A controllable gene-expression system for the pathogenic fungus *Candida glabrata*

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A system for controlling gene expression was established in the pathogenic fungus *Candida glabrata* to elucidate the physiological functions of genes. To control the expression of the gene of interest, the *C. glabrata* cells were first transformed with the plasmid carrying the tetracycline repressor–transactivator fusion *tetR::GAL4*, then with the DNA fragment containing the controllable cassette, the tetracycline operator chimeric promoter (*tetO::SchOP1*). The peptide elongation factor 3 (*CgTEF3*) and DNA topoisomerase II (*CgTOP2*) genes from *C. glabrata* were cloned and their expression assessed using this system. When the promoter of *CgTEF3* or *CgTOP2* was replaced with *tetO::SchOP1*, doxycycline almost completely repressed the expression of both mRNAs, and impaired growth. Repression of the *TOP2* or *TEF3* gene by doxycycline also hampered the survival of *C. glabrata* cells in mice; in mouse kidneys the number of *C. glabrata* cells, in which the *TOP2* or *TEF3* promoter was replaced with the *tetO::SchOP1* controllable cassette, did not increase when the mice were given doxycycline. Thus, it appears that the gene repression mediated by doxycycline occurred not only in culture media but also in animals; therefore, this system can be used to elucidate the function of the gene in fungal infections and pathogenesis.

Keywords: *Candida glabrata*, gene expression, tetracycline-responsive element, peptide elongation factor 3, DNA topoisomerase II

INTRODUCTION

The incidence of fungal infections has been increasing, particularly in patients who are immunocompromised by HIV infection, and in patients who are receiving immunosuppressive therapy for organ transplantation or chemotherapy for cancer. Current therapies against pathogenic fungi rely mainly on two chemical groups: the polyenes, which disrupt membrane structure, and the azoles, which inhibit the synthesis of ergosterol (Gallis *et al.*, 1990; Georgopapadakou & Walsh, 1996). However, the toxicity of the polyenes and appearance of azole-resistant strains require the development of novel antifungal drugs (Gallis *et al.*, 1990; Rex *et al.*, 1995).

The recent release of the complete nucleotide sequence of *Saccharomyces cerevisiae* has allowed us to identify all the genes in this unicellular organism (Saccharomyces

Genome Database, Stanford University; The Munich Information Centre for Protein Sequences; Yeast Protein Database: Proteome); their physiological functions are currently being studied. A project to sequence the genome of the pathogenic fungus *Candida albicans* has been initiated. Among 6000 *S. cerevisiae* genes, approximately 600 have turned out to be essential for growth; deletion or disruption of any one of these genes is lethal. These essential genes are considered to be feasible antifungal targets, but such information alone is not sufficient to pinpoint the importance of the genes for infection, colonization, invasion and pathogenicity.

Unlike *C. albicans*, *Candida glabrata* always grows as a haploid yeast cell and does not form hyphae, which makes genetic manipulations easy. *C. glabrata* is phylogenetically similar to *S. cerevisiae* (Barns *et al.*, 1991) and is an opportunistic pathogen (Aisner *et al.*, 1976; Hickey *et al.*, 1983). It causes mucocutaneous and systemic mycoses in immunocompromised patients who are treated with immunomodulators or anti-cancer drugs (Wingard, 1995). As seen in patients with *C.*

Abbreviation: GAL4AD, GAL4 activation domain.

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albicans infection, extensive use of topical and systemic antifungal drugs has increased the incidence of mucous-membrane colonization and the appearance of azole-resistant *C. glabrata* (Hitchcock *et al.*, 1993; Newman *et al.*, 1994). Thus, *C. glabrata* seems to be a feasible model organism for establishing a system applicable to the functional analysis of fungal genes for infection and virulence.

