Candida albicans UPC2 is transcriptionally induced in response to antifungal drugs and anaerobicity through Upc2p-dependent and -independent mechanisms

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Many genes in the *Candida albicans* ergosterol biosynthetic pathway are controlled by the transcriptional activator Upc2p, which is upregulated in the presence of azole drugs and has been suggested to regulate its own transcription by an autoregulatory mechanism. The *UPC2* promoter was cloned upstream of a luciferase reporter gene (*RLUC*). *UPC2-RLUC* activity was induced in response to ergosterol biosynthesis inhibitors and in response to anaerobicity. Under both conditions, induction correlates with the magnitude of sterol depletion. Azole inducibility in the parental strain was approximately 100-fold, and in a *UPC2* homozygous deletion strain was 17-fold, suggesting that, in addition to autoregulation, *UPC2* transcription is controlled by a novel, Upc2p-independent mechanism(s). Curiously, basal *UPC2-RLUC* activity was fivefold higher in the deletion strain, which may be an indirect consequence of the lower sterol level in this strain, or a direct consequence of repression by an autoregulatory mechanism. These results suggest that transcriptional regulation of *UPC2* expression is important in the response to antifungal drugs, and that this regulation occurs through Upc2p-dependent as well as novel Upc2p-independent mechanisms.

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INTRODUCTION

The pathogenic yeast *Candida albicans* causes oral, vaginal and systemic disease in immunocompromised hosts, and vaginal infection in immune-competent hosts. Significant mortality is seen with systemic disease, which is most commonly seen in neutropenic patients, such as those receiving transplant chemotherapy. *Candida* infections are one of the most common opportunistic infections associated with AIDS, and usually manifest as oral disease in these patients (Pfaller & Diekema, 2004).

The most frequently used antifungals for treatment of oral candidiasis are the azoles, which inhibit ergosterol biosynthesis. Resistance to the azoles has emerged due to the fungistatic nature of these drugs and their frequent use for prophylaxis (Pfaller & Diekema, 2004). The azoles, such as fluconazole (FLC) and clotrimazole (CLO), act by targeting the ergosterol biosynthesis enzyme lanosterol 14- α -demethylase, which is encoded by *ERG11* (White *et al.*, 1998). Other ergosterol biosynthesis inhibitors act either up- or downstream of Erg11p. These include terbinafine

(TER), which inhibits the *ERG1* gene product, fenpropimorph (FEN), which inhibits Erg2p, and lovastatin (LOV), which inhibits Hmg1p. Inhibition of sterol synthesis at any of these points results in upregulation of many genes within the pathway (Arthington-Skaggs *et al.*, 1996; Dimster-Denk & Rine, 1996; Henry *et al.*, 2000; Song *et al.*, 2004). Expression of many of these genes has recently been shown to be controlled by the master sterol transcriptional regulator Upc2p (MacPherson *et al.*, 2005; Silver *et al.*, 2004).

C. albicans Upc2p (*CaUpc2p*) is a Zn₂Cys₆ cluster transcription factor and is homologous at the sequence and functional levels to the *Saccharomyces cerevisiae* paralogues *UPC2* and *ECM22* (*ScUPC2* and *ScECM22*) (MacPherson *et al.*, 2005; Silver *et al.*, 2004). *CaUpc2p* is required for upregulation of *ERG11* (Oliver *et al.*, 2007) and other sterol biosynthesis genes in response to sterol depletion (MacPherson *et al.*, 2005; Silver *et al.*, 2004), and it activates transcription of target genes by binding to a conserved core sequence known as the sterol response element (SRE) (MacPherson *et al.*, 2005). The *CaUPC2* homozygous deletion is hypersensitive to ergosterol biosynthesis inhibitors as well as to certain drugs that target the cell wall, demonstrating that this transcription

Abbreviations: CLO, clotrimazole; FEN, fenpropimorph; FLC, fluconazole; LOV, lovastatin; NAT, nourseothricin; NKZ, nikkomycin Z; SRE, sterol response element; TER, terbinafine.

factor is central to the response to many antifungal drugs (MacPherson *et al.*, 2005; Silver *et al.*, 2004).

