Topoisomerase I Is Essential in *Cryptococcus neoformans*: Role in Pathobiology and as an Antifungal Target

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Manuscript received December 8, 1998
Accepted for publication February 12, 1999

ABSTRACT

Topisomerase I is the target of several toxins and chemotherapy agents, and the enzyme is essential for viability in some organisms, including mice and drosophila. We have cloned the *TOP1* gene encoding topoisomerase I from the opportunistic fungal pathogen *Cryptococcus neoformans*. The *C. neoformans* topoisomerase I contains a fungal insert also found in topoisomerase I from *Candida albicans* and *Saccharomyces cerevisiae* that is not present in the mammalian enzyme. We were unable to disrupt the topoisomerase I gene in this haploid organism by homologous recombination in over 8000 transformants analyzed. When a second functional copy of the *TOP1* gene was introduced into the genome, the topoisomerase I gene could be readily disrupted by homologous recombination (at 7% efficiency). Thus, topoisomerase I is essential in *C. neoformans*. This new molecular strategy with *C. neoformans* may also be useful in identifying essential genes in other pathogenic fungi. To address the physiological and pathobiological functions of the enzyme, the *TOP1* gene was fused to the *GAL7* gene promoter. The resulting *GAL7::TOP1* fusion gene was modestly regulated by carbon source in a serotype A strain of *C. neoformans*. Modest overexpression of topoisomerase I conferred sensitivity to heat shock, γ-rays, and camptothecin. In contrast, alterations in topoisomerase I levels had no effect on the toxicity of a novel class of antifungal agents, the dicationic aromatic compounds (DACs), indicating that topoisomerase I is not the target of DACs. In an animal model of cryptococcal meningitis, topoisomerase I regulation was not critically important to established infection, but may impact on the initial stress response to infection. In summary, our studies reveal that topoisomerase I is essential in the human pathogen *C. neoformans* and represents a novel target for antifungal agents.

CRYPTOCOCCUS neoformans is a common cause of life-threatening central nervous system infection in immunocompromised patients (White *et al.* 1992; Powderly 1993). Unfortunately, despite progress with present antifungal agents, a significant number of treatment failures and relapses still occur (Kelly *et al.* 1994; Parugam *et al.* 1994; Birley *et al.* 1995; Mitchell and Perfect 1995; Armengou *et al.* 1996; Berg *et al.* 1998). New antifungal agents with fungicidal activity are needed to more effectively manage systemic cryptococcosis in severely immunosuppressed hosts.

Over the past few years, studies into the molecular biology of *C. neoformans* have advanced, and there are now molecular tools available to examine this pathogenic yeast (Mitchell and Perfect 1995), including transformation by electroporation (Edman and Kwon-Chung 1990) and biolistics (Toffaletti *et al.* 1993). Thus, it is now possible to use molecular strategies and animal models to identify and characterize potential drug targets for this pathogen (Perfect 1996).

Topoisomerases are enzymes that modulate the topology of DNA during replication (Brill *et al.* 1987), transcription (Tsao *et al.* 1989), recombination (Holm *et al.* 1989; Rose *et al.* 1990), and repair (Bohr 1988). Topoisomerase I is a monomeric, ATP-independent enzyme that transiently nicks one DNA strand by forming a covalent enzyme-DNA complex (Gupta *et al.* 1995; Redinbo *et al.* 1998). Camptothecin inhibits topoisomerase I by stabilizing the enzyme-DNA intermediate of the topoisomerase reaction in which the enzyme is covalently bound to DNA by a phosphotyrosine linkage. Camptothecin inhibits the religation reaction of topoisomerase in this complex, leading to elevated levels of cleavage complexes and irreversible cellular DNA damage (Liu *et al.* 1996; Stewart *et al.* 1998). For example, stabilization of a covalent intermediate, thereby converting the topoisomerase I into a cellular poison, is the most plausible explanation for camptothecin antifungal activity since camptothecin is toxic to wild-type *Saccharomyces cerevisiae* cells, but *S. cerevisiae* mutants lack-

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ing topoisomerase I activity are viable and camptothecin resistant (Goto and Wang 1985).

Although topoisomerase I is not essential for viability in *S. cerevisiae*, *in vivo* studies revealed that it is essential both in the mouse and in *Drosophila melanogaster* (Lee *et al.* 1993; Morham *et al.* 1996). Furthermore, Jiang *et al.* (1997) showed in a mouse model of candida infection that a *Candida albicans* strain heterozygous for a topoisomerase I deletion (*top1/TOP1*) was attenuated for virulence compared to wild type. Moreover, when the *C. albicans TOP1* gene was differentially expressed with the use of a regulated maltose promoter, virulence was reduced under the repressed conditions found in the host. A complete topoisomerase I null mutant could not be obtained in *C. albicans*, suggesting that topoisomerase I might be essential in this organism.

Our recent studies in antifungal drug development have identified a new series of molecules classified as dicationic aromatic compounds (DACs). These compounds include pentamidine analogues, dicationic substituted bis-benzimidazoles, carbazoles, furans, and benzimidazoles. Some compounds in these classes have potent broad-spectrum antifungal activity, including potent fungicidal activity against *C. neoformans* (Dykstra *et al.* 1994; Del Poeta *et al.* 1998a,b). In a particular group of DACs, the bis-benzimidazoles, we have shown an apparent selective inhibition of the catalytic activity of the topoisomerase I enzyme from *Pneumocystis carinii* compared to the mammalian enzyme (C. C. Dykstra, personal communication). Sun *et al.* (1994) have also shown that certain bis-benzimidazoles exhibit tumor cytotoxicity that is correlated with relative potency as topoisomerase I inhibitors. These previous findings led us to focus on topoisomerases as potential targets for anticryptococcal activities (Shen *et al.* 1992) and, specifically, for DACs that are known to have a prerequisite for DNA binding to activate their broad antimicrobial activity (C. C. Dykstra, personal communication).

The combination of our preliminary studies with DACs that showed a possible effect on topoisomerase I function, the importance of the topoisomerase I enzyme for viability in several different organisms, and the potential effects of topoisomerase I on *C. albicans* virulence stimulated our efforts to identify the topoisomerase I gene in *C. neoformans*. Similarly, the potential importance of topoisomerase I as an antifungal target for *C. neoformans* and other pathogenic fungi created an interest in the structure and function of the enzyme. For instance, overexpression of topoisomerase I occurs in several different human tumors, allowing these tumors to be targeted by new drugs against topoisomerase I (Husain *et al.* 1994; Bronstein *et al.* 1996). In fact, the topoisomerase I inhibitors topotecan and irinotecan are now in clinical use as novel chemotherapeutic agents for leukemia and lymphoma.

In this study, we cloned the *C. neoformans TOP1* gene and demonstrated that it is essential for viability. Increases in topoisomerase I levels were found to effect responses to environmental stress in the host. Our studies further reveal that the target of DACs is not topoisomerase I and remains to be identified.

MATERIALS AND METHODS

Strains, media, and compounds: *C. neoformans* strain H99, a serotype A clinical isolate, and M001 strain, an *ade2* isogenic derivative of H99, were used in this study. A *GAL7::TOP1* strain was created from M001. *C. neoformans* M001 strain was routinely grown on yeast extract peptone dextrose (YEPD) medium. *C. neoformans* H99 and *GAL7::TOP1* strains were routinely grown on yeast nitrogen base (YNB) supplemented with 20 mg/ml glucose or galactose (YNB-glucose and YNB-galactose, respectively).