We have established a system for controlling gene expression in *S. cerevisiae* by using the tetracycline-responsive element (Nagahashi *et al.*, 1997). In this study, this technique was applied to *C. glabrata*. The peptide elongation factor 3 (*TEF3*) and the DNA topoisomerase II (*TOP2*) genes were cloned from *C. glabrata* and their expression examined using this method. Because both *TEF3* and *TOP2* in *S. cerevisiae* have been shown to be essential (Sandbaken *et al.*, 1990; Goto & Wang, 1984); they are thought to be potential targets of antifungal drugs (Triana-Alonso *et al.*, 1995; Figgitt *et al.*, 1989; Shen *et al.*, 1992). Here, we report the effects of the repression of *TEF3* or *TOP2* of *C. glabrata* on its growth in media and survival in mice. A role for these genes in systemic infections is proposed.

METHODS

Generation of the test strain and transactivator-expressing strains. The plasmid pINTG4 (Fig. 1a) was constructed by ligating the 2.0 kb *Sph*I fragment containing the repressor-transactivator fusion *tetR::GAL4AD* (*GAL4* activation domain) from pINFAGAL4 (Nagahashi *et al.*, 1997) and the 1.5 kb *Kpn*I fragment from pT2/*Sal*I (Kitada *et al.*, 1995) carrying the *C. glabrata* *TRP1* gene (*CgTRP1*). pINTG4 was digested with *Eco*RV and used to transform the *C. glabrata* strains 2001HT (*his3 trp1*) and 2001HTU (*his3 trp1 ura3*) (Kitada *et al.*, 1995). The resulting *TRP*⁺ transformants, ACG4 (*his3 trp1 PSchOP1::tetR::GAL4AD::TRP1*) and ACG22 (*his3 ura3 trp1 PSchOP1::tetR::GAL4AD::TRP1*), constitutively expressed the *tetR::GAL4AD* fusion activator.

To generate the test strain, 97ScHIS3, p97tScHIS3U was constructed by introducing the 97t fragment, the *ScHIS3* ORF and the *CgURA3* gene into pRS414 (Stratagene; cloned into the *Sac*I/*Eco*RI sites, the *Eco*RI/*Sal*I sites and the *Xho*I site, respectively), which was used to transform ACG22 after being digested with *Xho*I at the 5' flanking region of *CgURA3*. The 97t fragment was the 0.6 kb *Sac*I-*Eco*RI fragment derived from p97tCC (Nagahashi *et al.*, 1997). The *ScHIS3* ORF was amplified by PCR from *S. cerevisiae* genomic DNA using primers HIS01 and HIS02 (Table 1), and the *CgURA3* gene was amplified from *C. glabrata* genomic DNA using primers PCGURA5 and PCGURA3 (Table 1).

Cloning the *TEF3* and *TOP2* genes of *C. glabrata*. An approximately 0.6 kb fragment of *CgTEF3* was amplified from *C. glabrata* genomic DNA by PCR, using primers YEF3-2373 and YEF3-2996 (Table 1), which were designed based on the sequence of the *S. cerevisiae* *YEF3* gene, encoding peptide elongation factor 3. The amplified fragment was cloned in pT7blue (Novagen). Because Southern blot analysis of *C. glabrata* genomic DNA using the above PCR fragment as a probe revealed that a *Bam*HI fragment of about 9 kb strongly hybridized with the fragment, a *C. glabrata* genomic DNA library, which contained mainly 9 kb *Bam*HI fragments, was generated using pBluescript II SK⁺ (Stratagene). The full-

Fig. 1. Plasmid construction. (a) Schematic representation of the fusion transactivator *tetR::GAL4* and integration vector pINTG4. The *Eco*RV site (marked with an asterisk), which is located in the 3' region of *CgTRP1*, was used for the linearization of the plasmid. (b) Schematic representation of the controllable promoters and the plasmid carrying a controllable cassette. The black boxes in the *SchOP1* region represent upstream activator (UAS) or upstream repressor (URS) sequences. These plasmids were derivatives of p97tCC, p98tCC and p99tCC (Nagahashi *et al.*, 1997). Unique restriction sites in these plasmids are marked with asterisks.

length *TEF3* gene was identified from the library by screening with the above PCR fragment. Genomic DNA was extracted from the *C. glabrata* strain ATCC 2001 as described by Rose *et al.* (1990).