Interestingly, the *CaUPC2* promoter itself contains a putative SRE (MacPherson *et al.*, 2005), suggesting transcriptional self-regulation. It is generally accepted that transcriptional self-activation accounts for most of the control of *ScUPC2* expression, but to date this hypothesis has been supported by indirect experimental evidence only. Transcriptional profiling of a mutant containing a hyperactive allele of *ScUPC2* (*UPC2-1*) revealed an increase in *ScUPC2* mRNA when compared with the wild-type, suggesting that *ScUPC2* is self-activated in the *UPC2-1* strain (Wilcox *et al.*, 2002). Another study using a *ScUPC2-lacZ* fusion showed that deletion of the SRE causes a significant, although not complete, reduction in the anaerobic inducibility of the reporter, some of which appears to be due to an increase in basal activity of the promoter lacking the SRE (Abramova *et al.*, 2001). Both of these studies were conducted using *S. cerevisiae* strains containing the *ScUPC2* paralogue *ScECM22*, and inducibility of *ScUPC2* may be affected by the presence of *ScECM22*. Studies showing that *ScUPC2* expression is induced by azole drugs have not shown whether inhibition of the ergosterol biosynthetic pathway with other antifungal drugs also results in a *ScUPC2* transcriptional response. The work in this study characterizes the transcriptional activation profile of *CaUPC2* in response to sterol depletion mediated by sterol synthesis inhibitors and anaerobicity, and investigates the hypothesis that *CaUPC2* expression is self-regulated.

METHODS

Strains and growth conditions. *C. albicans* strain BWP17 (*ura3::λ434ura3::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*) and its derivative D-6 (*upc2::URA3/upc2::ARG4*) were transformed with *UPC2-RLUC* expression constructs containing the nourseothricin (NAT)-resistance marker *SAT1* (generously provided by Dr Joachim Morchauser, University of Würzburg) to create strains *CaUPC2-750WT* (strain TW16201) and *CaUPC2-750D* (strain TW16202). Strains were maintained on YEPD (per litre: 10 g Difco yeast extract, 20 g Bacto peptone and 20 g glucose) containing 200 µg NAT ml⁻¹. Inocula prepared for luciferase assays and ergosterol quantification were grown in complete supplement mixture (CSM) [per litre: 0.75 g CSM (Bio 101) 5.0 g ammonium sulfate, 1.7 g yeast nitrogen base without amino acids or ammonium sulfate, and 20 g glucose] with 200 µg NAT ml⁻¹ to provide selection. Growth during

assays was carried out in CSM lacking NAT to avoid pleiotropic effects of the selective agent.

Creation of *UPC2* constructs containing the *Renilla reniformis* luciferase reporter. The plasmid pCRW3 containing the *Renilla* luciferase reporter plasmid was generously provided by D. R. Soll (University of Iowa) (Srikantha *et al.*, 1996). To construct the reporter plasmid containing the NAT-resistance marker, the plasmid pA83 (Reuss *et al.*, 2004) was used to amplify the *SAT1* marker with the oligonucleotides SAT1Kpn and SAT1EcoRV (Table 1). The resulting PCR fragment was cloned into the vector pCR-Topo (Invitrogen), after which the *SAT1* marker was excised and ligated into *EcoRV*- and *KpnI*-digested pCRW3 to create pCRW3-SAT1. This was done such that the *SAT1* marker would be transcribed in the opposite direction to the reporter gene, to avoid potential *RLUC* activity that could result from incomplete termination of *SAT1* transcription. To create the *CaUPC2-RLUC* fusion, 750 bp of *CaUPC2* sequence upstream of the initiating ATG was amplified from the plasmid pGEM-HIS-UPC2 (Silver *et al.*, 2004) using oligonucleotides UPC2Kpn and UPC2Sma (Table 1). The resulting fragment was cloned into *KpnI/XmaI* digested pCRW3-SAT1 to create pUPC2-RLUC. This plasmid was then linearized using *NsiI* and integrated at the *ADE2* locus of *C. albicans* strains according to an integration strategy used previously in this laboratory (Song *et al.*, 2004).