Camptothecin, obtained from Sigma (St. Louis), was dissolved in 1 m NaOH to obtain a stock solution of 25 mg/ml and stored at −70°. Drug dilutions were made from 500 to 0.09 μg/ml in RPMI-1640 following the schema in document M27-T (National Committee for Clinical Laboratory Standards 1995), and the final concentration of NaOH was 10 mm in all the tubes, including the control tube. DACs were from the chemistry laboratory of Dr. David Boykin (Georgia State University, Atlanta) as pure powders. Stock solutions of 10 mg/ml were made in sterile distilled water or dimethyl sulfoxide (DMSO), depending on solubility, filter sterilized by passage through a 0.22-μm Millex Durapore membrane filter, and stored at −70° until use. Drug dilutions were made from 100 to 0.09 μg/ml in RPMI-1640 following the schema in document M27-T (National Committee for Clinical Laboratory Standards 1995).

***In vitro* susceptibility testing of camptothecin and DACs:** Minimum inhibitory concentration (MIC) experiments were performed using the broth macrodilution method following the recommendation of document M27-T (National Committee for Clinical Laboratory Standards 1995).

Preparation of genomic DNA: DNA was isolated from *C. neoformans* strains as follows: 10 ml of mid- to late-log phase *C. neoformans* grown in YNB-glucose or YNB-galactose was pelleted and washed three times in sterile distilled water. Cells were suspended in 0.5 ml TENTS [10 mm Tris, pH 7.5, 1 mm EDTA, pH 8.0, 100 mm NaCl, 2% X-Triton, 1% sodium dodecyl sulfate (SDS)]. Then, 0.2 ml of 0.5-mm glass beads and 0.5 ml phenol:chloroform were added, and samples were vortexed for 2 min. After centrifugation for 10 min in a microfuge at 13,000 rpm, the aqueous phase was transferred to a fresh tube, 2 volumes of 100% ethanol was added, and samples were placed at −20° for 10 min. Precipitated DNA was collected, resuspended in 0.5 ml TE, pH 8.0, containing 10 μg/ml of RNAse A, and incubated at 37° for 20 min. The DNA was then extracted once with phenol:chloroform, reprecipitated, washed with 70% ethanol, resuspended in 100 μl of TE, and stored at −20°.

Isolation and characterization of the *C. neoformans* gene encoding topoisomerase I (*TOP1*): Amplification of a part of the *C. neoformans TOP1* gene from genomic DNA was performed by the following strategy: polymerase chain reaction (PCR) was used on *C. neoformans* genomic DNA with two degenerate primers containing inosine, from two conserved sites identified in several other known *TOP1* gene sequences. Primer Aa-Inos 5′-GA(AG) CCI CCI G(AG)I (CT)TI TT(CT) I(GT)IGG-3′ encoded EPPGLFR, and F-Inos 5′-C(GT)(CT) TG(AG) TG(AG) TT(AG) CAI A(AG)(AGT) ATI GCIAC-3′ encoded the complement of VAILCNHQ. PCR conditions were 95° for 5 min (1 cycle); 93° for 50 sec, 50° for 50 sec,

72° for 80 sec (25 cycles); 72° for 2 min (1 cycle). This amplification strategy produced a 1-kb fragment that was cloned into a KS^+ pBluescript plasmid and sequenced.

For isolation and sequencing of the entire *TOP1* gene, the following strategy was used. A genomic library of H99 in EMBL3 and a cDNA library from serotype D strain B3501 (Stratagene, La Jolla, CA) were screened with the amplified 1-kb *C. neoformans TOP1* probe fragment. Positive plaques were then purified through three rounds of repeated screening. For the cDNA library of *C. neoformans* strain B3501, the plasmid DNA was isolated from a single clone by the *in vitro* excision protocol using ex-assist helper phage with the SOLR strain (Stratagene). For the genomic DNA of *C. neoformans* strain H99, one strongly positive λ clone was purified using the plate lysate method, as described by Fritsch (Sambrook *et al.* 1989). The genomic clone was further digested with *Xho*I and *Pst*I, generating a 4-kb hybridizing fragment, and also *Sal*I, producing two separate hybridizing fragments of 1.9 and 2.3 kb. These fragments were then subcloned separately into SK^+ pBluescript plasmid. Double-stranded DNA from both *Sal*I clones was sequenced in both directions and shown to be truncated at the 5′-end. The same probe was then used to further screen the genomic clone digested with *Kpn*I and *Pst*I. A positive 6.2-kb fragment was isolated, cloned, sequenced, and shown to contain the 5′-end of the *TOP1* gene. This plasmid was named p*CnTOP1*. A positive clone from the cDNA library was isolated and the sequence analysis confirmed the predicted intron-exon borders and the start site of the gene. The entire sequence of the *C. neoformans TOP1* gene was submitted and deposited in the National Center of Biotechnology Information, National Library of Medicine, Bethesda, MD (accession no. AF009325).

Molecular biological techniques: DNA probes were labeled with [^{32}P]dCTP (New England Nuclear, Boston) using the random primer labeling kit (GIBCO-BRL, Gaithersburg, MD) with the 1-kb *TOP1* fragment as a template.

The EMBL3 genomic DNA library of *C. neoformans* has been previously described (Cox *et al.* 1995). The *C. neoformans* cDNA library of strain B3501 was constructed by Stratagene.

Southern blots, plaque lifts, and hybridization of nylon filters were performed according to Sambrook *et al.* (1989). DNA was sequenced by the dideoxy chain termination method (Sanger *et al.* 1977) using Sequenase version 2.0 (USB, Life Sciences, St. Petersburg, FL).

Karyotype analysis of *C. neoformans* variety *neoformans* (serotype A, D strains) and *C. neoformans* variety *gattii* (serotype B, C) strains was performed according to Perfect *et al.* (1993).

Construction of the plasmids for *TOP1* gene disruption: Three plasmid constructs, which carried three different disruption cassettes containing the *TOP1* gene and the selectable *ADE2* marker, were generated by (1) inserting the *ADE2* in a *Sal*I-filled-in site located in the middle of the *TOP1* gene (not shown); (2) replacing a 1250-bp, *Sal*I- and *Xba*I-restricted fragment with the *ADE2* gene (Figure 3B); and (3) replacing *Eco*RV-restricted 581- and 1212-bp fragments located at the 5′-end of the gene with the *ADE2* cDNA positioned in frame with the *TOP1* promoter (not shown). The *pGAL7::TOP1/ADE2* construct was generated by employing a series of molecular manipulations involving the *TOP1* gene, the 585-bp *C. neoformans GAL7* promoter from serotype D, strain B3501, and the 3000-bp *ADE2* marker genomic DNA fragment obtained from serotype A, strain H99:

1. Fragment A (309 bp) was generated by PCR using p*CnTOP1* plasmid DNA as a template and the primers 3, 5′-AGA TGG ATA CTC AAG CCT TCCT-3′, and 6B, 5′-TGC TCA TTT TCG CTC ATA CTA TC**G GGC CC**A TTC TTG ACA GCA ATT TGT GGGA-3′, which contain sequences homologous to the 5′-end of the promoter and the area flanking the ATG start codon, respectively. Because the 6B primer also contains an internal *Apa*I site (bold and underlined), an exogenous *Apa*I site was introduced in fragment A located 6 bp upstream from the ATG start codon of *TOP1*.
2. Fragment B (1505 bp) was generated using p*CnTOP1* plasmid DNA as a template and the primers 6A, 5′-TCC CAC AAA TTG CTG TCA AGG AT**G GGC CC**G ATA GTA TGA GCG AAA ATG AGCA-3′, and E20, 5′-CAA GCT TGT CCT TCT CCG CTT-3′, which contain sequences homologous to the 5′ and internal regions of *TOP1*, respectively. The 6A primer also contains an internal *Apa*I site (bold and underlined); likewise, an exogenous *Apa*I site was introduced in the 5′ region of fragment B.
3. Fragments A and B were combined and used as a template for PCR overlapping with the primers 3 and E20, generating fragment C (1814 bp), which contains a new *Apa*I site. This fragment was then digested with *Apa*I and *Xba*I, producing fragment D (1505 bp).
4. The *GAL7* promoter was amplified from the plasmid pAUG-MF, made by Wickes and Edman (1995), using primers 6C, 5′-CAG G**GA ATT C**GT GGA AAG AAG CAG GTC TTG TCGA-3′ and 6D, 5′-ATT A**GG GCC C**TC TCA AGA GGG GAT TGA GCG CTGA-3′, containing an *Eco*RI and *Apa*I site, respectively, generating fragment E (585 bp). This fragment was then cleaved with *Eco*RI and *Apa*I.
5. Fragments E (*GAL7* promoter) and D (*TOP1*) were ligated and cloned into the *Eco*RI and *Xba*I sites of p*CnTOP1* after deletion of 1705-, 1787-, and 600-bp fragments. The resulting plasmid contains the *GAL7* promoter in frame with the ATG start codon of the 3′-truncated *TOP1*. The *Sma*I::*Apa*I blunt-ended *ADE2* fragment was then inserted in an *Eco*RI filled-in site located at the 5′-end of the *GAL7* promoter to form *pGAL7::TOP1/ADE2* (Figure 3A). This *GAL7::TOP1* fusion construct was sequenced to make sure that no mutations were introduced by PCR manipulations.

Biolistic transformation: Constructs were transformed into *C. neoformans* strain M001 using biolistic delivery of DNA, following the protocol as described by Toffaletti *et al.* (1993). Transformants obtained from transformation of the first, second, and third contructs were selected on synthetic medium containing 6.7 g/liter of YNB without amino acid (YNB w/o), 1.3 g/liter of amino acid mix lacking adenine, 180 g/liter of sorbitol, 20 g/liter of glucose, and 20 g/liter of agar. Transformants obtained from the transformation of *pGAL7::TOP1/ADE2* were selected on synthetic medium containing 6.7 g/liter of YNB w/o, 1.3 g/liter of amino acid mix lacking adenine, 180 g/liter of sorbitol, 20 g/liter of galactose, and 20 g/liter of agar. Plates were incubated at 30°. Colonies were first purified and then 10 ml YNB-glucose or YNB-galactose broth cultures were inoculated with a single colony and incubated at 30°. Genomic DNA preparations for PCR and/or Southern blot analysis of transformants were performed as described above.

Introduction of a second functional copy of *TOP1* gene into *C. neoformans ade2* strain M001: The entire *TOP1* gene was amplified by PCR from H99 genomic DNA using the following primers containing *Kpn*I site (in boldface and underlined): 3: 5′-GTGC **GGTACC** ATA GAT GGA TAC TCA AGC CTT CCT; 4: 5′-GCTG **GGTACC** AAA GTA TGC GAT ACC ACT CCC ATT. Next, the fragment was digested with *Kpn*I and cloned in *Kpn*I site of the *pACT::HYG* plasmid (Figure 3), and the resulting plasmid was named *pACT::HYG/TOP1*. The *pACT::HYG* was previously made by cloning the 863-bp actin promoter and the 1.7-kb hygromycin B gene selectable marker (HYG) in the SK pBluescript plasmid. The *pACT::HYG/TOP1* was then linearized with *Not*I and the resulting 9.2-kb fragment

was introduced ectopically by biolistic transformation into *C. neoformans ade2* strain M001. Hygromycin-resistant transformants was selected and undigested genomic DNA of transformants were screened by Southern analysis using hygromycin B probe. There was no evidence of extrachromosomal transforming DNA, indicating that the stable transformants carried only integrated copies (data not shown). One transformant was chosen and named *C. neoformans TOP1 + TOP1*.

RNA extraction and RT-PCR: An isolated colony of each of four groups, H99-glu, H99-gal, *GAL7::TOP1*-glu, and *GAL7::TOP1*-gal, was inoculated in 10 ml YNB broth supplemented with 20 mg/ml of either glucose or galactose and incubated at 30° in a shaker incubator for 48 hr. Next, total RNA was prepared as described by FastRNA Kit-Red protocol (BIO 101, La Jolla, CA), and first-strand synthesis of cDNA was performed using 1 μg of total RNA for each group following the protocol described by SuperScript preamplication system (GIBCO-BRL). First-strand synthesis was made with *TOP1* specific primer RT-1 (5′-GCC CAA GGG AAC TTT TCT CGC AAAG). Second-strand synthesis of the *TOP1* gene was with RT-1 and an internal specific primer RT-2 (5′-GTC TTA CCG CCA AAG TCT TCC GTAC), yielding a 745-bp fragment. Because of the presence of a 54-bp intron in this region, this pair of primers yields a 799-bp fragment when genomic DNA was used as a template for PCR reaction. Actin specific primer AC-2 (5′-CAG CTG GAA GGT AGA CAA AGA GGC) was used for the first-strand synthesis of the cryptococcal actin gene, as a control. Second-strand synthesis of the actin gene was with AC-2 and AC-1 (5′-CGC TAT CCT CCG TAT CGA TCT TGC), yielding a 543-bp fragment. Because of the presence of a 51-bp intron in this region, this pair of primers yields a 594-bp fragment when genomic DNA is used as a template for PCR. Evaluation of *TOP1* and actin mRNAs was performed using ^{33}P (New England Nuclear) in the second-strand synthesis and quantified by phosphoimaging (Storm 860, Molecular Dynamics, Sunnyvale, CA). The *GAL7* gene expression was evaluated using 1 μg of total RNA for each group. First-strand synthesis was made with *GAL7* specific primer obtained from serotype D strain *GAL7-N* (5′-GGG ATT GTA TGG CTT GTC GTT TGA-3′); second-strand synthesis of *GAL7* gene was with *GAL7-N* and *GAL7-M* internal specific primer (5′-CCT CAT GGT CAA GTG TGG ACC ACT-3′), yielding a 529-bp fragment. Because of the presence of 299-bp intronic sequences in this region, this pair of primers yields an 828-bp fragment when genomic DNA was used as template for PCR.

Ionizing radiation: Survival studies after ionizing radiation with a ^{137}Cs irradiator using 850 rad/min were performed as follows. From overnight YNB broth cultures supplemented with 20 mg/ml of either glucose or galactose, cells from four groups, H99-glu, H99-gal, *GAL7::TOP1*-glu, and *GAL7-TOP1*-gal, were pelleted, resuspended, and diluted into 10 ml fresh YNB broth, containing either glucose or galactose, to a final density of 10^6 cells/ml and placed into the irradiator for 2 hr. Samples were taken every 30 min, diluted with PBS, and plated onto YNB-glucose or YNB-galactose plates for assessment of quantitative yeast survival. The experiment was repeated three times.

Heat shock: From overnight YNB broth cultures supplemented with 20 mg/ml of either glucose or galactose, cells from four groups, H99-glu, H99-gal, *GAL7::TOP1*-glu, and *GAL7::TOP1*-gal, were pelleted, resuspended, and diluted into 10 ml fresh YNB broth with either glucose or galactose to a final density of 10^6 cells/ml and incubated at 45°. Samples were taken from the tubes at various time points (0, 1, 2, 3, and 4 hr) diluted with PBS and plated onto YNB-glucose or YNB-galactose plates for assessment of quantitative yeast survival. All cultures were performed in triplicate.