To isolate the *TOP2* gene from *C. glabrata*, we used the *S. cerevisiae* temperature-sensitive mutant SD 1-4 [*MATa ade1 ade2 ura3-52 top2-1(ts)*] (DiNardo *et al.*, 1984) for the functional screening. Another *C. glabrata* genomic DNA library was constructed by ligating 5–10 kb fragments of *C. glabrata* genomic DNA, that had been partially digested with *Sau*3AI, into the *Bam*HI site of pRS416 (Stratagene). After transforming the SD 1-4 cells, with the above DNA library, the cells that grew at a non-permissive temperature (37 °C) were collected and the plasmid, which reproducibly supported the growth of SD 1-4 cells at 37 °C were recovered.

Table 1. Primers used in this study

Name	Sequence (5'–3')	Introduced site
HIS01	GGCCGAATTCGCAAAGATGACAGAGCAG	<i>EcoRI</i>
HIS02	GGCCGTCGACCCGTTCTCCATCTCTT	<i>Sall</i>
PCGURA5	CCACACGTCGACCCATAAGAGAGGTCAAAGG	<i>Sall</i>
PCGURA3	AATCTGTGCTAGTGAAGTCAAGAGCTTGCC	<i>Sall</i>
CGHIS5EV	TTTTTTGATATCTGGCCCCAGTCACC	<i>EcoRV</i>
CGHIS3SM	GGCCGGCCCCGGGACAAGCTCATCGCCC	<i>SmaI</i>
YEF3-2373	GTAAGTTGGTCAAG	–
YEF3-2996	CATCAACTTGACCGTTGGC	–
TEF3AF	TTTTTTGAGCTCTGGTATTGGTGTGAGCC	<i>SacI</i>
TEF3AR	TTTTTTCCCGGGTGGATTAGAATATTGTTTAGCTTG	<i>SmaI</i>
TEF3BF	TTTTTTGAATTCTGAATGACTGACTCT	<i>EcoRI</i>
TEF3BR	TTTTTTCTCGAGATCCAAGACGTCCTTGCCG	<i>XhoI</i>
C5TEF3	GAGACGTAAGGACGACCAC	–
C3TEF3	GATCATTCTTCAGTGAAACCG	–
CGTOPA	GTGAGTTCAGGCAAGAAGTAGATCCTACGCGCTC	–
	AATTAGGAAAGCAAAATTGACGTGGGCCCCGGGAC	
	AAGCTCATCG	
CGTOPB	AATATGCTCTAACTGAGATATCTTCTGATATCTTTC	–
	AGAAGCGGTTTTAGGTTCACTCATTTGAACGAATT	
	CTTTTCTGAGATAAAGC	
P5TOP2	TTGATAGAATTCGTTCAAATGAGTGAACCTAAAAC	<i>EcoRI</i>
P3TOP2	TCATCAGTCGACTTCGAAAACGTCAACAAGTGTC	<i>Sall</i>
C5TOP2	GGGAAAATCGAGTTGTGATTTG	–
C3TOP2	GGCGGATGACACACATTC	–
P5ERG11	TTTTTTGAATTCAATAACATGTCCACTGAAAAAC	<i>EcoRI</i>
P3ERG11	TTTTTTGTGACGTGACCCTTTGGACCCAAG	<i>Sall</i>

Generation of strains containing the *TEF3* and *TOP2* controllable cassettes. The plasmids p97CGH, p98CGH and p99CGH (Fig. 1b) were generated by replacing *ScURA3* cloned in p97tCC, p98tCC or p99tCC (Nagahashi *et al.*, 1997) with the *C. glabrata HIS3* (*CgHIS3*) gene. These plasmids comprise the *ScHOP1* promoter derivatives. p97 has both the upstream activation sequence (UAS; nt –198 to –190 in the *ScHOP1* gene) and the upstream repression sequence (URS; nt –173 to –165 in the *ScHOP1* gene), p98 carries only the URS region and p99 harbours no UAS or URS (Vershon *et al.*, 1992; Fig. 1b). The *CgHIS3* gene was amplified from *C. glabrata* genomic DNA by PCR using primers CGHIS5EV and CGHIS3SM (Table 1).

