast. It is known that, whereas human cells and *S. cerevisiae* utilize the universal genetic code, in *C. albicans*, the CUG codon is decoded as serine and not leucine as would normally be expected [44–46]. The *C. albicans TOP2* gene has six CUG codons (Figure 1), which presumably result in Ser-to-Leu protein substitutions when expressed in *S. cerevisiae*. Conceivably, one or more of these Ser-to-Leu substitutions present in the recombinant fungal protein could differentially affect decatenation but not relaxation activity. Whatever the effects, if any, the recombinant protein is active and complements in *S. cerevisiae*.

Response of the fungal enzyme to topoisomerase II inhibitors that form a cleavable complex

It is known that amsacrine, doxorubicin and other topoisomerase II inhibitors form a ternary complex with enzyme and DNA (sometimes called the cleavable complex) that on addition of detergent generates permanent double-stranded breaks in DNA [39]. Formation of the cleavable complex is thought to be the lesion that underlies the cytotoxicity of many topoisomerase-targeting drugs. This feature would explain the killing by amsacrine and doxorubicin of yeast strains growing by virtue of the fungal topoisomerase II. To test this idea directly, the purified enzyme was incubated with supercoiled pBR322 DNA and ATP in the presence or absence of drug, and DNA cleavage was induced by the addition of SDS. After proteinase K treatment to remove topoisomerase II subunits covalently linked to DNA ends, DNA products were examined by agarose-gel electrophoresis (Figure 8). In the absence of drug, *C. albicans* topoisomerase II induced DNA relaxation but did not promote measurable DNA cleavage (lanes 1 and 2). Inclusion of 10 μg/ml amsacrine stimulated DNA breakage giving approx. 50% con-

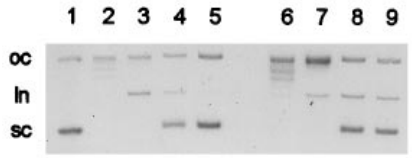

Figure 8 Amsacrine-mediated DNA cleavage by *C. albicans* topoisomerase II and by the human α isoform

Supercoiled pBR322 was incubated with *C. albicans* enzyme (600 ng) (lanes 2–5) or human topoisomerase IIα (130 ng) (lanes 6–9) in the presence of 0, 10, 50 and 100 μg/ml amsacrine respectively. No enzyme or drug additions were made to lane 1. After incubation with SDS and proteinase K, samples were analysed by electrophoresis in 0.8% agarose. oc, open circular pBR322; sc, supercoiled pBR322; ln, linear pBR322.

version of DNA into the linear form using 600 ng (1.8 pmol) of *C. albicans* enzyme (lane 3). Higher drug levels diminished cleavage and inhibited DNA relaxation (lanes 4 and 5). DNA cleavage by human topoisomerase IIα (130 ng; 0.4 pmol) was compared in lanes 6–9. Approx. 10% of the DNA was linearized. Allowing for differences in enzyme levels, the *C. albicans* and human topoisomerases were trapped with similar efficiencies by the drug. Thus these data obtained *in vitro* are in broad agreement with the kill curves seen for the yeast transformants in Figure 5.

DISCUSSION

Topoisomerase II of *C. albicans* is of considerable interest as a target for anti-fungal agents. However, little is known about the protein, and selective anti-fungal topoisomerase inhibitors have yet to be developed. To further our understanding of the fungal topoisomerase II, we have cloned and characterized the *C. albicans TOP2* gene, and, by using overexpression in yeast, we have for the first time purified the recombinant enzyme to near-homogeneity. These studies have allowed the interactions of inhibitors to be studied *in vitro*. Moreover, we have found that the fungal *TOP2* gene complements in yeast, rescuing growth of a drug-permeable *S. cerevisiae* mutant bearing a temperature-sensitive mutation in topoisomerase II. These yeast cells, growing by virtue of the *Candida* enzyme, constitute a novel system for the study of fungal topoisomerase II inhibitors.