Animal model of cryptococcal meningitis: New Zealand white rabbits weighing 2–3 kg were housed in separate cages and provided with water *ad libitum* and Purina rabbit chow. Wild-type H99 and the isogenic *GAL7::TOP1* strains were prepared by growth for 48 hr at 30° in YNB-glucose. The cells were pelleted, washed once, and suspended in PBS at a concentration of 3.3×10^8 cells/ml. After sedation with ketamine (Fort Dodge) and xylazine (Vedco), $\sim 10^8$ viable cells of each strain in a volume of 0.3 ml were inoculated intracisternally into 16 rabbits that had received an intramuscular injection of cortisone acetate at 2.5 mg/kg (Merck, West Point, PA) 1 day earlier and then daily for 14 days. Eight rabbits received the wild-type H99 strain and 8 rabbits received the *GAL7::TOP1* strain. Rabbits were sedated with ketamine and xylazine on days 2, 6, and 14 after inoculation, and cerebrospinal fluid (CSF) was withdrawn. Quantitative yeast cultures were performed by diluting the CSF in PBS, plating on YEPD medium, and incubating at 30° for 72 hr.

Statistical analysis: Comparisons of *in vitro* and *in vivo* yeast cfu/ml between each strain were performed by Student's *t*-test.

RESULTS

Cloning the *C. neoformans TOP1* gene encoding topoisomerase I: To study the role of the topoisomerase I gene in virulence and as a drug target, the *C. neoformans TOP1* gene encoding topoisomerase I was cloned. Two degenerate oligonucleotides (see materials and methods) corresponding to highly conserved areas of other topoisomerase I genes were synthesized and used as primers with *C. neoformans* genomic DNA as a template. This primer pair yielded a fragment of the predicted size that comigrated with control PCR products derived from *S. cerevisiae* genomic DNA. Sequence analysis revealed a unique sequence with homology to known topoisomerase I genes from other organisms. This fragment was sequenced and used as a probe to clone the entire *TOP1* gene from a *C. neoformans* genomic library. Strands of both the open reading frame and the 5′- and 3′-untranslated regions were sequenced, revealing a 3193-bp gene with nine predicted introns and an open reading frame of 2532 bp, encoding an 844-amino-acid protein (GenBank accession no. AF009325). Presumptive TATAAA and CAAT boxes were present at −269 bp and −223 bp from the ATG start site, respectively. To confirm the sequence and exon-intron borders, a *TOP1* cDNA clone from *C. neoformans* serotype D strain B3501 was isolated and sequenced. Sequence polymorphisms were present at 91 nucleotides (3.6%) in the coding region of the *TOP1* gene from strain B3501 (serotype D) compared with strain H99 (serotype A), but the predicted amino acid sequences for the topoisomerase I protein were identical in the two serotypes. By Southern blot of isolated chromosomes from the four *C. neoformans* serotypes, the *TOP1* gene was located on a 1130-kb chromosome in the A and D serotypes (variety *neoformans*), and on a larger 1550-kb chromosome in serotypes B and C (variety *gattii*; data not shown).

The comparative analysis of amino acid sequences of topoisomerase I from *C. neoformans* showed 48 and 40%

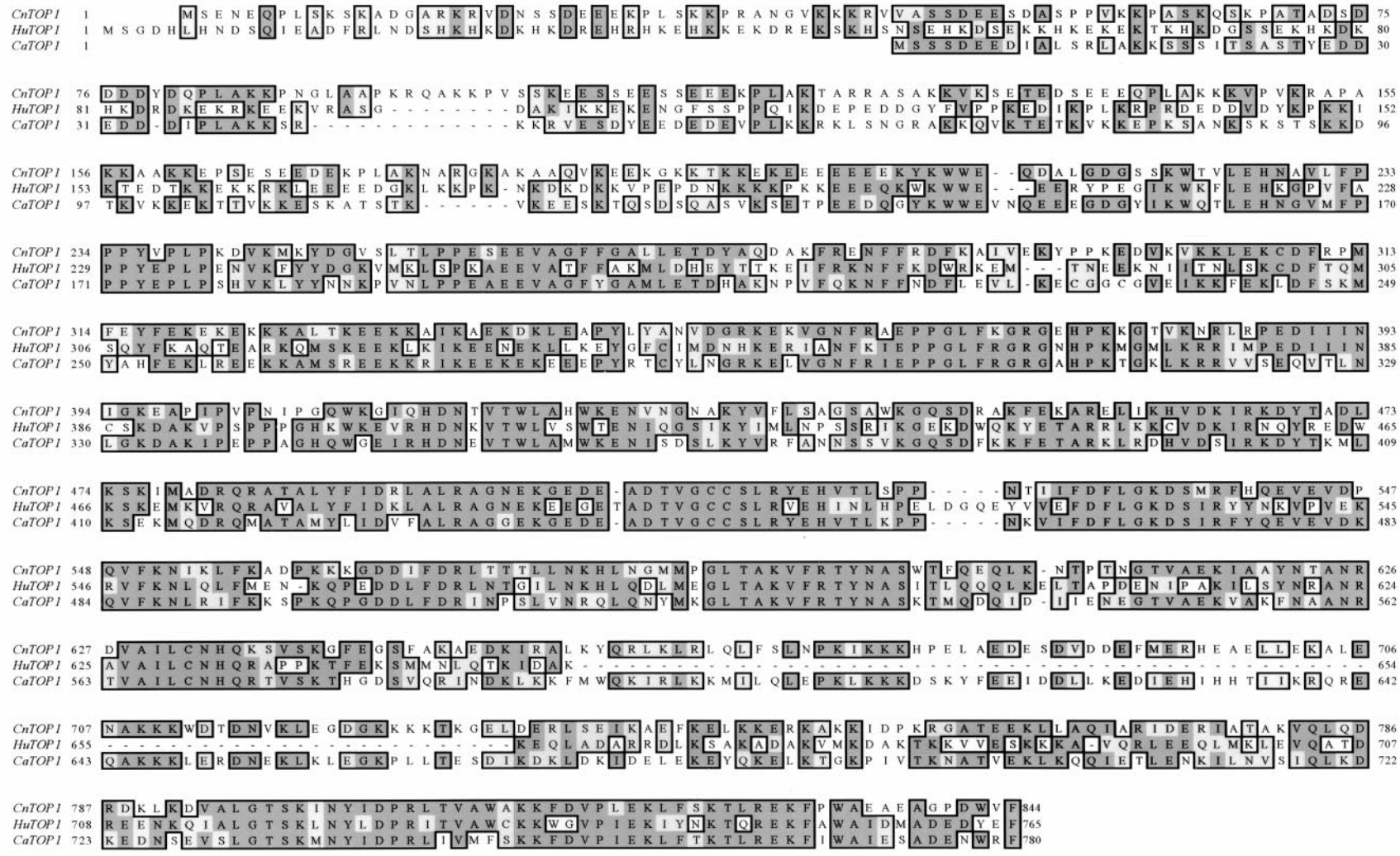

Figure 1.—*C. neoformans* topoisomerase I amino acid sequence comparison with those from *C. albicans* and human. Percentages of similarity, derived by the ClustalW alignment using the MACVector program, version 6.01, are 48 and 40% to *C. albicans* and human, respectively. Tyrosine (Y) active site is at positions 802, 723, and 738 in *C. neoformans*, human, and *C. albicans TOP1*, respectively. Fungal insert is shown from residues 657 to 732 and from residues 590 to 668 in *C. neoformans* and *C. albicans*, respectively. *Cn-TOP1*, *C. neoformans* topoisomerase I; *Hu-TOP1*, Human topoisomerase I; *Ca-TOP1*, *C. albicans* topoisomerase I.

identity with topoisomerase I of *C. albicans* and human, respectively (Figure 1). The predicted amino acid sequence of the *C. neoformans* topoisomerase I differs from topoisomerase I from other organisms, especially at the N and C termini. The active site of all topoisomerases contains a tyrosine (Y) residue that becomes covalently linked to DNA during catalysis, and a tyrosine residue is present at the expected location in *C. neoformans* topoisomerase I (residue 802). In the conserved region, in close proximity to the tyrosine residue that is known to attach transiently to DNA, *C. neoformans* has a more typical isoleucine/leucine for topoisomerase proteins than that of *C. albicans*, which has a methionine. This conserved area is also the active site for camptothecin binding, and a mutation in this site confers camptothecin resistance in other eukaryotic genes (Nitiss and Wang 1988). Interestingly, as described by Taylor *et al.* (1996), all known yeast topoisomerase I amino acid sequences contain an insertion not found in the mammalian enzyme. This fungal insert is also present in both *C. neoformans* and *C. albicans* topoisomerase I (Figure 1).