Region A (nt –598 to –234) or region B (nt –6 to +1215) of *CgTEF3* (see Fig. 2) was amplified with PCR using primers TEF3AF and PTEF3AR, or TEF3BF and TEF3BR, respectively. For the homologous recombination, the A and B regions of *CgTEF3* were cloned into the *SacI*/*SmaI* sites (for region A) and *EcoRI*/*XhoI* sites (for region B) of p97CGH, 98CGH and 99CGH, generating p97TEF3, p98TEF3 and p99TEF3, respectively. These three plasmids were linearized with *Bss*HII and were used to transform ACG4; two strains, 98TEF3 and 99TEF3, were obtained. For *TOP2*, the controllable cassettes carrying the A and B regions of *CgTOP2* were amplified from p97CGH, p98CGH, or p99CGH by PCR using the primers CGTOPA and CGTOPB. These primers include part of the sequence of the *C. glabrata TOP2* gene (*CgTOP2*) (CGTOPA contains nt –149 to –89 and CGTOPB includes nt –6 to +60 of *CgTOP2*) and part of p97CGH, p98CGH and p99CGH. These three controllable cassettes were used to

transform ACG4; the resulting strains were designated 97TOP2, 98TOP2 and 99TOP2, respectively. All the synthetic oligonucleotides used for PCR are listed in Table 1.

Southern and Northern blot analyses. Ten micrograms of genomic DNA from *C. glabrata* ATCC 2001 was digested with restriction endonucleases and separated on an agarose gel. The DNA was transferred to a nylon membrane (Hybond-N⁺, Amersham), hybridized with the radiolabelled probe and visualized by autoradiography or by image analyser BAS1000 (Fuji).

Total RNA was extracted by the glass-bead lysis method (Rose *et al.*, 1990). Approximately 10 µg total RNA was separated on an agarose gel, transferred onto a nylon membrane (Hybond-N, Amersham) and hybridized with radiolabelled probes. DNA fragments used for hybridization were the 0.5 kb *EcoRV*–*EcoRV* fragment of *CgTEF3*, the 0.4 kb fragment of *CgTOP2* (nt –6 to +394) and the 0.3 kb fragment of *CgERG11* (nt –6 to +328). The signal obtained from *CgERG11* was used to normalize the mRNA signals. The RNA bands that hybridized with the radiolabelled probes were visualized by autoradiography. Radiolabelling of the probe DNA was carried out by the random priming method using [α^{32} P]dCTP.

Determination of cell number *in vitro*. Approximately 1×10^5 99TEF3 cells were inoculated into YPD (1% yeast extract, 2% peptone, 2% glucose) containing the indicated concentrations of doxycycline (0–10 µg ml^{–1}). After 14 h at 37 °C, the OD₆₆₀ was determined. For the time-course experiments, approximately 1×10^5 cells of 98TOP2 or 99TEF3 were inoculated

and cultured in YPD at 37 °C with or without doxycycline (at a final concentration of 10 µg ml⁻¹); their growth was monitored by measuring OD₆₆₀ at the indicated times after the addition of doxycycline. The number of viable cells was also determined by spreading 20 µl of the diluted cultures at the indicated times after the doxycycline addition and by counting the number of colonies that had appeared after cultivation of the cells for 24 h at 37 °C.

Determination of doxycycline concentration and *C. glabrata* cells in mouse organs. Male CD-1 mice were immunocompromised by injecting them with 200 mg cyclophosphamide kg⁻¹ 3 d before infection and 100 mg kg⁻¹ 0, 3, 7 and 11 days after infection. In addition to cyclophosphamide, mice were injected with 125 mg cortisone acetate kg⁻¹ at the time of the cyclophosphamide injection. Each mouse was intravenously inoculated with 1 × 10⁵ *C. glabrata* cells, and was administered doxycycline (2 mg ml⁻¹), dissolved in 5% sucrose solution, as drinking water from 6 d before the infection. On the day indicated, the mice were killed, and their liver and kidneys were removed and homogenized in 4 vols 30 mM H₃PO₄ using Sonofier 250 (Branson) at 4 °C. To determine the concentrations of doxycycline in each kidney and liver, 45 µl of 3 mM EDTA and 150 µl of CH₃CN were added to 5 µl of the 20% homogenate. The resulting diluted homogenates were centrifuged at 3500 r.p.m. for 10 min at 4 °C and 150 µl of 10 mM phosphate solution containing 1 mM EDTA was added to 50 µl of the supernatant. The concentration of doxycycline was determined by reverse-phase HPLC using a Capcell pak C18 column (S-5 mm, 4.6 × 150 mm; UG 120A; Shiseido) in a 20% CH₃CN solution containing 8 mM phosphate solution and 0.8 mM EDTA. To estimate the number of surviving *C. glabrata* cells in kidneys, the isolated kidneys at each time point were homogenized and the homogenates spread on YPD plates containing penicillin G (200 units ml⁻¹) and streptomycin (200 µg ml⁻¹). After a 24 h incubation at 37 °C, the number of yeast colonies that had appeared on the plates was counted.