C. albicans TOP2 was cloned by a PCR method utilizing oligonucleotide primers based on *S. cerevisiae TOP2* sequence coding for protein motifs that are also conserved in *Sch. pombe* topoisomerase II (Figure 1). The 1461-residue *C. albicans* topoisomerase II protein is organized in a similar fashion to other type-II topoisomerases, showing greatest identity with its counterparts in *S. cerevisiae* and *Sch. pombe*, and less homology to the human α and β isoforms (Table 1). The *C. albicans* protein had in common several motifs characteristic of topoisomerases II, namely PLRGK and MIMTDQD sequences [47]. Unexpectedly, the EGDSA motif, present in all known type-II topoisomerases described heretofore, was replaced in the *Candida* protein by EGLSA. This motif corresponds to a region of topoisomerase II that shares homology with the DNA gyrase GyrB protein [48], and which in the crystal structure of an *S. cerevisiae* topoisomerase II fragment lies above the DNA breakage–reunion domain [10]. Mutation of the GyrB EGDSA motif to EGNSA has been reported to confer resistance to quinolone anti-bacterial agents in *E. coli* [49]. Thus it appears that the conserved aspartate in the EGDSA structure can be replaced with other residues without complete impairment of enzyme function.

Expression of a plasmid-borne *C. albicans TOP2* gene rescued the lethal phenotype of yeast *top2ts* mutants and also allowed purification of a functional protein product. Evidently, the fungal gene promoter is active in yeast (Figure 3), and fungal topoisomerase II can substitute for its yeast counterpart in discharging essential functions in chromosome condensation and segregation. Complementation occurs despite the fact that the *C. albicans TOP2* gene carries six CUG codons (Figure 1) which are read as Ser in the non-universal code used in *C. albicans* [44–46] but are decoded as Leu in *S. cerevisiae*. The full-length fungal protein made in yeast therefore carries six leucine substitutions (five when expressed from YEpWCa10). With the exception of the highly conserved Ser-476, these substitutions occur at non-conserved positions. However, three residues are located in the C-terminal and N-terminal regions (Figure 2), segments of the protein that in human and *S. cerevisiae* topoisomerases II contain phosphorylation sites for serine/threonine kinases that appear to regulate enzyme activity (reviewed in ref. [8]). Conceivably, these changes, or indeed the EGLSA motif, could differentially reduce decatenation activity (Figure 7), leaving the relaxation activity unaffected. These issues remain to be resolved.

Recombinant fungal topoisomerase II induced DNA cleavage in the presence of amsacrine and was comparably efficient with the human α isoform (Figure 8). These results are in agreement with previous studies using a partially purified topoisomerase II preparation from *C. albicans* and calf thymus topoisomerase II as a representative mammalian enzyme [26]. Differential DNA cleavage was reported for four compounds, and similar or lower levels of DNA breakage were observed for the fungal enzyme in most cases, e.g. using amsacrine or etoposide. However, a complex synthetic pentacyclic difluorinated quinolone was described, which thus far is the only reported example of an agent selective for *C. albicans* topoisomerase II. This situation reflects the infancy of the field, but it would appear that selective inhibitors of the fungal enzyme are there to be discovered. *In vitro* experiments as in Figure 8, comparing drug inhibition of highly purified *C. albicans* topoisomerase II and human topoisomerase isoforms, will be important in pointing up potential problems of host toxicity.

Our observation that the fungal *TOP2* gene rescues growth of a drug-permeable yeast *top2* mutant allows the study of fungal topoisomerase II inhibitors *in vivo*. Given that selective fungal inhibitors are not readily available, we used amsacrine and doxorubicin as model compounds to establish the point that topoisomerase II inhibitors are able to kill yeast growing by virtue of the fungal topoisomerase II (Figure 5). Two lines of evidence strongly suggest that yeast killing arises from cleavable complex-formation involving the fungal protein. First, amsacrine, which induces similar levels of DNA breakage with the topoisomerases *in vitro*, was comparably toxic to yeast cells expressing fungal or human topoisomerase II (Figure 5). Secondly, etoposide, which forms cleavable complexes *in vitro*, with mammalian topoisomerase II but much less efficiently with the recombinant fungal enzyme, killed yeast cells expressing human α but not those producing *Candida* topoisomerase II (results not shown). Therefore cleavable complex-formation by drugs *in vitro* was in accord with cell killing *in vivo*. In concert with studies on human topoisomerase isoforms expressed in the same yeast genetic background, this approach should be valuable in identifying anti-fungal inhibitors.

In summary, we have characterized for the first time the *C. albicans TOP2* gene and its highly purified protein product and have established a yeast system in which cell growth depends on

the expression of the fungal topoisomerase II. This system should prove useful not only in testing known and novel topoisomerase inhibitors but also, by exploiting the genetic dominance of the fungal *TOP2* gene, should facilitate mutational analysis of the *C. albicans* topoisomerase II protein.

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