The *TOP1* gene is essential in *C. neoformans*: To determine the function of topoisomerase I in *C. neoformans*, we sought to disrupt the *TOP1* gene by homologous recombination. For this purpose, we made a series of *top1::ADE2* gene disruption constructs (see *Construction of the plasmids for TOP1 gene disruption*, in materials and methods), which were introduced by biolistic transformation into the *ade2 C. neoformans* strain M001. Transformants were screened by PCR using an internal and an external primer specific for the novel junction that would be created by homologous recombination. We initially screened 500 individual ADE$^+$ transformants and failed to detect a single transformant in which the *TOP1* gene had been replaced by the *top1::ADE2* disruption allele. Next, we screened an additional 7500 transformants in 750 PCRs in which 10 transformants were combined in each pool. Again, we found no isolates in which the *TOP1* gene had been replaced by the *top1::ADE2* allele. We note that these experiments involved three different *top1::ADE2* disruption constructs (see materials and methods), mitigating concerns that the failure to achieve homologous recombination was attributable to an unusual feature of the *top1::ADE2* allele. These findings suggest that the *TOP1* gene is essential for viability in *C. neoformans*. We note that, in previous studies in which several nonessential genes have been disrupted in this strain, homologous recombination has been shown to occur at a rate of ~3–10% (Alspaugh *et al.* 1997; Odom *et al.* 1997). Thus, if the *TOP1* gene were nonessential in *C. neoformans*, we would have expected to observe 240–800 *top1::ADE2* isolates in our studies.

To establish unequivocally that the *TOP1* gene is essential in *C. neoformans*, we developed a novel approach. First, another functional copy of the *TOP1* gene, linked to the hygromycin B-resistant gene as a selectable marker, was inserted ectopically into the genome of the *C. neoformans ade2* strain M001 (Figure 2, A and C). Second, this transformant, now containing two functional *TOP1* genes, was then used as the recipient for a second transformation event using the *top1::ADE2* disruption construct 2, which had also been employed in the preceding transformations (Figure 2B; see also materials and methods). In this case, the wild-type *TOP1* gene was readily disrupted in 2 out of the first 30 transformants analyzed, for an overall frequency of homologous recombination of 7%. Figure 2C shows the Southern analysis of one transformant using the *TOP1* and *ADE2* genes as probes, demonstrating that the endogenous *TOP1* locus has been replaced by the *top1::ADE2* disruption construct and the ectopic second copy of the *TOP1* gene is still present. Because the *TOP1* gene

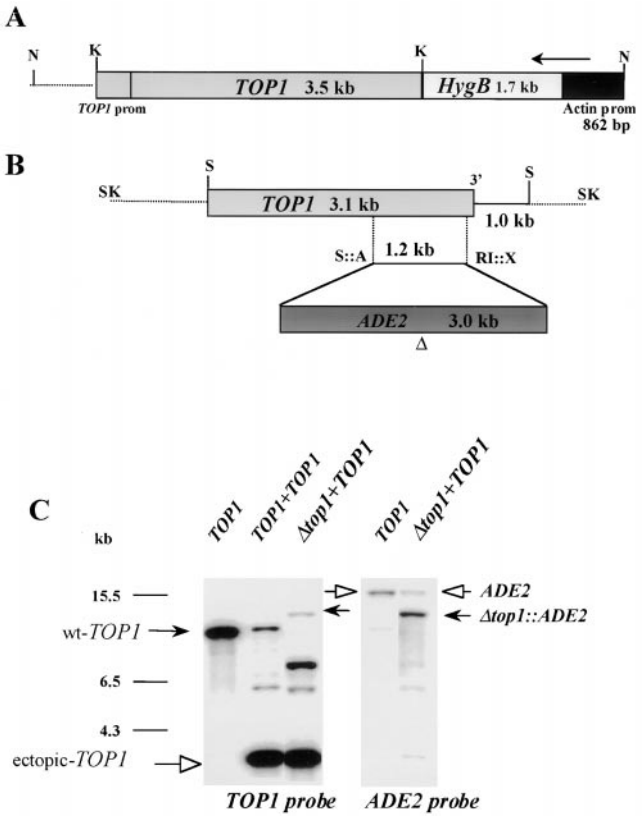

Figure 2.—Disruption of the wild-type *C. neoformans TOP1* gene. A Diagram of the 9.2-kb linearized fragment containing a functional copy of the *TOP1* gene and hygromycin B gene expressed by the actin promoter. B Diagram of *top1::ADE2* gene disruption construct (2, see materials and methods). (C) Southern analysis of transformant genomic DNA digested with *Kpn*I. *TOP1*, wild-type *TOP1* gene in *C. neoformans* strain H99; *TOP1* + *TOP1*, wild-type *TOP1* gene and the ectopic integration of the second copy of *TOP1* in *C. neoformans TOP1* + *TOP1* gene (solid arrow and open arrow on the left, respectively); Δ*top1* + *TOP1*, disruption of the wild-type *TOP1* gene, confirmed by probing with both *TOP1* and *ADE2* (solid arrow on the right). N, *Not*I; K, *Kpn*I; S, *Sal*I; A, *Apa*I; RI, *Eco*RI; X, *Xba*I.

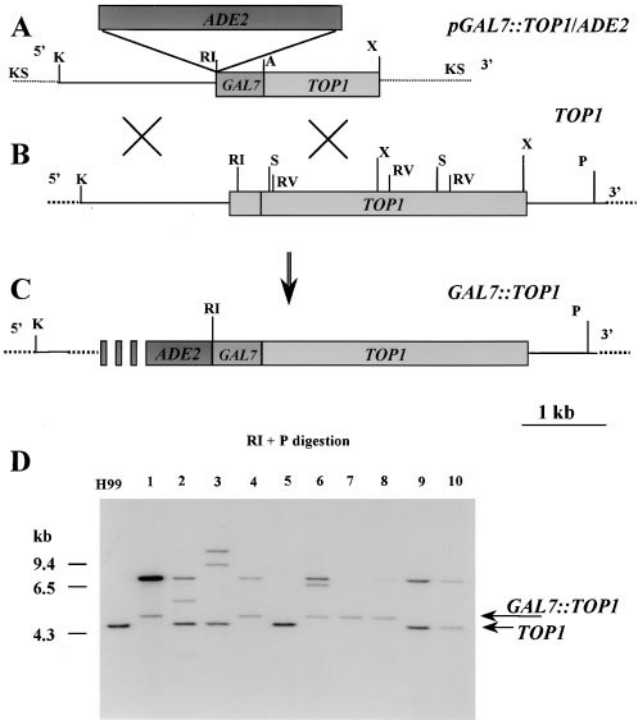

Figure 3.—*TOP1* gene expressed by the *GAL7* promoter. (A) Map of *pGAL7::TOP1/ADE2* plasmid. (B) Map of the wild-type *TOP1* gene; (C) Replacement of the endogenous wild-type *TOP1* gene with *GAL7::TOP1*. (D) Southern analysis of transformants with genomic DNA digested using *Eco*RI and *Pst*I and the *TOP1* probe. H99, wild-type *TOP1* gene in *C. neoformans* strain H99; lanes 1–10, transformants. Transformants in lanes 1, 4, 6, 7, and 8 result from double crossover events. Transformants in lanes 1, 4, 6, and 8 also show ectopic integrations of the *TOP1* gene. Confirmation of the *GAL7::TOP1* gene replacement was also performed by PCR analysis using four different primer combinations (data not shown). In the transformant shown in lane 7, the wild-type *TOP1* gene was replaced by the *GAL7::TOP1* allele with no ectopic integrations; this strain was named *GAL7::TOP1*. K, *Kpn*I; RI, *Eco*RI; A, *Apa*I; X, *Xba*I; S, *Sal*I; RV, *Eco*RV; P, *Pst*I.