RESULTS

Generation of a transactivator-expressing strain of *C. glabrata*

To control the expression of a certain gene in *C. glabrata*, we applied the tetracycline-mediated transcription system, which we previously demonstrated in *S. cerevisiae* (Nagahashi *et al.*, 1997). This system requires the controllable promoter, which consists of the *tetO* and *ScHOP1* promoters, and the fusion transactivator, which was generated by connecting *tetR* with *S. cerevisiae* GAL4AD. The transactivator gene was cloned in an integration plasmid, pINTG4, and then used to transform cells of *C. glabrata* strains 2001HT and 2001THU (Kitada *et al.*, 1995), generating strains ACG4 and ACG22. Because *ScHIS3* complements a *his3* null mutation of *C. glabrata* (Kitada *et al.*, 1995), we first created the controllable strain 97ScHIS3 (see Methods), in which *ScHIS3* was linked to the controllable promoter 97t (Nagahashi *et al.*, 1997) and was integrated at the 5' flanking region of the *URA3* locus of ACG22, to examine whether the transactivator is functional in the transformants. When 97ScHIS3 was cultured in a histidine-depleted medium, the growth was severely impaired by the addition of tetracycline (data

not shown), demonstrating that tetracycline efficiently suppressed the transcription of *ScHIS3* driven by the *tetO::ScHOP1* chimeric promoter in *C. glabrata*.

Isolation of the *C. glabrata* *TEF3* and *TOP2* genes

To assess the importance of certain genes for the growth and infection, we isolated the *C. glabrata* *TEF3* and *TOP2* genes, which encode peptide elongation factor 3 and DNA topoisomerase II, respectively. As the PCR with *C. glabrata* genomic DNA and the primers designed from the sequence of *S. cerevisiae* *YEF3* (Qin *et al.*, 1990) yielded a single band of 0.6 kb, this fragment was used for the further screening. After screening 2000 independent colonies, one clone termed B4, that harboured an approximately 9 kb *Bam*HI fragment of *C. glabrata* genomic DNA, was obtained. Nucleotide sequencing of B4 revealed that it contained a long ORF of 3.5 kb; the amino acid sequence of the expected product is 87.4% and 78.5% identical to that of *S. cerevisiae* Yef3p and *C. albicans* Tef3p, respectively (Table 2). In addition, B4 supported the growth of a *YEF3*-deficient *S. cerevisiae* strain (data not shown). These results demonstrate that the cloned gene is the *C. glabrata* homologue of *S. cerevisiae* *YEF3*; therefore it was designated CgTEF3 (*C. glabrata* translation elongation factor 3).

The *C. glabrata* DNA topoisomerase II gene was functionally cloned using the *S. cerevisiae* *top2* temperature-sensitive mutant, SD 1-4 (DiNardo *et al.*, 1984). By screening 20000 independent colonies, one clone which grew at a non-permissive temperature was obtained. Deletion analysis of the recovered DNA from the colony revealed that the 7.0 kb *Xho*I-*Spe*I fragment was sufficient to rescue SD 1-4 at a non-permissive temperature. This fragment contained an ORF of 4.2 kb, which can encode a protein of 161 kDa with significant sequence identity to *S. cerevisiae* Top2p. We therefore designated this gene as CgTOP2 (*C. glabrata* DNA topoisomerase II). CgTop2p shows significant sequence homology with DNA topoisomerases from other organisms (Table 2). Among them, ScTop2p shows the highest sequence identity to CgTop2p. The tyrosine residue, which is considered to be a catalytic site in other topoisomerases (Worland & Wang, 1989), is also conserved in CgTop2p at position 780.

