Microbiology (1997), **143**, 377–386 Printed in Great Britain

The topoisomerase I gene from *Candida albicans*

Weidong Jiang,[1] David Gerhold,[2] Eric B. Kmiec,[2] Melinda Hauser,[3] Jeffrey M. Becker[3] and Yigal Koltin[1]

Author for correspondence: Yigal Koltin. Tel: +1 617 374 9090. Fax: +1 617 225 2997. e-mail: koltin@chemgenics.com

[1] ChemGenics Pharmaceuticals Inc., One Kendall Square, Bldg 300, Cambridge, MA 02139, USA

[2] Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, 233 S. 10th Street, Philadelphia, PA 19107, USA

[3] Department of Microbiology and Program in Cellular, Molecular, and Developmental Biology, University of Tennessee, Knoxville, TN 37996-0845, USA

We report here the cloning of the *Candida albicans* genomic topoisomerase I gene (*TOP1*) by use of PCR and subsequent hybridization. The predicted protein sequence shared 58·8% identity with the *Saccharomyces cerevisiae* topoisomerase I and 30–50% identity with other eukaryotic topoisomerase I proteins. A conditional gene disruption strain (CWJ477) was constructed so that one copy of *TOP1* was deleted and the other copy of *TOP1* was placed under a regulatable promoter. Under repressed conditions, cells grew slowly and cell morphology was abnormal. The virulence of CWJ477 was markedly reduced in a mouse model system, and that of the single gene knockout strain was slightly attenuated, indicating that *TOP1* might play a role in the infection of *C. albicans* in mice in a dose-dependent manner. Despite the reduced virulence of both the single and double knockout strains, viable cells of the pathogen were recovered from the kidneys as late as 22 d post-infection.

Keywords: *Candida albicans*, *TOP1*, null mutant, virulence

INTRODUCTION

DNA topoisomerases are key enzymes that resolve topological problems incurred during the cellular processes of replication, transcription, recombination and chromosome segregation. Based on their mechanism of action, they are classified into two types: type I DNA topoisomerases can break and rejoin one DNA strand at a time, whereas type II enzymes work by making reversible breaks in both strands, in a somewhat concerted manner, and by passing another segment of DNA duplex through this transient break (reviewed by Roca, 1995). Type I topoisomerases work as monomeric and ATP-indepenent enzymes. Based on their common enzymic properties and protein sequence analysis, the type I topoisomerases can be divided into two subtypes: type I-5′ and type I-3′. Type I-5′ topoisomerases bind, cleave and open transient gates in single-stranded DNA segments, in order to allow the passage of another single-stranded or double-stranded DNA segment. At the DNA cleavage stage, a protein–DNA covalent intermediate is formed between a tyrosyl residue and the 5′-phosphate at the DNA break site. Examples of type I-5′ topoisomerases are bacterial DNA topoisomerase I and III and eukaryotic DNA topoisomerase III. In contrast to type I-5′, type I-3′ topoisomerases bind preferentially to double-stranded DNA, and cleave one of the DNA strands of the duplex by forming a protein–DNA covalent intermediate between a tyrosyl residue and the 3′-phosphate at the break site. This family includes all eukaryotic DNA topoisomerase I enzymes and prokaryotic DNA topoisomerase IV.

The *Saccharomyces cerevisiae TOP1* gene is a member of the highly conserved group of type I-3′ topoisomerases (Goto & Wang, 1985; Roca, 1995). Despite the evidence for involvement of topoisomerase I in essential functions such as transcription and DNA replication, *TOP1* is not required for growth, and deletion of this gene results in only a modest growth defect. Deletion of *TOP1* does, however, cause a large increase in mitotic recombination in the tandemly repeated ribosomal DNA arrays, a chromosomal domain that is normally suppressed for recombination (Christman *et al.*, 1988). Null mutations in the topoisomerase I gene are also not lethal in *Schizosaccharomyces pombe* (Uemura *et al.*, 1987) or *Ustilago maydis* (Gerhold *et al.*, 1994), but are lethal to a developing *Drosophila melanogaster* embryo (Lee *et al.*, 1993).

The GenBank accession number for the nucleotide sequence reported in this paper is U41342.

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Drugs against DNA topoisomerase I, such as camptothecin, have been developed as potential anticancer agents. The cytotoxicity of the drug is due to the conversion of eukaryotic DNA topoisomerase I into a DNA-damaging agent (poison), rather than the inhibition of an essential function (reviewed by Chen & Liu, 1994). Therefore, although *TOP1* is not essential in many fungi, fungal topoisomerase I could still be considered as a potential antifungal drug target. The *Candida albicans* DNA topoisomerase I enzyme has been recently isolated and characterized (Fostel *et al.*, 1992; Fostel & Montgomery, 1995). *In vitro* experiments showed that stabilization by camptothecin of the fungal topoisomerase I cleavage complex requires a concentration 10-fold greater than the minimum concentration of camptothecin needed to enhance nicking by the human topoisomerase I (Fostel *et al.*, 1992). Conversely, the fungal topoisomerase I is more susceptible to the aminocatechol A-3253 than the human topoisomerase I (Fostel & Montgomery, 1995). This evidence suggests that there are structural differences between the two enzymes which may allow for the identification of new antifungal agents which target the fungal topoisomerase I and not the human enzyme. To begin to verify the structural differences of topoisomerase I from fungal pathogens and humans, we characterized *TOP1* from *C. albicans*. Here we report the cloning and sequencing of *TOP1*, and the use of an inducible/repressible promoter to study the phenotype of gene shut-off. We also demonstrate here that the *TOP1* gene disruption strain has reduced virulence in a mouse model.

METHODS

Strains. *C. albicans* strains CAF2-1 (*ura3Δ::imm434/URA3*) and CAI4 (*ura3Δ::imm434/ura3Δ::imm434*) were obtained from W. Fonzi (Fonzi & Irwin, 1993). DNA cloning was carried out in *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacI* (F′ *proAB lacI*^{q} ΔM15, Tn*10* [*tet*^{r}]); Stratagene] or DH5α [F$^-$ *φ80dlacZ*ΔM15 Δ(*lacZYA–argF*)*U169 deoR recA1 endA1 phoA hsdR17* ($r_K^- m_K^+$) *supE44 λ*$^-$ *thi-1 gyrA96 relA1*; Gibco-BRL].

Gene cloning and sequencing. The same conditions and primers were used here to amplify a *TOP1* gene fragment from *C. albicans* genomic DNA as were described for cloning of the *U. maydis TOP1* gene by PCR (Gerhold *et al.*, 1994).

The amplified PCR fragment was subcloned into pT7Blue vector (Novagen), forming plasmid pCaT53. The clone was verified by sequencing. Using this PCR fragment, genomic clones were obtained from a YEp13-based *C. albicans* DNA library (Rosenbluh *et al.*, 1985). A 3138 bp *Eco*RI fragment isolated from a clone with the largest insert was subcloned into pBC KS(−) (Stratagene), resulting in plasmid pCaT1-R12. The sequence of this DNA fragment from both strands was determined by an ABI373 sequencer.

Construction of plasmids and strains for gene disruption and promoter replacement of *Candida TOP1*. The 3138 bp *Eco*RI DNA fragment containing the *C. albicans TOP1* gene was subcloned into the *Eco*RI site of the pBluescript SK(−) plasmid, forming plasmid pSK+TOP1. An 861 bp *Nco*I–*Eco*RI fragment of the 3′-end of the *Candida TOP1* gene was subcloned from pCAT1-R12 into the *Sma*I site of plasmid pUC19 after it was blunted with Klenow and nucleotide triphosphates, resulting in plasmid pUC19T1. A 646 bp *Sma*I–*Eco*RV fragment of the 5′-end of the *C. albicans TOP1* gene was subcloned from pCAT1-R12 into the *Xba*I site of plasmid pUC19T1 after blunting with Klenow and nucleotide triphosphates, forming plasmid pUC19TΔ. Finally, a blunted *Bam*HI–*Bgl*II fragment of the *hisG–URA3–hisG* cassette from pCUB6 (Fonzi & Irwin, 1993) was cloned into the *Bam*HI site of pUC19TΔ, forming plasmid pUC19TΔU. Plasmid pUC19TΔU was linearized by *Hin*dIII and *Sac*I and used to transform strain CAI4 to replace *TOP1*. *URA3* prototrophs were restreaked on minimal medium and on 5-fluoroorotic acid to identify *ura3* auxotrophs typical of strains in which *URA3* loopout occurred. Both the primary transformant (CWJ429) and a loopout strain (CWJ431) derived from CWJ429 were verified by Southern blot hybridization.