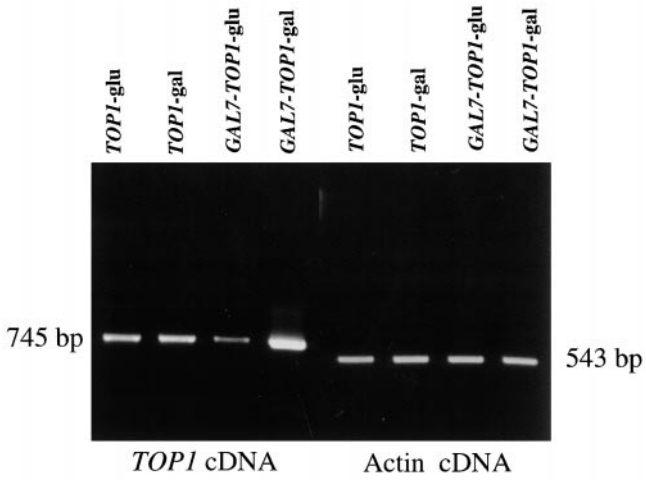

Figure 4.—*In vitro* quantitative analysis of *C. neoformans* *TOP1* mRNA in H99 and *GAL7::TOP1* strains. The isogenic *TOP1* wild-type strain (H99) and the *GAL7-TOP1* strain were grown in YNB medium containing glucose (repressing conditions) or galactose (inducing conditions) as a carbon source. RNA was extracted, converted to cDNA, and amplified with primers directed against the *TOP1* or actin genes. RT-PCR products were fractionated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. The *TOP1* and the actin gene RT-PCR products are 745 and 543 bp, as indicated.

was not disrupted in 8000 transformants in a strain with a single copy of the *TOP1* gene, whereas the *TOP1* gene could be readily disrupted at a frequency of 7% in a strain with two copies of the *TOP1* gene, we conclude that the *TOP1* gene is essential for viability in *C. neoformans*.

Cloning the *TOP1* gene under *GAL7* regulation: Because *TOP1* is essential in *C. neoformans*, we could not study a strain lacking topoisomerase I. As an alternative approach to studying the function of this gene, the *TOP1* gene was placed under the control of a regulated galactose promoter by homologous recombination in the *ade2* strain M001 using the plasmid *pGAL7::TOP1/ADE2* (Figure 3A). Remarkably, in 50% of the resulting ADE^+ transformants, the endogenous *TOP1* gene had been replaced by the *pGAL7::TOP1/ADE2* construct (Figure 3C). One strain in which the *GAL7::TOP1/ADE2* fusion gene was integrated at the *TOP1* locus, and at no other ectopic sites, was chosen and designated the *GAL7::TOP1* strain (Figure 3D).

We have employed the *GAL7::TOP1* strain in which *TOP1* expression can be regulated to study the effects of different levels of topoisomerase I activity on pathobiology and drug action in *C. neoformans*. According to previous studies with the *GAL7* promoter in serotype D strain (Wickes and Edman 1995), we expected that TOP1 activity would be strongly induced in galactose medium and strongly repressed in glucose medium. However, the *GAL7::TOP1* strain was viable on glucose medium, revealing that even under repressing conditions, enough topoisomerase I is expressed to support cell viability. By semiquantitative RT-PCR, we assayed *TOP1* mRNA levels to determine if the *GAL7::TOP1* strain produces *TOP1* mRNA under glucose-repressed conditions. Figure 4 shows that even under glucose-repressed conditions the *GAL7::TOP1* strain still produces a small amount of *TOP1* mRNA. Moreover, a modest overexpression of the *TOP1* gene was detected in galactose medium. Actin served as a control gene. Because these portions of the 3′ regions of these two genes contain two small introns (54 bp for *TOP1* gene and 51 bp for *actin* gene), we can establish that the RT-PCR products are derived from cDNA and not contaminating genomic DNA on the basis of the size of the RT-PCR products. Using the same method, we then assayed *GAL7* mRNA levels for serotype A to determine the *GAL7* gene expression under glucose-repressed and galactose-induced conditions in H99 strain. In contrast to differences of 500-fold observed by Wickes and Edman

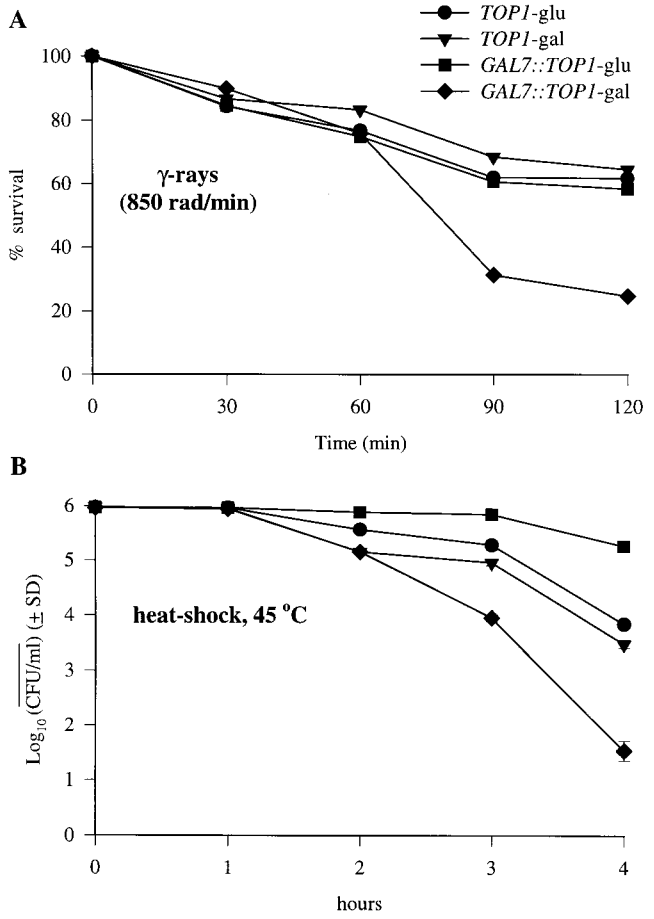

Figure 5.—*In vitro* stress responses. (A) Effect of ionizing radiation on *C. neoformans TOP1* wild-type strain H99 and the *GAL7::TOP1* strain in synthetic medium (YNB) with glucose or galactose. Percentages of survival (% survival) are geometric means of three separate experiments. (B) Heat shock performed at 45° for 4 hr. Colony-forming units/ml (cfu/ml) are geometric means ± SD of three separate experiments.