Induced repression of CgTEF3 and CgTOP2

To study the roles of *TEF3* and *TOP2* in growth and infection, we replaced the promoters of CgTEF3 and CgTOP2 with 97t, 98t and 99t (Nagahashi *et al.*, 1997) by means of homologous recombination (Fig. 2a). By the transformation of each one of the above three controllable cassettes into ACG4, the five strains 98TEF3, 99TEF3, 97TOP2, 98TOP2 and 99TOP2 were generated. 97TEF3, which contains *TEF3* connected to 97t, was not obtained. The correct integration of the controllable promoters to the expected locus was confirmed by PCR using the primers C5TEF3 and C3TEF3 for *TEF3*, and C5TOP2 and C3TOP2 for *TOP2* (Fig. 2b, c). 99TEF3 and 98TOP2 were chosen for

Table 2. Pairwise sequence identities

Values are shown as percentage amino acid identities.

(a) Peptide elongation factor 3

	<i>S. cerevisiae</i> *	<i>C. albicans</i> †	<i>Pneumocystis carinii</i> ‡
<i>C. glabrata</i>	87.4	78.5	56.6
<i>S. cerevisiae</i>	.	77.2	56.8
<i>C. albicans</i>	.	.	57.5

(b) DNA topoisomerase II

	<i>S. cerevisiae</i> §	<i>C. albicans</i>	<i>Schiz. pombe</i> ¶	Human α #	Human β #	<i>Drosophila melanogaster</i> **
<i>C. glabrata</i>	73.1	56.0	48.1	48.0	42.7	41.9
<i>S. cerevisiae</i>	.	55.9	47.7	42.0	42.0	42.1
<i>C. albicans</i>	.	.	48.5	42.6	43.3	43.1
<i>Sch. pombe</i>	.	.	.	43.6	46.6	43.4
Human α	.	.	.	.	68.1	54.1
Human β	.	.	.	.	.	60.2

* Qin *et al.* (1990).

|| Keller *et al.* (1997).

† Di Domenico *et al.* (1992)

¶ Uemura *et al.* (1986).

and Myers *et al.* (1992).

Tsai-Pflugfelder *et al.* (1988) (α)

‡ Ypma-Wong *et al.* (1992).

and Jenkins *et al.* (1992) (β).

§ Giaever *et al.* (1986).

** Wyckoff *et al.* (1989).

Fig. 2. Generation of controllable strains. (a) Schematic representation of the integration of the controllable promoter, *tetO::ScHOP1*. Region A represents the 5' flanking region of the target gene and region B indicates the 5' end of the ORF. The DNA fragment encompassing the controllable cassette, where the *CgHIS3* gene and *tetO::ScHOP1* are flanked by the A and B regions, was used to transform the *C. glabrata* strain ACG4. (b) Confirmation of the correct integration of the *tetO::ScHOP1* in the *TEF3* locus. PCR was carried out with genomic DNA from the transformants and the wild-type ATCC 2001, with the primers for the 5'-flanking region and ORF. The expected length of the PCR products for *tetO::ScHOP1::TEF3* and the wild-type *TEF3* are 4.6 kb and 2.6 kb respectively. Three different clones of 99TEF3 were examined. (c) Confirmation of the correct integration of *tetO::ScHOP1* in the *TOP2* locus. The expected length of the PCR products for *tetO::ScHOP1::TOP2* and the wild-type *TOP2* are 2.3 kb and 0.9 kb, respectively. Lanes: 4, 9, 13, DNA size markers; 1-3, three independent clones of 97TOP2; 5-8, four independent clones of 98TOP2; 10-12, three independent clones of 99TOP2; 14, wild-type. Asterisks indicate the random integration of *tetO::ScHOP1*.