***C. albicans* transformation.** *C. albicans* strains were transformed using the lithium acetate/heat shock method described elsewhere (Sanglard *et al.*, 1996), with modifications. Briefly, 500 µl of YEPD overnight culture was diluted into 50 ml fresh YEPD and grown for 5 h at 30 °C with shaking at 180 r.p.m. Cells were prepared by pelleting, washing once with sterile water, and resuspending in 0.1 M lithium acetate in Tris-EDTA, pH 7.5 (Li-TE). *NsiI*-linearized plasmid DNA (5–10 µg) and 10 µg carrier DNA (sheared herring sperm DNA, Invitrogen) were used for each transformation. Cells were incubated overnight with DNA in 40 % PEG 3350 in Li-TE, heat-shocked for 30 min at 42 °C, and washed once in 1 M sorbitol. This was followed by 4 h growth at 30 °C with shaking at 180 r.p.m. in YEPD in the absence of NAT selection, after which cells were plated on YEPD plates containing 200 µg NAT ml⁻¹, as described elsewhere (Reuss *et al.*, 2004).

PCR and Southern blot screening of transformants. Genomic DNA from pUPC2-RLUC-transformed BWP17 and D6 was prepared from cells grown overnight in YEPD-NAT using glass bead lysis as described elsewhere (Hoffman & Winston, 1987). Transformants were initially screened for positive integration of *NsiI*-digested pUPC2-RLUC at the *ADE2* locus using the oligonucleotides ADE2 and RLUC (Table 1). PCR-positive transformants were then confirmed, by Southern blotting, to contain the pUPC2-RLUC construct as a single copy. Briefly, ~10 µg genomic DNA was digested with *KpnI* overnight, run on a 0.7 % agarose gel and blotted overnight to a nitrocellulose membrane. The blot was probed with ³²P end-

Table 1. Oligonucleotides used for reporter plasmid construction and transformant screening

Oligonucleotide	Sequence (5'–3')
SAT1Kpn	TACAACGGTACCCAGCGTCAAACTAGAGAATAATAAG
SAT1EcoRV	TACAACGATATCGATTCTAGAAAGACCAC
UPC2Kpn	CTCTCGGTACCATGGATGTTGGTATATCAGG
UPC2Sma	CTCTCCCCGGGAAATGGCTTTTTTGTGAAAAA
RLUC	CACCACTGCGGACCAGTTATCATCCGTTTCC
ADE2	CAGTTAAATAGTCTTCATATC

labelled *RLUC* oligonucleotide probe (Table 1). Transformants containing pUPC2-*RLUC* in single copy at the *ADE2* locus were used for the luciferase assay.

Drugs and conditions for *UPC2-RLUC* activity. Drugs used for induction of the *UPC2* reporter construct included the azoles FLC (Pfizer; stock concentration 3 mg ml⁻¹ in water) at final concentrations of 0.1–100 µg ml⁻¹, and CLO (Sigma-Aldrich; stock concentration 10 mg ml⁻¹ in DMSO) at a final concentration of 10 µg ml⁻¹. TER (Novartis; stock concentration 10 mg ml⁻¹ in DMSO) was used at a final concentration of 100 µg ml⁻¹, FEN (Sigma-Aldrich; stock concentration 10 mg ml⁻¹ in DMSO) at a final concentration of 100 µg ml⁻¹, nikkomycin Z (NKZ) (Sigma-Aldrich; stock concentration 5 mg ml⁻¹ in water) at a final concentration of 10–100 µg ml⁻¹, and LOV (Calbiochem; stock concentration 10 mg ml⁻¹ in ethanol) at a final concentration of 20 µg ml⁻¹. In assays in which a non-water vehicle was used, the no-drug controls were also treated with vehicle. For anaerobic conditions, AnaeroPack anaerobic catalysts (Mitsubishi Gas Chemical) were used in GasPack anaerobic jars (BD). All drug- or anaerobicity-induction experiments were carried out in 5 ml volumes in 50 ml conical tubes at 30 °C with shaking at 180 r.p.m. for 6, 24 or 48 h.

Luciferase assay of *UPC2-RLUC* activity. Luciferase assays were performed as described elsewhere (Srikantha *et al.*, 1996), with modifications as described in Song *et al.* (2004). Because of inter-assay variability, data presented are representative of three independent experiments. Intra-assay variability was assessed by growing three independent colonies of both *UPC2-RLUC* strains in the presence and absence of 100 µg FLC ml⁻¹. This experiment confirmed that within a given assay, variability was low (under 10%).