The N-terminal DNA fragment of the *TOP1* gene (encoding the first 198 amino acids) was amplified by PCR using primers TOP1N1 (5′-ATGGATCCATGAGTTCATCAGACGAA) and TOP1N2 (5′-TCAAGCTTGCAACTTCTTCTGCTTC) and cloned into the pT7Blue vector. A. *C. albicans* promoter induced by maltose and repressed by glucose (*MRP1*; 1085 bp without ATG) was also amplified by PCR using primers MRP1-X1 (5′-ACTCTAGATCACCAATTCCATCACC) and MRP1-B1 (5′-ATGGATCCGTCGATCAAGTTACTTAC) and cloned into the pT7Blue vector. The characterization of *MRP1* will be described elsewhere (P. Riggle, personal communication). The *Xba*I–*Bam*HI promoter fragment was subcloned from the pT7Blue vector into the same sites of plasmid pKW16, which is based on a pBluescript plasmid, pSKII, with a *URA3* marker at the *Sac*I site, resulting in plasmid pUM2. The *Bam*HI–*Hin*dIII fragment of *TOP1* was then subcloned from the pT7Blue vector into the same sites of pUM2, forming plasmid pUMT2. *Asp*I-linearized pUMT2 was transformed into the heterozygous *TOP1* gene disruption strain CWJ431. Transformants were selected on a minimal medium containing maltose as sole carbon source. Again transformants were verified by Southern blot hybridization. After verification by Southern analysis, one transformant was designated CWJ477. This strain contained one disrupted, nonfunctional copy of *TOP1* and one copy of *TOP1* under the control of the maltose-inducible promoter.

DNA topoisomerase I assays. Glass beads were used to prepare *C. albicans* cell extracts as described for *S. cerevisiae* (Levin *et al.*, 1993). Cells from a 50 ml culture at late-exponential phase were collected, washed once with protein buffer (50 mM Tris/HCl, pH 7·5; 1 mM EDTA; 0·3 M KCl; 10%, v/v, glycerol; 2 mM PMSF), and resuspended in the same buffer with a volume equal to the volume of the cell pellet. Acid-washed glass beads (2 vols) were added to the suspension. The suspension was vortexed at 4 °C for 60 s three times. Supernatant was collected after an additional wash of beads with 1 vol. of the protein buffer and centrifuged at maximum microfuge speed for 20 min. The volume of the final clear protein extract was about 1 ml, which was stored at −70 °C for later use. Protein extract was diluted 1:10 with protein buffer and 1 μl was used in each reaction containing 200 ng supercoiled pUC19 in 20 μl of the topoisomerase assay buffer (20 mM Tris, pH 7·5; 100 mM KCl; 10 mM EDTA; 50 μg gelatin ml^{-1}). After 30 min incubation at 30 °C, reactions were stopped with 1 μl 20 mg proteinase K ml^{-1} and 2 μl 20% (w/v) SDS. DNA was examined in a 0·8% agarose gel containing ethidium bromide after incubation at 37 °C for 30 min and extraction with phenol/chloroform.

Virulence test of *TOP1*-deleted *C. albicans* strains. Three

(a)

