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 EcoRI fragment that mapped to chromosome 4. It was used to construct a plasmid in which TOP2 expresses a recombinant enzyme (residues 57–1461 of C. albicans topoisomerase II fused to the first five residues of Saccharomycoses 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 amscarcine and doxorubicin. Parallel experiments in yeast expressing human topoisomerase IIΔ 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 amscarcine 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 Saccharomycoses cerevisiae and the fission yeast Schizosaccharomycoses 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 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
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

Amascrine 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. EcoRI–XhoI adaptor was from Stratagene. Synthetic oligonucleotides were from Osuwel, Southampton, Hants, U.K. or were made in our department. The Sequenase version 2.0 sequencing kit, [α-32P]dCTP and [α-32P]dUTP (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 (top-2–4), JN394t2–4 (MATα ISE2 ura3–52 top2–4 rad52::LEU2) and JEL1 (a leu2 trpl ura3–52 pbio1–1122 pep3–4 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, and colony hybridization and DNA sequencing identified a plasmid, pBK3, that carried a 6.3 kb full-length TOP2 insert.

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'-TGTGTGGGTAGAGATTATGAG (nucleotide positions 1829–1852) and reverse primer 5'-CCATCATGATCTTGATC-3' (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 MgCl₂ 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 TOP2 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-32P]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 EcoRI 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 x SSC (where 1 x 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 EcoRI inserts were cloned individually into pBluescript SK+, 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 GALI promoter, amp and URA genes and the replication origins for the 2 μm plasmid and pBR322, was isolated as a BamHI–XhoI fragment. A second fragment carrying BamHI and AgeI ends was made by annealing two synthetic self-complementary oligonucleotides, 5'-GATCCCGTAACCATGTCAACTGAA and 5'-CCGGTTCA-GTTGACATGGTTACGG. This fragment specifies sequence from the GALI 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 HpaI site internal to TOP2 was obtained by PCR. The forward primer was 5'-CCTAGGCTACAACCGGTATAAT GCT TCA GAG ACA TAT in which the AgeI site is underlined and the sequence in brackets encodes C. albicans topoisomerase II beginning at codon 57; the reverse primer was 5'-CCCCATTTCTACCCACC-AGT corresponding to nucleotide positions 944–961, i.e. downstream of the HpaI site in the C. albicans TOP2 gene. Using pBK3 as template, and 2 units of Vent DNA polymerase at 1.5 mM MgCl₂, 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 AgeI
and HpaI to yield a 358 bp fragment which was gel-purified. Ligation of fragments 2 and 3 through the Agel site was expected to join the \textit{C. albicans TOP2} gene sequence to the beginning of the \textit{S. cerevisiae TOP2} coding sequence. Finally, a HpaI–XhoI fragment bearing the 3' region of \textit{C. albicans TOP2} and its natural stop codon was obtained by ligating EcoRI–XhoI adaptors to the ends of the 6.3 kb EcoRI TOP2 insert isolated from pBK3 (Figure 1), digesting with HpaI and XhoI and isolating the 5390 bp HpaI–XhoI TOP2 fragment. In addition to TOP2 sequence (nucleotides 551–4765), the fragment contains a partial \textit{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 \textit{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 \textit{Bam}H1, \textit{Age}I and \textit{Hpa}I restriction sites.

\textbf{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 \textit{A} \textit{600} of 0.4 and grown in the absence or presence of different concentrations (0–100 \textmu g/ml) of amarsamine 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).

\textbf{\textit{C. albicans} topoisomerase II}

The enzyme was purified by a modification of the method of Worland and Wang [22] that has been developed for \textit{S. cerevisiae} topoisomerase II. \textit{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 \textit{A} \textit{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 \textmu g/ml leupeptin and 10 \textmu g/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 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) (Polymion 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 Polymion 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 (NH₄)₂SO₄ was added to the eluate with stirring, and additional solid (NH₄)₂SO₄ 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 \textit{S. cerevisiae} DNA topoisomerase II and human topoisomerase IIz were purified from \textit{S. cerevisiae} JEL1 strains transformed with plasmids bearing the appropriate \textit{TOP2} genes under \textit{GAL1} control as described previously [32]. The purification protocol was essentially identical with that outlined above for the \textit{C. albicans} enzyme and resulted in proteins that were more than 95 % pure by SDS/PAGE analysis.

\textbf{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 MgCl₂, 1 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 30 \textmu g/ml BSA and 0.4 \textmu g of supercoiled pBR322 DNA (total volume 20 \mu l). 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 \mu g/ml BSA and substituting 0.25 \mu g of kinetoplast DNA (from \textit{C. fasciculata}).