Fig. 3. Effects of doxycycline on the expression of *TEF3* and *TOP2* and on the growth of 99TEF3 and 98TOP2. (a) Repression of *TEF3* by doxycycline. Ten million 99TEF3 cells were inoculated into YPD and cultured in the absence (lanes 1–8) or presence (lanes 10–17) of 10 μ g doxycycline ml⁻¹ for the indicated time. Ten micrograms of total RNA extracted from each strain was separated on an agarose gel and hybridized with the *TEF3* and *ERG11* probes. Lane 9 indicates the level of the *TEF3* transcript in wild-type cells. (b) Repression of *TOP2* by doxycycline. Ten million cells of 98TOP2 were inoculated into YPD and cultured in the presence or absence of 10 μ g doxycycline ml⁻¹ for 2 h. Ten micrograms of the total RNA extracted from each strain was separated on an agarose gel and hybridized with the *TOP2* and *ERG11* probes. Lanes: 1; 97TOP2; 2, wild-type; 3 and 4, 98TOP2; 5, 99TOP2. (c) The number of viable 99TEF3 and 98TOP2 cells, cultured without (○) or with (■) 10 μ g doxycycline ml⁻¹. (d) Morphology of 98TOP2 grown without (top) or with (bottom) 10 μ g doxycycline ml⁻¹ for 12 h.

further experiments, because they expressed the *TEF3* and *TOP2* mRNAs at levels similar to those observed in the wild-type cells, and their growth in the absence of doxycycline was indistinguishable from that of the wild-type (Fig. 3a, lanes 1–8 for 99TEF3, lane 9 for wild-type; and Fig. 3b, lane 1 for 97TOP2, lane 2 for wild-type, lane 3 for 98TOP2, lane 5 for 99TOP2). We used doxycycline instead of tetracycline, because it was superior to tetracycline in terms of suppressing the expression of *TEF3* and *TOP2* (data not shown). By Northern blotting, it was demonstrated that the *TEF3* mRNA declined to an undetectable level within 30 min of the addition of doxycycline (Fig. 3a, lanes 12–17). The *TOP2* mRNA also became undetectable within 2 h of doxycycline addition (Fig. 3b, lane 4). In contrast, doxycycline did not significantly affect the level of lanosterol 14-demethylase (*ERG11* gene) mRNA, indicating the specificity of doxycycline to the *tetO::SchOP1* promoter (Fig. 3a, b). Seven hours after

doxycycline addition, the 98TOP2 cells displayed aberrant morphology and the number of viable cells started decreasing (Fig. 3c). Most of the 98TOP2 cells became elongated and aggregated within 12 h (Fig. 3d). The growth of the 99TEF3 cells was also severely hampered by doxycycline; the number of viable cells remained very small at any time after the doxycycline addition (Fig. 3c). However, we did not observe any morphological changes in 99TEF3 cells, even when they were cultured in the presence of doxycycline. The growth impairment of 99TEF3 by doxycycline was reproducibly observed at doxycycline concentrations higher than 2 μ g ml⁻¹ (Fig. 4a); the same result was obtained with 98TOP2 cells (data not shown). Together, these results demonstrate that both *TEF3* and *TOP2* are essential for the growth of *C. glabrata*, and that the depletion of *TEF3* leads to immediate cell death, while the effect of *TOP2* repression on viability becomes apparent after several generations.

Fig. 4. Effects of doxycycline on the survival of 99TEF3 and 98TOP2 in mouse kidneys. (a) Determination of the minimal concentrations of doxycycline required to inhibit the growth of 99TEF3. One hundred thousand 99TEF3 cells were inoculated into YPD and cultured for 14 h. OD₆₆₀ is indicated as the percentages of those of doxycycline-untreated cells. Each value is the mean from three independent experiments. ▲, TEF3 cells; ▽, wild-type cells. (b) Concentrations of doxycycline in liver (●) and kidneys (■) of the immunocompromised mice. Each value is the mean from the three independent experiments. (c) Number of viable cells recovered from mouse kidneys. Mice that were infected with wild-type (top), 99TEF3 (middle) and 98TOP2 (bottom) were killed, and the *C. glabrata* cells were recovered from the kidneys. Error bars indicate standard deviation from three independent kidneys. Experiments were repeated three times. ○, Number of cells recovered from doxycycline-untreated mice; ■, number of cells recovered from doxycycline-treated mice. Error bars are not shown where the symbol is bigger than the error bar.