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1     GAA TTC CTC AAA CAC GGT CAA AAA AAT ACC AAC TAT CTT CTG TTT CTC CCC ACT CAC ACG
61    ACC CAA CTA TTT TTT TGG TGA TGG TTT TAG GCG CGA CGT TAA TCA TTT TTA CTA TTG AGA
121   ATG ATT ACT CCC ACA TTC TAT TAC ACC TCA TCT TCA TCT TCA TCT TTC ATC TTT CAC ATC
181   ACT AAA TAT AAC CTT GCG ACC TTC ACA AAT TTT TTT TTT TGA CAA GCA ATC CAA AAT TAC
241   AAT TTT CAT TTC ATT TCT TTT ATA TAT AAA AGT TTT TCA CCA TTA ATT TCA CCA CAC ATC
301   TCA TTA GCA ATT GGG CAA AAA TAG AAA GTA ATT TTA TAA CTT ATA ACC AAA AAC AAT TCA
361   AGA ACA ATA TCA TTA TTA TTA AAT TTA TCA CGG AAT TTG TTT TGC AAA TCA AGT AAG AAC
421   AAT TTC CAT CAA TTT ACT CAT CAG TTT GGT TGT AAT AAT AAA AAC AGA TTA TTT TTC TTA
481   TCA TCA CCA CCA AGA GTA TTC CGT TAT TTA AAT CCA TTA TTT GTT CGT TCA TAT AGC ATA
541   ATT CCT ATG AGT TCA TCA GAC GAA GAA GAC ATT GCC TTG TCT AGA CTC GCT AAA AAA TCA
          M   S   S   S   D   E   E   D   I   A   L   S   R   L   A   K   K   S   18
601   TCC TCG ATC ACT TCA GCT TCC ACT TAT GAA GAC GAT GAA GAT GAT GAT ATC CCT TTA GCT
          S   S   I   T   S   A   S   T   Y   E   D   E   D   D   D   I   P   L   A   38
661   AAA AAA TCC AGG AAA AAG AGG GTT GAA TCT GAT TAT GAA GAA GAT GAA GAC GAA GTC CCA
          K   K   S   R   K   K   R   V   E   S   D   Y   E   E   D   E   D   E   V   P   58
721   TTG AAA AAG AGA AAA TTG TCT AAT GGT AGA GCA AAA AAA CAA GTT AAA ACC GAA ACT AAA
          L   K   K   R   K   L   S   N   G   R   A   K   K   Q   V   K   T   E   T   K   78
781   GTT AAA AAG GAA CCT AAA AGT GCC AAT AAA TCC AAA TCT ACA TCT AAA AAG GAC ACC AAA
          V   K   K   E   P   K   S   A   N   K   S   K   S   T   S   K   K   D   T   K   98
841   GTT AAG AAA GAG AAA ACT ACA GTC AAG AAG GAA TCT AAA GCC ACA AGC ACT AAA GTG AAA
          V   K   K   E   K   T   T   V   K   K   E   S   K   A   T   S   T   K   V   K   118
901   GAA GAA TCC AAA ACT CAA TCA GAT TCA CAA GCA TCG GTT AAA TCT GAA ACT CCT GAA GAA
          E   E   S   K   T   Q   S   D   S   Q   A   S   V   K   S   E   T   P   E   E   138
961   GAT CAA GGG TAC AAA TGG TGG GAA GTG AAT CAA GAA GAA GAA GGT GAT GGT TAT ATC AAA
          D   Q   G   Y   K   W   W   E   V   N   Q   E   E   E   G   D   G   Y   I   K   158
1021  TGG CAA ACA CTA GAA CAT AAC GGT GTT ATG TTT CCA CCA CCA TAT GAA CCA TTA CCA TCT
          W   Q   T   L   E   H   N   G   V   M   F   P   P   P   Y   E   P   L   P   S   178
1081  CAT GTC AAA TTA TAT TAT AAC AAT AAA CCA GTT AAT TTA CCT CCA GAA GCA GAA GAA GTT
          H   V   K   L   Y   Y   N   N   K   P   V   N   L   P   P   E   A   E   E   V   198
1141  GCC GGA TTT TAT GGA GCA ATG TTA GAA ACT GAT CAT GCT AAA AAC CCA GTT TTC CAA AAG
          A   G   F   Y   G   A   M   L   E   T   D   H   A   K   N   P   V   F   Q   K   218
1201  AAT TTT TTC AAT GAT TTT TTG GAA GTT TTA AAA GAA TGT GGT GGT TGT GGT GTT GAA ATT
          N   F   F   N   D   F   L   E   V   L   K   E   C   G   G   C   G   V   E   I   238
1261  AAA AAA TTT GAA AAA TTA GAT TTT AGT AAA ATG TAT GCT CAT TTT GAA AAA TTA CGT GAA
          K   K   F   E   K   L   D   F   S   K   M   Y   A   H   F   E   K   L   R   E   258
1321  GAG AAA AAG GCC ATG AGT AGG GAA GAA AAG AAA AGA ATC AAA GAA GAA AAA GAA AAA GAA
          E   K   K   A   M   S   R   E   E   K   K   R   I   K   E   E   K   E   K   E   278
1381  GAA GAA CCT TAT AGG ACT TGT TAT CTT AAT GGT AGA AAA GAA TTA GTG GGG AAT TTC CGT
          E   E   P   Y   R   T   C   Y   L   N   G   R   K   E   L   V   G   N   F   R   298
1441  ATT GAA CCT CCA GGT TTA TTC CGT GGT CGT GGT CCA CAT CCT AAA ACT GGG AAA TTA AAA
          I   E   P   P   G   L   F   R   G   R   G   A   H   P   K   T   G   K   L   K   318
1501  CGT CGA GTA GTG CTG GAA CAA GTG ACT TTG AAT TTA GGT AAA GAT GCT AAA ATA CCT GAA
          R   R   V   V   L   E   Q   V   T   L   N   L   G   K   D   A   K   I   P   E   338
1561  CCA CCT GCA GGC CAT CAA TGG GGG GAA ATT AGA CAT GAT AAT GAA GTC ACT TGG TTA GCC
          P   P   A   G   H   Q   W   G   E   I   R   H   D   N   E   V   T   W   L   A   358
1621  ATG TGG AAA GAA AAT ATT TCT GAT TCA TTG AAA TAC GTT AGA TTT GCT AAT AAT TCT TCA
          M   W   K   E   N   I   S   D   S   L   K   Y   V   R   F   A   N   N   S   S   378
1681  GTT AAA GGT CAA TCC GAT TTC AAA AAA TTT GAA ACG GCG AGA AAA TTA AGA GAT CAC GTT
          V   K   G   Q   S   D   F   K   K   F   E   T   A   R   K   L   R   D   H   V   398
1741  GAT TCT ATT AGA AAA GAT TAT ACC AAA ATG TTA AAA TCA GAG AAA ATG CAA GAT AGA CAA
          D   S   I   R   K   D   Y   T   K   M   L   K   S   E   K   M   Q   D   R   Q   418
1801  ATG GCC ACG GCT ATG TAT CTT ATT GAT GTT TTT GCA TTG AGG GCT GGT GGT GAA AAA GGT
          M   A   T   A   M   Y   L   I   D   V   F   A   L   R   A   G   G   E   K   G   438
1861  GAG GAT GAA GCC GAT ACC GTT GGT TGT TGT TCA TTA CGA TAT GAA CAT GTA ACT TTA AAA
          E   D   E   A   D   T   V   G   C   C   S   L   R   Y   E   H   V   T   L   K   458
1921  CCA CCC AAC AAG GTT ATT TTC GAT TTT TTG GGT AAA GAT TCA ATT AGA TTT TAT CAA GAA
          P   P   N   K   V   I   F   D   F   L   G   K   D   S   I   R   F   Y   Q   E   478
1981  GTT GAA GTT GAT AAA CAA GTT TTC AAA AAT CTA CGA ATT TTC AAA AAA TCT CCT AAA CAA
          V   E   V   D   K   Q   V   F   K   N   L   R   I   F   K   K   S   P   K   Q   498
2041  CCT GGT GAT GAT TTA TTT GAT CGT ATA AAC CCT TCA TTA GTC AAT CGA CAA TTA CAA AAT
          P   G   D   D   L   F   D   R   I   N   P   S   L   V   N   R   Q   L   Q   N   518
2101  TAT ATG AAA GGA TTA ACA GCA AAA GTT TTC CGT ACA TAT AAT GCC TCG AAA ACC ATG CAA
          Y   M   K   G   L   T   A   K   V   F   R   T   Y   N   A   S   K   T   M   Q   538
2161  GAT CAA ATT GAT ATA ATG GAA AAT GAA GGT ACA GTG GCG GAA AAA GTG GCT AAA TTC AAT
          D   Q   I   D   I   I   E   N   E   G   T   V   A   K   V   A   K   F   N   558
2221  GCT GCC AAT AGA ACG GTG GCT ATT TTA TCT AAT CAC CAG CGT ACG GTC AGT AAA ACC CAT
          A   N   R   T   V   A   I   L   C   N   H   Q   R   T   V   S   K   T   H   578
2281  GGT GAT AGT GTT CAG AGA ATT AAT GAC AAA TTG AAA AAA TTC ATG TGG CAA AAG ATT AGA
          G   D   S   V   Q   R   I   N   D   K   L   K   K   F   M   W   Q   K   I   R   598
2341  TTA AAG AAA ATG ATC TTA CAA TTA GAA CCC AAA TTG AAA AAG AAA GAT TCG AAA TAT TTT
          L   K   K   M   I   L   Q   L   E   P   K   L   K   K   K   D   S   K   Y   F   618
2401  GAA GAA ATT GAT GAT TTA CTC AAA GAA GAT ATT GAA CAT ATT CAT CAT ACT ATA ATT AAA
          E   E   I   D   D   L   L   K   E   D   I   E   H   I   H   H   T   I   I   K   638
2461  AGA CAA CGA GAA CAA GCT AAA AAA AAA TTA GAA CGT GAT AAT GAA AAA TTG AAA CTT GAA
          R   Q   R   E   Q   A   K   K   K   L   E   R   D   N   E   K   L   K   L   E   658
2521  GGT AAA CCA TTA TTA ACT GAA TCA GAT ATA AAA GAT AAA TTA GAT AAA ATT GAT GAA TTA
          G   K   P   L   L   T   E   S   D   I   K   D   K   L   D   K   I   D   E   L   678
2581  GAA AAA GAA TAT CAA AAA GAA TTG AAA ACT GGT AAA CCA ATA GTC ACC AAA AAT GCT ACC
          E   K   E   Y   Q   K   E   L   K   T   G   K   P   I   V   T   K   N   A   T   698
2641  GTT GAA AAA TTA AAA CAA CAA ATT GAA ACT CTT GAA AAT AAA ATT CTT AAT GTT TCA ATT
          V   E   K   L   K   Q   Q   I   E   T   L   E   N   K   I   L   N   V   S   I   718
2701  CAA TTA AAA GAT AAA GAA GAT AAT TCT GAA GTT TCT TTA GGA ACT TCA AAA ATG AAT TAT
          Q   L   K   D   K   E   D   N   S   E   V   S   L   G   T   S   K   M   N   Y   738
2761  ATT GAT CCA AGA TTA ATT GTT ATG TTT TCT AAA AAA TTT GAT GTT CCT ATT GAA AAA TTA
          I   D   P   R   L   I   V   M   F   S   K   K   F   D   V   P   I   E   K   L   758
2821  TTT ACC AAA ACT TTA AGA GAA AAG TTC ATT TGG GCT ATT GAA TCA GCT GAT GAA AAT TGG
          F   T   K   T   L   R   E   K   F   I   W   A   I   E   S   A   D   E   N   W   778
2881  AGA TTC TAA AAT TAG GGG TTT GTT TCT TAG CTT ATT ATT ATA TAC TAT ATG CTG TAG AGT
          R   F   *                                                               780
2941  AAA ATT TTG TAC CTT GTA ATA TAT ATA TAT ACA TTG TTT CAA CAT AGA AAA ATA GAT TGA
3001  TAC TGC AGT ATG AAA AAG AAT ATG CAC ACA CCA AGC AAG TGT ATT TTA GAT AAA GGA TTG
3061  GTG TTT TGA TAT TGG AAG GGT GAA AGA TGA AGG GGG TAT CAC ACA GAC ACG TAC AAT CAA
3121  GAA ATT GAA ATT TCT CCG AAT TC
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(b)