\textbf{DNA cleavage}

Reaction mixtures contained 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 5 % DMSO, 0.4 \mu g of supercoiled pBR322 DNA human topoisomerase IIz or recombinant \textit{C. albicans} topoisomerase II, and reactions were carried out in the presence or absence of amarsamine. Samples were incubated for 30 min at 37 °C, and then further incubated with 1 \mu l of 10 % (w/v) SDS and 1 \mu l of 0.5 mg/ml proteinase K for 30 min at 37 °C. Samples were analysed by electrophoresis in 0.8 % agarose.

\textbf{RESULTS}

\textbf{Topoisomerase II encoded by \textit{C. albicans TOP2} shares closest homology with the \textit{S. cerevisiae} enzyme}

A PCR-based approach was used to isolate a full-length \textit{C. albicans TOP2} gene. First, a 213 bp \textit{C. albicans TOP2} PCR product was amplified from \textit{C. albicans} strain 2402E genomic DNA using two oligonucleotide primers made to the \textit{S. cerevisiae TOP2} sequence corresponding to VVGRDYYG and MTDQDHDG motifs (residues 464–471 and 525–532) (see the
Figure 1  For legend see facing page.
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 EcoRI fragment (not shown). Plasmid clone pBK3 containing the 6.3 kb EcoRI fragment was obtained by colony hybridization using the radiolabelled PCR product to probe a size-selected library of 2404E EcoRI 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 an incomplete open reading frame that we designated

Figure 1 Restriction map (top) and nucleotide sequence (bottom) of a 6290 bp EcoRI fragment containing the TOP2 gene of \textit{C. albicans}

E, H, K, R and X denote restriction sites for EcoRI, HindIII, KpnI, EcoRI and Xbal 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 polyserine sequences. Table 1 compares the relative sequence identities of the three yeast topoisomerases with the human topoisomerase IIα and IIβ isoforms. \textit{C. albicans} topoisomerase II was most closely related to its counterpart in \textit{S. cerevisiae}, i.e. 55% identity compared with 42-44% identity with the other proteins.

Chromosomal mapping of the \textit{C. albicans} TOP2 and SDHA genes was carried out independently using gene-specific PCR products as probes. Both genes mapped to chromosome 3 of \textit{C. albicans}. Syngeneity does not extend to \textit{S. cerevisiae}, the corresponding TOP2 and SDH1 genes of which map to chromosomes XIV and XI respectively [37,38].

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

Previous studies have elegantly shown that \textit{S. cerevisiae} topoisomerase II is the target in yeast for a variety of anti-cancer agents including amascrine and etoposide [33,39–41]. Moreover, plasmids expressing either the human TOP2α or TOP2β gene can complement a temperature-sensitive topoisomerase II mutant in \textit{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 \textit{C. albicans} TOP2 gene for its ability to rescue the temperature-sensitive growth of the \textit{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 \textit{URA3} genes yielding construct pBK50. Strain JCW6 was separately transformed with YCP50, pBK50 or plasmid YCPDEDWOB10 bearing the \textit{S. cerevisiae} TOP2 gene under the control of the constitutive \textit{S. cerevisiae} \textit{DE1} gene promoter [32], and transformants were selected on uracil-minus plates. Transformants on YPDA plates were incubated for 3 days at 25°C.
B. A. Keller, S. Patel and L. M. Fisher

Figure 2 For legend see facing page.
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 GAL1 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

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

Values are percentage amino acid identities.

<table>
<thead>
<tr>
<th></th>
<th>S. cerevisiae</th>
<th>Sch. pombe</th>
<th>Human α</th>
<th>Human β</th>
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<td>47.6%</td>
<td>41.6%</td>
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<td>—</td>
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<td>43.1%</td>
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**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 4 Structure and restriction map of YEpWCa10, a plasmid designed to allow overexpression of C. albicans topoisomerase II in S. cerevisiae**

SalI and XhoI sites are unique in the plasmid.
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
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 (NH₄)₂SO₄ 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 IIz 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 × 10⁶ 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 × 10⁶ 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 MgCl₂, 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-
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 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
topo 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 topoi-

somerase 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 topoi-

gerase 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 isofrom (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 de-
scribed, which thus far is the only reported example of an agent
selective for C. albicans topoisomerase II. This situation re-

flects 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 topoi-

serase 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
topo 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
topo II inhibitors are able to kill yeast growing by virtue of the
fung al 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
to the topoisomeras es in vitro, was comparably toxic to yeast cells
expressing fungal or human topoisomerase II (Figure 5). Sec-
ondly, 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
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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REFERENCES