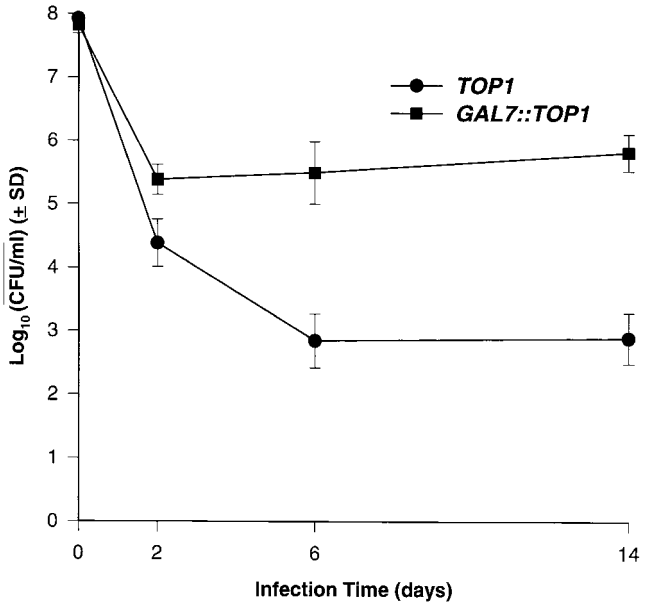

Figure 6.—*C. neoformans* wild-type strain H99 and the *GAL7::TOP1* strain produce infection in immunosuppressed rabbits. Rabbits (eight for each strain) were immunosuppressed with corticosteroids and then inoculated intrathecally with 10^8 cells of the isogenic *TOP1* wild-type strain (H99) and the isogenic *GAL7-TOP1* strain. CSF was removed on day 2, 6, and 14 following inoculation, and the number of surviving organisms was determined by serial dilution and plating on YEPD medium. Each data point represents the mean of all cultures for each strain, and the standard deviation of the mean is indicated. Counts were significantly higher in *GAL7::TOP1* strain at day 6 ($P < 0.001$) and curves for counts between day 6 and 14 of infection were the same.

(1995) in a serotype D strain, we found only a modest repression of the *GAL7* gene in glucose medium compared to galactose (two- to fivefold) (data not shown).

Increased topoisomerase I levels confer sensitivity to heat shock and γ-rays: Overexpression of topoisomerase I in *S. cerevisiae* increases sensitivity to DNA-damaging agents, such as ionizing radiation (Nitiss *et al.* 1996). Therefore, we tested whether induction of topoisomerase I confers a similar phenotype in *C. neoformans*. When grown in galactose-inducing conditions, the *GAL7::TOP1* strain was more sensitive to ionizing radiation than the isogenic wild-type *TOP1* strain H99 and the corresponding *GAL7::TOP1* strain grown on glucose ($P < 0.01$; Figure 5A). To further explore the stress-response phenotype of strains with altered topoisomerase I expression, we examined the response to heat stress. After heat shock (45° for 4 hr), the *GAL7::TOP1* strain, in which *TOP1* expression was induced by galactose, was more sensitive to heat shock than the isogenic *TOP1* wild-type strain H99, or *GAL7::TOP1* cells grown on glucose ($P < 0.001$; Figure 5B). Thus, induction of topoisomerase I renders cells more sensitive to heat shock and DNA damage.

***C. neoformans TOP1* gene might be important during the initial stress response in the host:** With these *in vitro* functional data, it is clear that topoisomerase I expression could affect cell viability under environmental stresses. Therefore, we examined the effect on yeast survival in the *GAL7::TOP1* strain in the host. *C. neoformans* wild-type strain H99 and the isogenic *GAL7::TOP1* strain were inoculated intracisternally in rabbits. During the first week of infection, the *GAL7::TOP1* strain survived better than the *TOP1* wild-type strain H99 ($P < 0.001$). However, as the infection progressed, the survival rate of the two strains was similar (Figure 6).

Topoisomerase I is the target of camptothecin but not of dicationic aromatic compounds in *C. neoformans*: In previous studies we found that several DACs have potent *in vitro* activity (MIC < 1 μg/ml) against *C. neoformans* (Del Poeta *et al.* 1998a,b). Therefore, mammalian and fungal cell extracts were used in an inhibitory assay of topoisomerase I by the DACs. Several bis-benzimidazole DACs showed topoisomerase I inhibitory concentrations (IC50) of 67 to >100 μm for *C. neoformans* extracts (Dykstra and Tidwell 1991; Dykstra *et al.*

TABLE 1

Topoisomerase I is the target of camptothecin, but not of dicationic aromatic compounds (DACs) in *C. neoformans*

Compound	*TOP1*-glucose	*TOP1*-galactose	*GAL7::TOP1*-glucose	*GAL7::TOP1*-galactose
Camptothecin[a]	>500	>500	>500	6.25
DACs[b]				
DB 346	3.12	3.12	3.12	3.12
DB 337	0.78	0.39	0.78	0.78
DB271	0.78	0.39	0.39	0.78
DB 190	1.56	1.56	3.12	1.56
DB 188	0.19	≤0.09	0.19	0.19
DB 378	≤0.09	0.19	≤0.09	0.19
DAP 132	1.56	1.56	1.56	1.56
40.820	0.19	≤0.09	0.19	0.19
DB 329	0.39	0.19	0.39	0.39
DB 332	0.78	0.78	0.78	0.78
DB 244	0.78	0.78	0.78	0.78
DB 325	0.39	≤0.09	≤0.09	0.19

[a] Drug dilutions from 500 to 0.09 μg/ml.
[b] Drug dilutions from 100 to 0.09 μg/ml.

1994). The furan, DB244, had an IC50 (inhibition concentration) of 10 μm for *C. neoformans* topoisomerase I activity. Despite the inhibitory activity against both topoisomerase I function *in vitro* and direct antifungal activity, DACs may not target topoisomerase I in the intact yeast cell. For instance, a comparison of antifungal activity of camptothecin, whose known target is topoisomerase I, showed that overexpression of topoisomerase I in the *GAL7::TOP1* strain under inducing conditions increased the killing activity of camptothecin from >100 μg/ml to 6.25 μg/ml (Table 1). These results suggest that camptothecin has limited penetration into *C. neoformans* cells but under inducible conditions more target is available for the limited intracellular camptothecin to bind topoisomerase I and form lethal enzyme-DNA complexes. In contrast, the minimum inhibitory concentrations for all of the DACs were identical in the *GAL7::TOP1* strain grown under inducing (galactose) or repressing (glucose) conditions, indicating that these agents do not inhibit topoisomerase I by a camptothecin-like mechanism (Table 1). Moreover, if the DACs were simple inhibitors of topoisomerase I, one would expect that decreased expression of *TOP1* in the *GAL7::TOP1* strain grown under glucose-repressing conditions would increase antifungal activity of DACs. This is not the case, further indicating that the *in vivo* target of the DACs is not topoisomerase I.

DISCUSSION

In this study, we isolated and cloned the *C. neoformans TOP1* gene encoding topoisomerase I. Our findings reveal that the *TOP1* gene is essential for viability in *C. neoformans*. In contrast, although topoisomerase I is involved in essential functions such as transcription and DNA replication, it is not required for growth in *S. cerevisiae* (Thrash *et al.* 1985; Christman *et al.* 1988). However, mutations in several genes that render topoisomerase I essential for viability in *S. cerevisiae* have been identified (Castano *et al.* 1996). In several fungi, such as *Ustilago maydis* (Gerhold *et al.* 1994) and *Schizosaccharomyces pombe* (Vemura *et al.* 1987), topoisomerase I is also not essential for viability. On the other hand, topoisomerase I is essential in *D. melanogaster* (Lee *et al.* 1993) and mammalian embryos (Morham *et al.* 1996). These results were the first indications that the product of the topoisomerase I gene can be essential for cell viability.