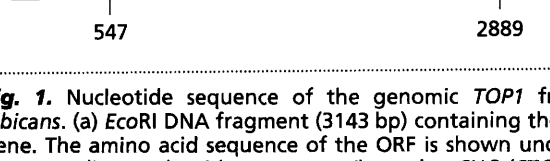

Fig. 1. Nucleotide sequence of the genomic *TOP1* from *C. albicans*. (a) *Eco*RI DNA fragment (3143 bp) containing the *TOP1* gene. The amino acid sequence of the ORF is shown under the corresponding nucleotide sequence. The codon CUG (CTG in the DNA) encodes serine rather than leucine in *Candida* (Omaha *et al.*, 1993). The essential tyrosine residue in the catalytic domain and the nearby methionine residue which is different from those in most other eukaryotic cells (usually leucine or isoleucine) are indicated as bold letters. (b) DNA strider map of the *Eco*RI fragment. *Eco*RV and *Nco*I sites were used for gene disruption and their relative positions to the ORF (547–2889) are indicated.

strains were used to determine virulence in the mouse model system. CAF2-1 is a heterozygous *URA3* knockout strain, which is used as a control for wild-type *TOP1*. CWJ429 is a heterozygous *TOP1* knockout strain with one copy of *TOP1* replaced by a *hisG–URA3–hisG* cassette and one wild-type copy of *TOP1*. CWJ477 is derived from CWJ429, in which one copy of *TOP1* was disrupted and the second copy of *TOP1* was placed under the regulation of a maltose-inducible and glucose-repressible promoter. Both CWJ429 and CWJ477 have one copy of *URA3*, which is similar to the parental strain, CAF2-1. Single colonies from strains CWJ429 and CWJ477 were inoculated into the liquid culture media YEPD (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, dextrose) and YEPM (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, maltose) and grown overnight (12 h) at 30 °C. The parental strain (CAF2-1) was cultured only in YEPD. The virulence of these *C. albicans* strains was tested in a normal mouse model system as described previously (Becker *et al.*, 1995). Male ICR mice (22–25 g; Harlan Sprague–Dawley) were inoculated with 100 µl of a suspension of 10^7 cells ml^{-1} via the lateral tail veins. Kidneys were excised on the indicated days post-infection, homogenized and plated onto YEPD or YEPM to determine c.f.u. (g tissue)$^{-1}$. For histological examination, paraffin sections were prepared from infected kidneys preserved in neutral-buffered formalin. Sections were stained using a commercially available silver stain kit (Sigma) to detect the presence of *Candida*, and counterstained with haematoxylin and eosin.

Germ-tube formation test. *C. albicans* cells were grown in either YEPD or YEPM and washed once with water. Germ-tube formation was induced in 10% (v/v) foetal bovine serum (Gibco-BRL), RPMI 1640 medium (Mediatech) or 50% serum/50% Sabouraud Dextrose Broth (Difco) at 37 °C (Bulawa *et al.*, 1995). After 1·5, 3 or 14 h incubation, cells were examined microscopically.

RESULTS

Cloning of *TOP1*

Our strategy for cloning the *C. albicans TOP1* gene was to identify peptide domains conserved among eukaryotic topoisomerase I genes, to design oligonucleotide primers based on these domains, and to use PCR to amplify the intervening *TOP1* DNA fragment from genomic template DNA. The gene fragment recovered was then used to select a full-length gene from a genomic DNA library. We have successfully used this approach to clone the *U. maydis TOP1* gene (Gerhold *et al.*, 1994). The same oligonucleotide primers were used in the amplification of a fragment of *TOP1* from the *C. albicans* genomic DNA. The amplified DNA fragment was ligated into plasmid pT7Blue (Novagen). The DNA sequence of this fragment predicted an amino acid sequence showing strong homology to other topoisomerase I proteins. Using this fragment as a probe, we screened a genomic DNA library based on plasmid YEp13 (Rosenbluh *et al.*, 1985) by hybridization and obtained five positive clones. DNA sequence analysis demonstrated that the largest clone, pCaT1, contained the entire coding region of the *C. albicans TOP1* gene. A 3145 bp *Eco*RI fragment from pCaT1 was subcloned into pBC KS(−) (Stratagene), forming plasmid pCaT1-R12. The complete sequence from both DNA strands revealed the complete *C.*