Ergosterol quantification experiments. Total ergosterol levels were measured as described elsewhere (Arthington-Skaggs *et al.*, 1999). Cultures were inoculated such that the OD₆₀₀ was 0.2 in a total volume of 25 ml CSM. Cells were grown in the presence and absence of drug, or grown anaerobically for 48 h before harvesting. Equivalent OD units of each culture were used to allow for direct comparison of ergosterol levels between strains and conditions. Wavelength scans of samples were performed from 210 to 340 nm.

RESULTS

Creation of *C. albicans* strains expressing *CaUPC2-RLUC* fusions

Previous work has shown that exposure to azoles and other ergosterol biosynthesis inhibitors induces the expression of ergosterol biosynthetic genes as well as their transcriptional activator *CaUpc2p* (Silver *et al.*, 2004). In order to study the effect of these drugs over prolonged exposure, however, it was necessary to create reporter fusions to avoid difficulties in extracting high-quality RNA at late time points. Additionally, to address the role of *CaUpc2p* self-activation in *CaUPC2* transcriptional induction, it was necessary to express the *CaUPC2-RLUC* fusion in our $\Delta upc2/\Delta upc2$ strain D6. This allowed for monitoring of changes between the wild-type and the deletion strain to test the effect of endogenous *CaUpc2p* on *CaUPC2* transcriptional inducibility. The *R. reniformis* luciferase reporter from plasmid pCRW3 (Srikantha *et al.*, 1996) was used to monitor changes in *CaUPC2* transcriptional activation. To express *CaUPC2-RLUC* in both strains

tested in this study, the NAT-resistance marker *SAT1* was cloned into pCRW3, and 750 bp of the *CaUPC2* promoter was fused to the *RLUC* gene in this plasmid (see Methods for details).

CaUPC2 expression is highly regulated at the transcriptional level in response to azole drugs

To assess the role of *CaUPC2* transcriptional activation in response to ergosterol depletion in a wild-type strain, *CaUPC2-750WT* was grown in the presence and absence of various antifungal drugs that target the ergosterol biosynthesis pathway. The effect of a range of FLC concentrations (0.1–100 µg ml⁻¹) on *CaUPC2-RLUC* expression was tested at 6, 24 and 48 h. The drug concentrations were chosen to test the effect of concentrations of drug below, at and above the MIC of FLC for the wild-type strain. This analysis revealed that *CaUPC2* responds transcriptionally to FLC exposure at concentrations near or above the MIC (1.0 µg ml⁻¹; Fig. 1). Exposure to a subinhibitory concentration of FLC (0.1 µg ml⁻¹) did not induce *UPC2* transcription to a significant degree (Fig. 1). The presence of 1.0, 10 or 100 µg FLC ml⁻¹, however, caused activation of *CaUPC2-RLUC* activity, and this induction increased with time, to a maximum of about 100-fold inducibility at 48 h (Fig. 1). Interestingly, prolonged incubation with more than 1.0 µg FLC ml⁻¹ caused a greater induction at earlier time points, but by 48 h, 1.0 µg FLC ml⁻¹ was sufficient to induce a maximal transcriptional response. The effect of another azole, CLO, was similar to that of FLC at the concentration tested (10 µg ml⁻¹; Fig. 2). The concentration of CLO used was based on previous work (Song *et al.*, 2004), and is reflective of the MIC of CLO for the *C. albicans* strains used in this study.

Fig. 1. Time-course of *UPC2-RLUC* induction in the wild-type by a range of FLC concentrations. *CaUPC2-750WT* was grown in the presence and absence of FLC at 0.1 (●), 1.0 (■), 10 (▲) or 100 µg ml⁻¹ (◆). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, i.e. the luciferase activity in the presence of the drug relative to the luciferase activity in the absence of the drug. The results are representative of three independent experiments.

Fig. 2. Time-course of *UPC2-RLUC* induction by the addition of ergosterol biosynthesis inhibitors. CaUPC2-750WT was grown in the presence and absence of CLO at $10 \mu\text{g ml}^{-1}$ (◆), TER at $100 \mu\text{g ml}^{-1}$ (▲), FEN at $100 \mu\text{g ml}^{-1}$ (■) or LOV at $20 \mu\text{g ml}^{-1}$ (□). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, i.e. the luciferase activity in the presence of the drug relative to the luciferase activity in the absence of the drug. The results are representative of three independent experiments.