Further support for the essential features of *TOP1* in pathogenic fungi comes from the studies of Jiang *et al.* (1997). They found that topoisomerase I expression is important for normal *C. albicans* cellular morphology, germ-tube formation, and virulence in a mouse model. Our interpretation of their studies is that *TOP1* is essential for viability in *C. albicans*. For instance, a heterozygous *top1/TOP1* disruptant could be readily obtained, but no homozygous *top1/top1* mutant could then be obtained by a second round of transformation. Similarly, in our study we were able to disrupt the wild-type *C. neoformans TOP1* gene only after a second functional copy of the *TOP1* gene was introduced into the genome. This is the third example of a cryptococcal gene that has been shown to be essential for viability by molecular methods (Lodge *et al.* 1994; Thompson *et al.* 1999). Therefore, our results support the idea that molecular strategies can be used in *C. neoformans* for essential genes and this strategy could be used in other medically important fungi in which homologous recombination is infrequent.

Why is topoisomerase I essential in *C. neoformans*?

Topoisomerase I and II can substitute for each other in many cellular processes, and alterations in one topoisomerase can alter sensitivity to drugs acting against the other topoisomerase (Nitiss *et al.* 1993, 1996; Ishida *et al.* 1995). A second *C. neoformans* topoisomerase gene, *TOP2*, encoding topoisomerase II has been identified (J. R. Perfect, unpublished results). However, it appears that the interaction of these two genes is not sufficient to allow TOP2 to replace the function of TOP1 when it is not present. Moreover, we do not know whether *C. neoformans* has homologs of the topoisomerase-related function (*TRF*), or *TOP3* genes, or their functions in relationship to the *TOP1* gene. Studies to isolate and analyze interactions between *TOP1*, *TOP2*, *TOP3*, and *TRF* genes in *C. neoformans* and *C. albicans* would allow us to understand the functions and essential features of topoisomerases in pathogenic fungi.

In our studies to characterize *TOP1* and its protein as an antifungal target, we were able to regulate its expression. The *GAL7* promoter used in this study was originally isolated from the serotype D strain B3501. We observed a much more restricted range of induced *vs.* repressed expression levels in serotype A (two- to five-fold) compared to previous studies in serotype D, possibly because the two serotypes diverged over ~40 million years ago (T. Mitchell and R. Vilgalys, personal communication). In fact, our findings in H99 strain reveal only a modest upregulation of the endogenous *GAL7* gene on galactose medium, suggesting that the whole *GAL7* regulatory system is not as differentially regulated in this serotype A strain as in the serotype D strain. On the other hand, the purpose of *TOP1* regulation under *GAL7* promoter was mainly to induce the *TOP1* gene to evaluate whether *C. neoformans* topoisomerase I was the target of the DACs. Moreover, construction of the *GAL7::TOP1* strain provided an opportunity to examine the importance of topoisomerase I for the pathobiology of *C. neoformans*. With the *C. neoformans TOP1* gene-regulated strain, *GAL7::TOP1*, we performed a series of phenotype analyses and compared them to the isogenic *TOP1* wild-type strain H99. Both strains were similar with respect to capsule size and melanin production (data not shown). However, similar to findings in *S. cerevisiae* (Bernham *et al.* 1990), the *GAL7::TOP1* strain under induced TOP1 conditions was more sensitive to ionizing irradiation, and under repressed conditions was more resistant to heat shock. Other studies have also suggested a relationship between heat-shock gene expression (*HSP70*) and inhibition of topoisomerase I with camptothecin (Rowe *et al.* 1987; Kroeger and Rowe 1989). In *C. neoformans* we have found support for an interaction between heat-shock induction and the level of topoisomerase I activity that could have pathobiological relevance.

The ability to survive better *in vitro* under stress conditions such as heat shock may be similarly observed *in vivo*. For instance, the *GAL7::TOP1* strain survived better than the parental wild-type strain during the first week of infection in the rabbit model of cryptococcal meningitis. The low glucose concentrations (2–4 mg/dl), absence of galactose, and high temperature (39°–40°) in the subarachnoid space should repress expression of the *TOP1* gene in the *GAL7::TOP1* strain *in vivo*. On the other hand, it was clear that no difference was observed among the two strains during the second week of an established infection in the rabbit. From these observations, we hypothesize that *C. neoformans TOP1* would not need to be markedly regulated in the host during persistent infections but may be important in its initiation. However, the total impact of *TOP1* gene expression of *C. neoformans* in the host needs further study.

Our findings indicate that complete inhibition of *TOP1* in *C. neoformans* will be lethal. Although present topoisomerase I inhibitors use the enzyme as a cellular poison, drugs that target topoisomerase I production will likely be fungicidal. Furthermore, topoisomerase I from two major human pathogens, *C. albicans* and *C. neoformans*, contains amino-acid insertion not found in the mammalian enzyme. This fungal insert is located in the linker domain. In human topoisomerase I the linker domain consists of 77 amino acids, while in fungal topoisomerase I the linker domain contains 155 amino acids. Redinbo *et al.* (1998) have shown that the linker domain in human topoisomerase I is not required for catalytic or relaxation activities. The function of the fungal insert in pathogenic fungi is not yet known. The structure of human topoisomerase I is of medical importance in anticancer therapy because this enzyme is the unique target of camptothecin, topotecan, and irinotecan. In parallel, the possibility that the fungal insert in the topoisomerase I enzymes from pathogenic fungi could be a specific target for antifungal therapy should be investigated.

The DACs could act on *C. neoformans* topoisomerase I function in two ways. First, they could inhibit topoisomerase I. If this were the case, it would be lethal in *C. neoformans*. Second, a key aspect of most antitopoisomerase drug action is the notion that these drugs act by stabilizing the intermediate or cleavage complex of the topoisomerase reaction to act as a cellular poison. Nitiss and Wang (1988) and, more recently, Redinbo *et al.* (1998) demonstrated that this is the mechanism of action of camptothecin. Therefore, we tested whether the DACs inhibit topoisomerase I via either mechanism. In our experiments with camptothecin, we initially found no activity against *C. neoformans*, even at concentrations up to 500 μg/ml. However, when the topoisomerase I target is placed under an inducible promoter, the *GAL7::TOP1* strain became 100-fold more sensitive to the fungicidal properties of camptothecin. Drug permeability is a major obstacle to the action of this drug and derivatives in yeasts (Nitiss *et al.* 1996). The *GAL7::TOP1* inducible strain allowed us to examine whether DACs used the same mechanism as campto-

thecin. We found no change in *in vitro* susceptibility for all DACs tested under inducible (galactose) or repressing (glucose) conditions. Thus, the DACs do not inhibit topoisomerase I in *C. neoformans.*

In conclusion, topoisomerase I is an excellent antifungal target. It is essential in *C. neoformans*, and its regulation under stress conditions has an impact *in vitro* and may have some influence during initiation of infection. The identification of several chemical agents, such as quinizarin, HIAA, and A-3253 as selective inhibitors of *C. albicans* topoisomerase I provides further support for structural differences between the fungal and mammalian enzymes (Fostel *et al.* 1992, 1996; Fostel and Montgomery 1995). With our results, the fungal insert has now been found in both *C. albicans* and *C. neoformans*, which suggests a structural basis for inhibitor design. The genes for *C. albicans* and *C. neoformans TOP1* can now be used to express the proteins in *S. cerevisiae* to study the intracellular activity of the topoisomerase I in a genetically permeabilized cell. The proteins can also be isolated for screening purposes, biochemical analysis, and further molecular modeling compared to the mammalian enzyme.

We thank Gary Cox, Andy Alspaugh, and Aaron Mitchell for help and suggestions, David Messerly for assistance with karyotype analysis, and Mary Ann Howard for preparing the manuscript. This work was supported by Public Health Service Grants AI28388, AI41937, and AI-94-014 from the National Institute of Allergy and Infectious Diseases, and as part of the Veterans Administration Research Center on AIDS and Human Immunodeficiency Virus infection. Joseph Heitman is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

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Communicating editor: A. P. Mitchell