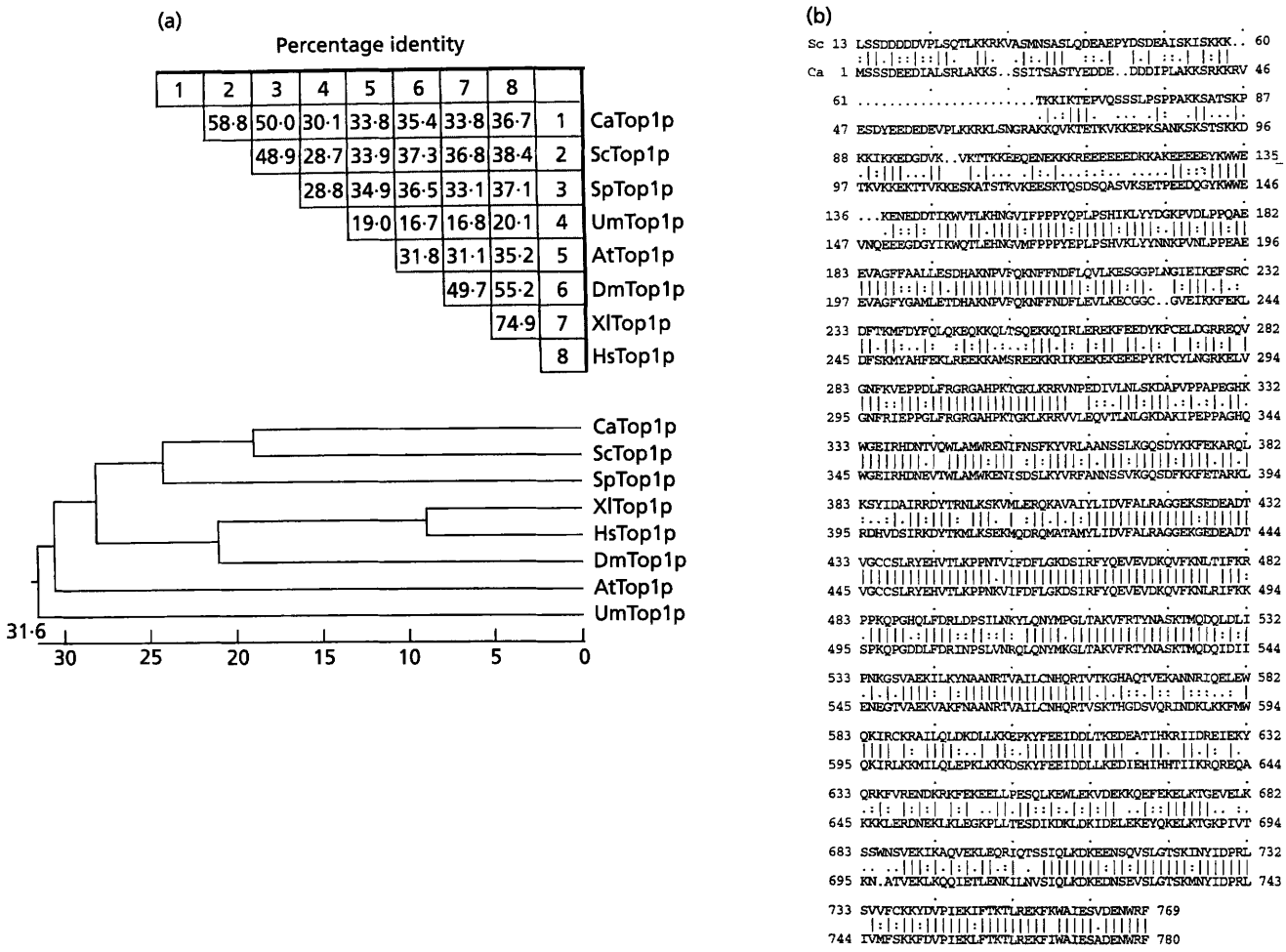

Fig. 2. Sequence comparison of Top1p from *C. albicans* with those from other eukaryotic cells by the Clustal method. (a) The upper panel shows the percentage identity in the pairwise comparison of Top1p from *C. albicans* (CaTop1p), *S. cerevisiae* (ScTop1p), *Schiz. pombe* (SpTop1p), *U. maydis* (UmTop1p), *D. melanogaster* (DmTop1p), *Arabidopsis thaliana* (AtTop1p), *Xenopus laevis* (XlTop1p) and *Homo sapiens* (HsTop1p). The lower panel depicts a phylogenetic tree of Top1p sequences from these organisms. (b) Alignment of the Top1p sequences from *S. cerevisiae* (upper sequence) and *C. albicans* (lower sequence). Vertical lines indicate amino acids that are conserved in both sequences.

albicans TOP1 ORF with highly significant homology to other *TOP1* genes (Fig. 1a).

Predicted *TOP1* peptide

The predicted *C. albicans TOP1* protein (Top1p) contains most of the peptide motifs conserved in Top1p from other eukaryotes (Fig. 2a). Peptide sequence comparison of Top1p revealed that *C. albicans* Top1p is most closely related to *S. cerevisiae* Top1p over the entire sequence (58·8% identity; see Fig. 2b). In the conserved region, in close proximity to the tyrosine (residue 738; Figs 1a and 2b) known to attach transiently to the DNA, the *C. albicans* enzyme is typified by a methionine rather than the isoleucine or leucine that is found in seven other eukaryotic enzymes. It is unclear whether this difference may lead to differential specificity of the *C. albicans* enzyme.

Conditional gene disruption of *TOP1*

The essentiality of *TOP1* has been tested in a variety of organisms. Thus far, it appears not to be essential in some organisms and can be considered a target for poisons rather than inhibitors (Chen & Liu, 1994). In *Drosophila* it is an essential enzyme that affects embryo development (Lee *et al.*, 1993). To test whether *TOP1* is essential in *C. albicans*, we initiated the disruption of *C. albicans TOP1* using the technology and procedure developed by Fonzi & Irwin (1993).

A gene disruption construct was made by multi-step cloning so that a 1630 bp internal coding sequence (encoding 543 amino acids; 70% of the ORF; Fig. 1b) of *TOP1* was replaced by a *hisG–URA3–hisG* cassette (Fig. 3; also see Methods). Linearized plasmid pUC19TΔU was transformed into *C. albicans* strain CAI4. Transformants were selected on minimal SD (0·67% Yeast Nitrogen base; 2%, w/v, glucose) plates. To identify

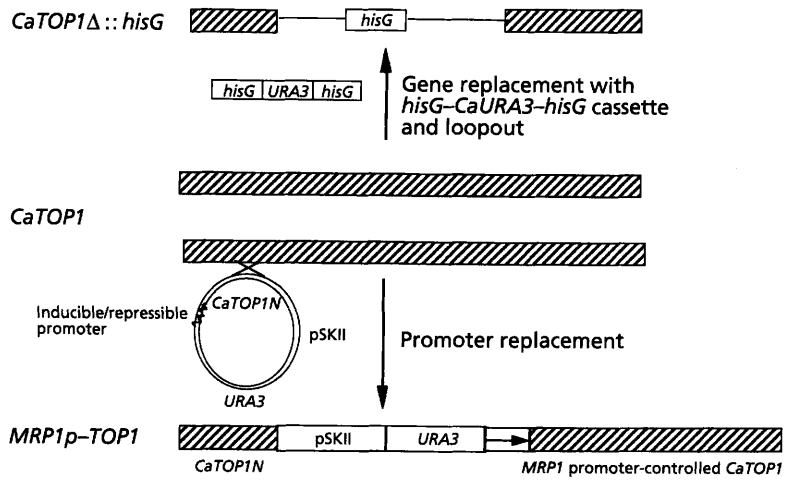

Fig. 3. Diagram of *TOP1* disruption and regulated gene expression. A 1630 bp DNA fragment encoding 543 internal amino acids of the *TOP1* ORF of the first copy of the gene was replaced by the *hisG–CaURA3–hisG* cassette, while the second copy of the gene was placed under a maltose-inducible/glucose-repressible promoter (*MRP1*) by plasmid integration (for details, see Methods).

the looping out of *URA3* the transformants were replated on 5-fluoroorotic acid plates supplemented with uridine. Transformants resistant to 5-fluoroorotic acid expected to be heterozygous *TOP1* disruptants (CWJ431; *TOP1/top1Δ::hisG*) were recovered. Both the primary transformant (CWJ429) and the loopout strain (CWJ431) were verified by Southern hybridization of the *Eco*RI-digested genomic DNA using a 900 bp *Eco*RI–*Nco*I fragment of the 5′-end of *TOP1* as a probe (data not shown). The same linearized plasmid DNA was used for the second round transformation of strain CWJ431 to obtain a homozygous knockout. No transformants were obtained after repeated transformation. This result suggests that *TOP1* may be essential or that *TOP1* deletion homozygosity may impair the growth of transformants. Therefore, we attempted another approach, i.e. using a regulatable promoter (Fig. 3) to test the essentiality of the gene.

A maltose-inducible/glucose-repressible promoter (*MRP1*) was cloned into an integrative *URA3* plasmid, forming plasmid pUM2. A DNA fragment encoding the N-terminal 198 amino acids of Top1p was placed under this promoter (see Methods), resulting in plasmid pUMT2. Integration of the *Asp*I-linearized pUMT2 into a wild-type copy of *TOP1* is expected to create a truncated Top1p with only a 54 N-terminal amino acid peptide and a full-length *TOP1* under the regulatable promoter. Transformants were selected on a maltose minimal medium and then streaked onto both maltose and glucose media. All the transformants grew on both media. However, some of them grew more slowly than others. The slower growing cells did not develop hyphae on rich medium after extended incubation (data not shown). These cells were subcultured and their morphology was further examined microscopically. Cells cultured in glucose medium were elongated and tended to lyse (Fig. 4a, lower panel) while cells from the maltose medium grew normally (Fig. 4a, upper panel). To determine whether the slow-growing cells lacked topoisomerase I activity, crude protein extracts were prepared from cells growing in both media and tested for DNA topoisomerase I activity using a supercoiled DNA relaxation assay (Gerhold *et al.*, 1994). In order to determine the sensitivity of this assay, protein extracts were diluted tenfold prior to use. Under these conditions, topoisomerae I activity was still detectable in cells grown in maltose, suggesting that the assay has a broad range of sensitivity (data not shown). No topoisomerase I activity was detectable in the slow-growing cells growing in medium containing glucose, while the activity was detectable in all cells grown with maltose (Fig. 4b). Each of the three strains demonstrating maltose-inducible topoisomerase I activity was characterized to verify their genetic construction by Southern hybridization using a 600 bp fragment of the 5′-end of *TOP1* (Fig. 4c). To further verify that the disruption of *TOP1* was specific, Southern hybridization performed using a *URA3* probe yielded a band of the expected size thereby ruling out additional integration events (data not shown). The results indicate that *C. albicans TOP1* is not essential for cell growth, but depletion of the protein leads to slow growth and a change in cell morphology.