Effects of *TEF3* and *TOP2* repression on the survival of *C. glabrata* in mice

Next, we intended to establish the roles of *TEF3* and *TOP2* on the survival of *C. glabrata* in mice. As mentioned earlier, expression of *CgTEF3* and *TOP2* was almost completely repressed by 2 µg ml⁻¹ or higher concentrations of doxycycline. When doxycycline was added to the drinking water and the mice were allowed

to drink freely from 6 d prior to infection, the doxycycline concentrations reached 8 µg (g kidney)⁻¹ and 10 µg (g liver)⁻¹, which are higher than the minimum concentration of doxycycline required to suppress *TEF3* and *TOP2* expression *in vitro* (Fig. 4b). Then, the number of viable *C. glabrata* cells in kidneys was determined by recovering the *C. glabrata* cells at various times after infection, because the *C. glabrata* cells exclusively colonized the kidneys (data not shown). The

number of viable 99TEF3 and 98TOP2 cells recovered from the kidneys was significantly lower in mice treated with doxycycline than in untreated mice. Although there was a transient increase in the number of recovered cells at 4 and 10 d post-infection, essentially 99TEF3 did not grow in mouse kidneys when doxycycline was present. The number of viable 98TOP2 cells increased up to day 4, which was followed by a rapid decline from day 10. The profiles of the doxycycline-mediated decrease in the number of viable 99TEF3 and 98TOP2 cells in the kidneys were similar to those of doxycycline-induced growth inhibition *in vitro*. Thus, it appears that the functions of *TEF3* and *TOP2* for viability are well preserved not only in cultures *in vitro*, but also in the organs of infected animals.

DISCUSSION

We have established a controllable gene expression system in *C. glabrata*. Doxycycline almost completely suppressed the expression of a gene whose transcription was under the control of the *tetO::SchOP1* promoter. Furthermore, transcriptional repression of the gene by doxycycline occurred relatively quickly. The *TEF3* and *TOP2* mRNA declined to an undetectable level within 2 h of the addition of doxycycline. The rapid response to doxycycline enabled us to study the roles of these genes in the survival of *C. glabrata* cells in animals.

By the doxycycline-induced repression, it appears that the effect of *TEF3* depletion occurred much earlier than that of *TOP2*. Because *TEF3* repression would lead to a serious defect in protein synthesis, it might cause a rapid arrest of the cell growth. 98TEF3, which expressed a lower level of the *TEF3* mRNA, showed a slight growth defect even in the absence of doxycycline (data not shown). This fact implies that proper levels of *TEF3* mRNA must be maintained to keep *C. glabrata* cells alive. The *TOP2* depletion, however, affected cell growth only after several generations. This is rather an unexpected result, because Top2p is required for the completion of DNA replication and for the segregation of sister chromatids. A defect in either one of these steps may result in rapid cell death. The results obtained from this study, however, suggest that inhibition of protein synthesis more effectively causes rapid cell death than that of DNA replication.

When *TOP2* was repressed, *C. glabrata* cells displayed an elongated morphology (Fig. 3c). A similar phenotype was also observed in the temperature-sensitive mutants of *S. cerevisiae* and *Schizosaccharomyces pombe* when they were cultured at a non-permissive temperature (DiNardo *et al.*, 1984; Holm *et al.*, 1985; Uemura & Yanagida, 1986). The aberrant morphology caused by a *top2* mutation has been reported to be associated with the appearance of abnormal chromosomes. Therefore, the elongated morphology caused by doxycycline in 98TOP2 cells is probably a consequence of the inhibition of DNA topoisomerase II.

The effects of doxycycline on the cell growth of 99TEF3 and 98TOP2 *in vitro* were well reflected in animal organs. This indicates that the physiological importance of *TEF3* and *TOP2* is not dependent on the conditions in which *C. glabrata* grows. Thus, this system can be applied to other genes whose function remains to be elucidated and also to the identification of novel virulence factors.

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