Virulence test of the *TOP1*-deleted *C. albicans* strains

The phenotypic change of the Top1p-depleted *C. albicans* strain indicates that *TOP1* may affect the infectious phase of the organism. To test the effect of *TOP1* deletion on virulence in an animal model, normal mice were infected with the parental strain (CAF2-1), *TOP1* heterozygous knockout strain (CWJ429), and the strain (CWJ477) with the only existing copy of *TOP1* under the maltose-inducible and glucose-repressible promoter. Prior experiments demonstrated that the maltose promoter used for gene deletions was not induced *in vivo* in the mouse model, presumably due to the absence of maltose in tissues and serum. Confirmation of the absence of induction of the maltose promoter was seen by placing *URA3* under control of the maltose promoter. This resulted in strains that were rapidly cleared from infected mice and as such were avirulent (data not shown). This outcome is similar to the lack of pathogenicity described for *ura3* homozygous knockout strains (Kirsch & Whitney, 1991).

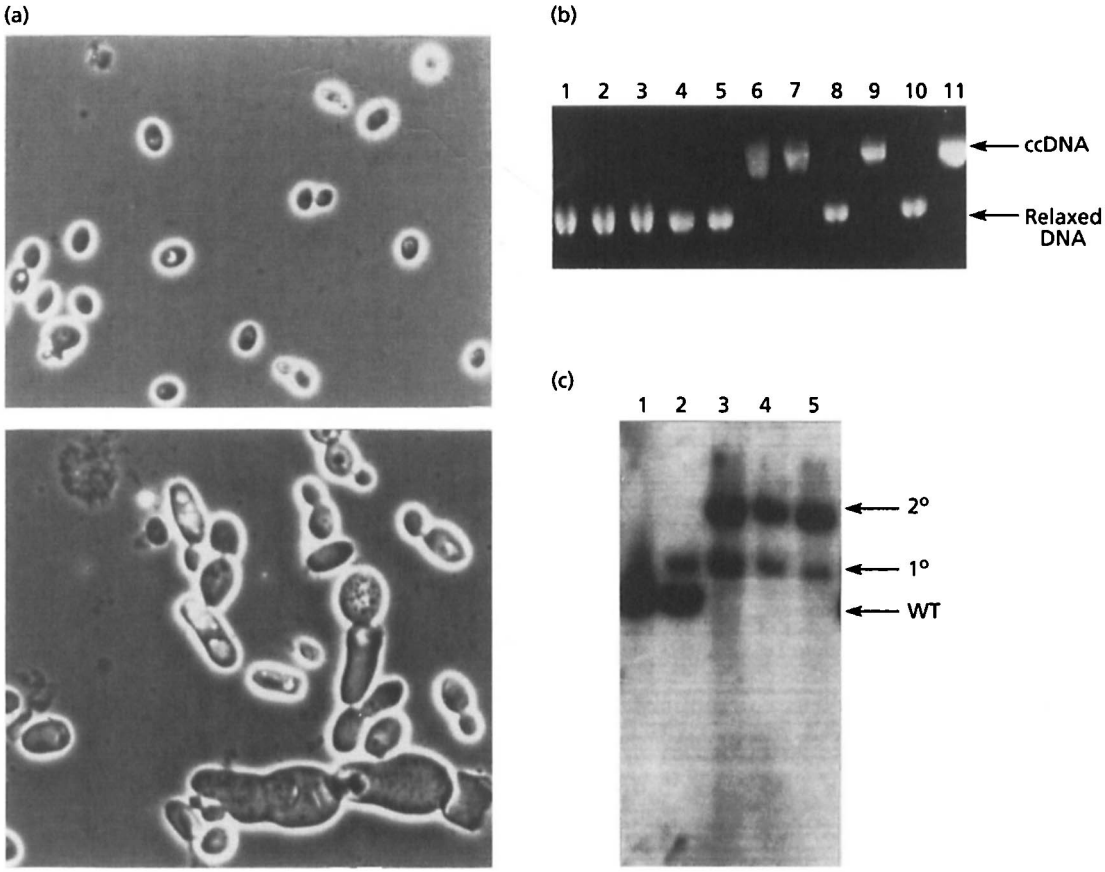

Fig. 4. Gene disruption of *C. albicans TOP1*. (a) Morphological change associated with *TOP1* gene shut-off. The top panel shows the transformant (CWJ477) lacking one of the *TOP1* alleles and expressing the second allele under a maltose/glucose-regulated promoter. The bottom panel shows the same transformant after *TOP1* shut-off (i.e. cultured on glucose). The cells divide somewhat slower, and display a novel morphology. Notice the increase in size and cell elongation. (b) Confirmation of *TOP1* shut-off by the enzyme assay. Top1p activity from four independent transformants which have the second copy of the gene presumably placed under a regulated promoter while the first copy has been disrupted was tested with the DNA plasmid relaxation assay. Lanes: 1–4, four transformants with the *TOP1* expression induced by maltose; 6–9, the same transformants as in lanes 1–4, but cultured in glucose, thus repressing *TOP1* expression. Lanes 3 and 8 show the same result, indicating that this transformant is different from the other three and that the *TOP1* gene is not placed under the control of the *MRP1* promoter. Lanes 5 and 10 show a primary transformant in which one of the two alleles of *TOP1* was disrupted. The same results were obtained under both repressive and nonrepressive conditions. Lane 11 shows the control reaction without the enzyme. (c) Southern hybridization to confirm *TOP1* disruption and promoter replacement. The DNA was digested with *Eco*RI and probed with a 600 bp fragment of 5′-end *TOP1*. Lanes: 1, wild-type (WT) *TOP1/TOP1*; 2, disruption of the first *TOP1* allele with the *hisG–CaURA3–hisG* cassette; 3–5, same strains used in lanes 6, 7 and 9 in Fig. 4(b), replacement of the *TOP1* promoter in the intact second allele with the *MRP1* promoter by plasmid integration.

Strains were cultured in both glucose and maltose media prior to injection. Since strain CWJ477 clumps and is fragile in glucose medium (Fig. 4a), it was difficult to determine cell density accurately. The suspension was vigorously vortexed for 30 s, which was sufficient to disrupt the cell clumps but not to lyse the cells, thus allowing accurate counting in the haemocytometer.

The survival curve for mice infected with the three strains indicated that inactivation of one or two copies of *TOP1* leads to dramatic attenuation of virulence (Fig. 5). There were no survivors after 7 d among mice infected with the parental strain, whereas about 50% of the mice survived infection with the heterozygous *TOP1* knockout strain, CWJ429, at day 11 from maltose-cultured cells and at day 13 from glucose-cultured cells. However, only one of nine mice infected with maltose-cultured CWJ477 cells had died by day 22, while about 50% mice infected with glucose-cultured CWJ477 cells survived.

To test whether the reduced virulence of *C. albicans* cells was due to either attenuation or clearance from the mice, c.f.u. $(g\ tissue)^{-1}$ was determined from homogenized kidney tissue on various days post-infection in survivors from each group, and on day 22 from all

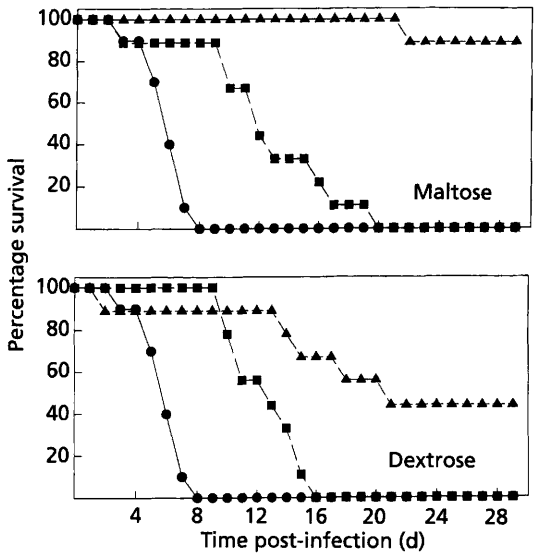

Fig. 5. Virulence test of *C. albicans TOP1* disruptants. Mice were inoculated with approximately 1×10^6 cells of three different *Candida* strains grown in YEPM (upper panel) and YEPD (lower panel). The percentage survival of mice was plotted against the days of observation. The experiment was repeated and the results are summarized in the plot. ●, CAF2-1 (control; $n = 10$); ■, CWJ429 (*TOP1/top1*; $n = 9$); ▲, CWJ477 (*top1/top1*; $n = 9$).

remaining mice. Large numbers of c.f.u. were recovered from the kidneys of mice infected with either *TOP1/top1* or *top1/top1* strains (Table 1). Results of a repeat experiment were also similar to the primary experiment (data not shown). Therefore, the survival rate of the host did not reflect the clearance of the pathogen or a significant decrease of c.f.u. in the kidneys of the infected host but rather reflected attenuated virulence of the strains.

TOP1-deleted cells have reduced capability to form germ tubes

Although *TOP1*-deleted *C. albicans* cells have reduced virulence, they were recovered from the kidneys of the infected mice. We tested the germ-tube formation of these strains under three different conditions. When cells were grown in maltose medium (induction conditions) and then tested for germ-tube formation, both heterozygous and homozygous *top1* strains (CWJ429 and CWJ477) were able to form germ tubes like the wild-type strain (CAF2-1) (data not shown). However, when cells were pre-grown in glucose medium, the homozygous *top1* strain (CWJ477) formed germ tubes with decreased frequency at the beginning of the serum induction (1·5 h) (Fig. 6, left panel). After extended incubation at 37 °C with serum, few germ tubes could be observed in the homozygous *top1* strain (Fig. 6, right panel). Similar results were also obtained from cells induced with 10% dialysed serum supplemented with 2% maltose or glucose. All strains could form germ tubes almost equally well in the 10% dialysed serum with maltose; however, the homozygous disruptant strain formed germ tubes poorly in the 10% dialysed serum with glucose. These results indicate that

Table 1. Recovery of *C. albicans* from mice infected with *TOP1* knockout strains

Strain* (growth conditions)	Days post-infection	Kidney mass (g)	c.f.u. (g tissue)$^{-1}$
CAF2-1 (dextrose)	5	0·62	$1{\cdot}3 \times 10^5$
CWJ429 (maltose)	5	0·57	$3{\cdot}6 \times 10^4$
	10	0·51	$1{\cdot}0 \times 10^6$
	11	0·59	$3{\cdot}8 \times 10^6$
CWJ429 (dextrose)	5	0·39	$1{\cdot}8 \times 10^4$
	10	0·43	$6{\cdot}1 \times 10^3$
	11	0·59	$3{\cdot}8 \times 10^6$
CWJ477 (maltose)	5	0·50	$2{\cdot}7 \times 10^4$
	10	0·47	$6{\cdot}1 \times 10^5$
	11	0·44	$7{\cdot}5 \times 10^5$
	22	0·45	$4{\cdot}1 \times 10^6$
	22	0·44	$1{\cdot}8 \times 10^6$
	22	0·33	$9{\cdot}7 \times 10^6$
	22	0·40	$7{\cdot}9 \times 10^6$
CWJ477 (dextrose)	5	0·44	$9{\cdot}4 \times 10^3$
	10	0·44	$1{\cdot}4 \times 10^6$
	11	0·45	$7{\cdot}8 \times 10^5$
	22	0·44	$1{\cdot}1 \times 10^6$

* CWJ429 = *top1::hisG–URA3–hisG/TOP1*; CWJ477 = *top1::hisG/MRP1:TOP1–URA3*.

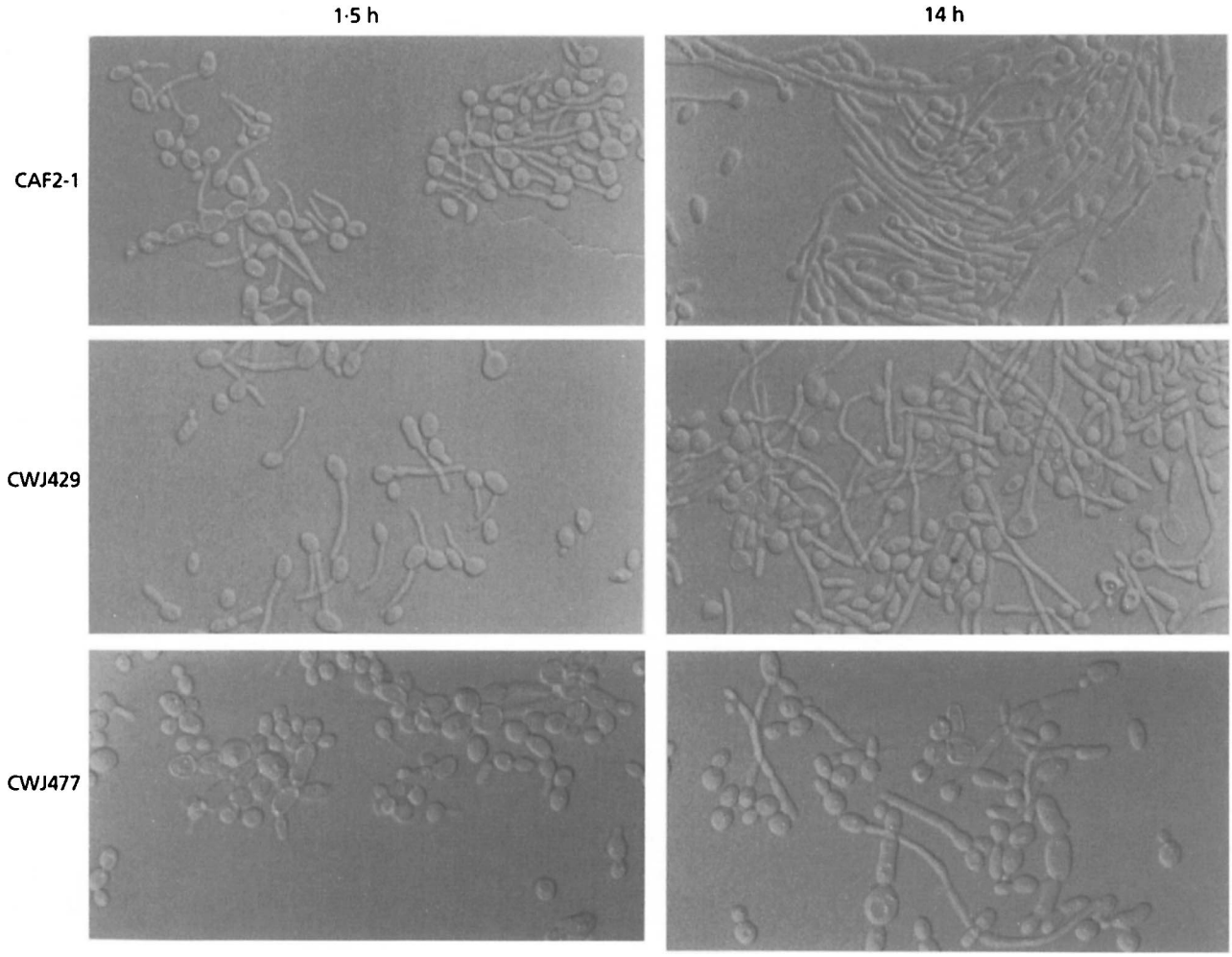

Fig. 6. Germ-tube formation of *C. albicans TOP1* disruptants. *C. albicans* cells were grown in YEPD glucose medium overnight and inoculated into 50% serum/50% Sabouraud Dextrose Broth at 37 °C. Cells were examined under the microscope after 1·5 h (left panel) and 14 h (right panel) incubation. Top, CAF2-1 (wild-type *TOP1*); middle, CWJ429 (*TOP1/top1*); bottom, CWJ477 (*top1/top1*).

topoisomerase I plays an important role in germ-tube formation.

TOP1-deleted cells form hyphae in the kidney

Histological examination of the kidneys of mice infected with the homozygous deletion strain (CWJ477) revealed that *Candida* was found in a hyphal form (Fig. 7). The characteristics of infection observed in kidneys containing the heterozygous knockout strain (CWJ429) were indistinguishable from CWJ477. At days 7–10 post-infection, kidneys obtained from mice infected with the wild-type *Candida* (CAF2-1) contained well-defined focal lesions, comprised of hyphal forms, primarily within the renal cortex (data not shown; see Kwon-Chung & Bennett, 1992). The kidneys infected with the deletion strains exhibited a very different pattern of infection. Renal tissue obtained 10 d post-infection revealed a dilated renal pelvis (Fig. 7a). Contained within the urinary space of the renal pelvis was a large mass of *Candida* hyphae, commonly referred to as a 'fungus ball' (Fig. 7b). The lack of lymphocytes in the region surrounding the inflammation suggests that this is an acute, suppurative response. Although there were indications of inflammation in the remainder of the renal tissue, no *Candida* (either yeast or hyphal forms) was observed in these regions.

DISCUSSION

We report here the cloning and characterization of the *C. albicans TOP1* gene. A conditional *TOP1* disruption mutant strain was constructed to investigate the role of topoisomerase I in cellular processes and in pathogenesis. Our data show that this gene is not essential for *in vitro* growth of *C. albicans*, but morphological changes are obvious in minimal medium when the protein is depleted under promoter-repressed condi-

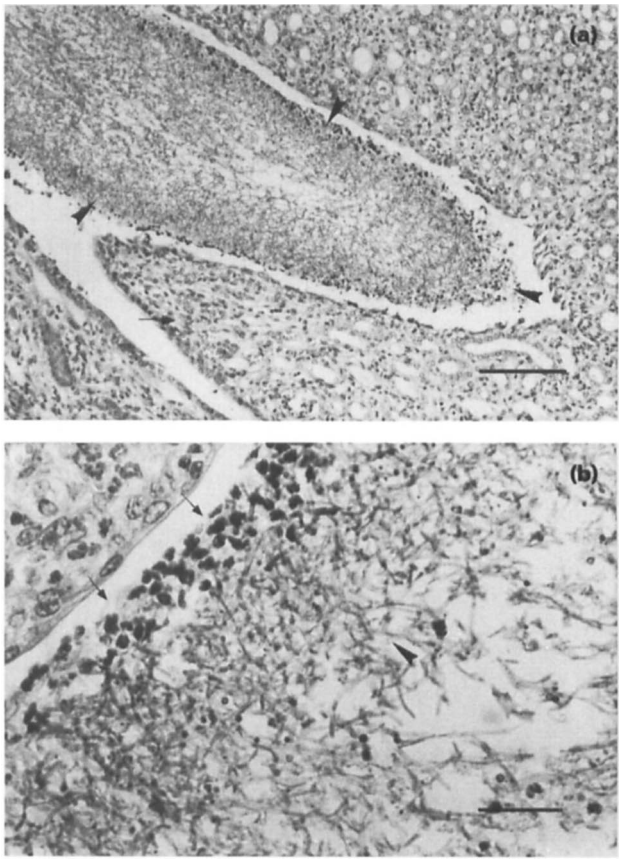

Fig. 7. Presence of hyphae in the kidneys of mice infected with the *TOP1* disruptant CWJ477. Kidneys infected with the *top1/top1* deletion strain were processed for histology on day 10 post-infection. (a) *Candida* hyphae were found exclusively in the renal pelvis (see arrowheads). The surrounding renal tissue (see arrow) shows evidence of inflammation, but no hyphae or yeast. Bar, 100 μm. (b) Enlargement of *Candida* hyphae from (a). The hyphal mass (see arrowhead) is surrounded by a layer of neutrophils (see arrows) associated with renal pyelitis. Bar, 25 μm.

tions, indicating that *TOP1* plays an important role in cellular processes. A similar effect in *TOP1*-disrupted cells of *S. cerevisiae* and *U. maydis* has also been observed (Thrash *et al.*, 1985; Gerhold *et al.*, 1994). In *S. cerevisiae*, deletion of this gene results in a modest growth defect and a large increase in mitotic recombination in the tandemly repeated ribosomal DNA arrays (Christman *et al.*, 1988). A *top1* gene disruption mutant of *U. maydis* displayed a subtle colouration phenotype evident during cell senescence and a much lower level of mitotic recombination elevation (Gerhold *et al.*, 1994).

We also showed that the *TOP1* deletion strains were deficient in their ability to form germ tubes and to grow in the hyphal form after serum induction *in vitro*. It is possible that the inability of the conditional knockout to form hyphae *in vitro* might be due to a general effect on cell viability rather than specifically to the lack of topoisomerase I activity. However, *in vivo*, clear histological evidence indicated that these same strains grew in the hyphal form in the renal pelvis; at day 10 post-infection it appears that *Candida* harboured in the kidney is limited to the urinary space with little or no evidence of hyphal growth in the tissues. These *in vivo* and *in vitro* observations are difficult to resolve. It is possible that there are additional factors present in the microenvironment of the murine urinary space which promote hyphal growth of these *Candida* mutants in the kidney. This possibility warrants further investigation.

Injection of *TOP1* deletion strains into mice indicates that deletion of one copy of *TOP1* causes some attenuation of virulence, while deletion of both copies leads to a more pronounced degree of attenuation. It is possible that these results might be due to the result of a gross general defect in growth, but the combined histological and survival curve data suggest otherwise. Cells which cannot grow in a host (*ura3/ura3* mutants; Kirsch & Whitney, 1991), for example, are simply cleared from the body before ever colonizing tissues. The *TOP1* deletion strains are maintained in the kidney for a prolonged period of time (up to 22 d) at quite high levels [$> 10^6$ c.f.u. (g tissue)$^{-1}$], and are able, in this environment, to form hyphae. Despite the obvious maintenance of the mutant *Candida* in the kidney, these strains are very much reduced in their capacity to cause death in infected mice. Similar results were reported recently for other gene disruptions (Bulawa *et al.*, 1995; Becker *et al.*, 1995).

Gene knockout of DNA topoisomerase I in *S. cerevisiae* (Goto & Wang, 1985; Thrash *et al.*, 1985), *Schiz. pombe* (Uemura *et al.*, 1987) and *Ustilago* (Gerhold *et al.*, 1994) shows that this enzyme is not essential for viability of these fungi. In contrast, topoisomerase I is essential in a developing *D. melanogaster* embryo (Lee *et al.*, 1993). Most of the potential drugs against human DNA topoisomerase I are poisons which do not inhibit the enzyme activity. Rather, these drugs damage cellular processes by trapping the putative covalent intermediate of the reaction (reviewed by Chen & Liu, 1994). We show here that *TOP1* is not essential for *in vitro* growth; however, its expression is important for normal cellular morphology, germ-tube formation and virulence in a mouse model.

ACKNOWLEDGEMENTS

This work was partly supported by an SBIR grant from NIH (AI 38133-0 ZRG5). The assistance of Eric Lo in plasmid construction is acknowledged. We thank Ken Winter and Perry Riggle for providing plasmids, Karen Kemmler for assisting with the animal studies, and Dr Clark S. Patton for assistance in the histological examination of the kidney tissue.

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Received 4 July 1996; revised 30 September 1996; accepted 8 October 1996.