CaUPC2 expression is transcriptionally induced in response to multiple ergosterol biosynthesis inhibitors

CaUpC2p has been shown to be a global regulator of sterol biosynthesis genes in response to levels of ergosterol or other late-stage intermediates of the sterol pathway (MacPherson *et al.*, 2005; Silver *et al.*, 2004). Because of this, it was of interest to test whether *CaUPC2* is transcriptionally induced in the presence of inhibitors of ergosterol biosynthesis enzymes other than the azoles that target *ERG11*. To test this, CaUPC2-750WT was grown in the presence or absence of FEN, TER, and LOV. These drugs were chosen because they target enzymes of both the early (LOV targets Hmg1p) and late (TER targets Erg1p, which is upstream of Erg11p; FEN targets Erg2p, downstream of Erg11p) parts of the ergosterol biosynthetic pathway. As shown in Fig. 2, TER and FEN (solid triangles and solid squares, respectively) treatment induced transcription of *CaUPC2* to levels similar to those seen with azole exposure (Fig. 1), and this induction increased with prolonged growth in the presence of the drug. Incubation with $20 \mu\text{g ml}^{-1}$ LOV also induced a *CaUPC2* transcriptional response, although the overall fold induction was lower than that seen with other ergosterol biosynthesis inhibitors (approximately fivefold; Fig. 2, open squares).

CaUPC2 expression is induced in response to ergosterol biosynthesis inhibitors in the absence of endogenous CaUpC2p

To test the hypothesis that *CaUPC2* expression is transcriptionally self-regulated, CaUPC2-750D6, the $\Delta\text{upc2}/\Delta\text{upc2}$ strain expressing *CaUPC2-RLUC*, was grown in the presence and absence of ergosterol biosynthesis inhibitors. This allowed for monitoring of the *CaUPC2*

reporter activity in the absence of endogenous *CaUpC2p*. Interestingly, growth of CaUPC2-750D6 in the presence and absence of 0.1 – $100 \mu\text{g FLC ml}^{-1}$ resulted in *UPC2* transcriptional induction, albeit to a lower level than that seen in CaUPC2-750WT (Fig. 3). The concentration of FLC required to induce a maximal transcriptional response beginning at early time points in this strain was $1.0 \mu\text{g ml}^{-1}$. Incubation of CaUPC2-750D6 with CLO, FEN, TER (Fig. 4, solid diamonds, solid squares and solid triangles, respectively) or LOV (Fig. 4, open squares) also resulted in induction of the *CaUPC2-RLUC* fusion, but with overall inducibility lower than that seen in the wild-type strain.

CaUPC2-RLUC basal activity is higher in the absence of endogenous CaUpC2p

The observation that *CaUPC2* fold induction is lower in the *CaUPC2* homozygous deletion suggested either that *CaUPC2* is autoregulated or that there are pleiotropic effects due to the genetic deletion. To address this, the basal *CaUPC2-RLUC* activities of both strains were compared, as well as the absolute level of activity in the presence of azoles, so that the two strains could be directly compared with each other. Interestingly, the decrease in fold induction seen in CaUPC2-750D6 was due primarily to an increased basal level of expression in the absence of drug (Fig. 5). The *CaUPC2* deletion strain exhibited approximately fivefold higher basal activity when compared with the wild-type strain. Additionally, the *CaUPC2-RLUC* activity was higher in the deletion strain at the lowest concentration of FLC tested ($0.1 \mu\text{g ml}^{-1}$). Most importantly, the maximal level of promoter activity in the presence of FLC was the same in both strains (Fig. 5), indicating that the *CaUPC2* promoter is activated transcriptionally in response to the drug to the same degree in both strains. The relationship of the *CaUPC2-RLUC*

Fig. 3. Time-course of *UPC2-RLUC* induction in $\Delta\text{upc2}/\Delta\text{upc2}$ at a range of FLC concentrations. CaUPC2-750D6 was grown in the presence and absence of FLC at 0.1 (●), 1 (◆), 10 (■) or $100 \mu\text{g ml}^{-1}$ (▲). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, i.e. the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

Fig. 4. Time-course of *UPC2-RLUC* induction in $\Delta upc2/\Delta upc2$ by additional ergosterol biosynthesis inhibitors. CaUPC2-750D6 was grown in the presence and absence of CLO at $10 \mu\text{g ml}^{-1}$ (◆), TER at $100 \mu\text{g ml}^{-1}$ (▲), FEN at $100 \mu\text{g ml}^{-1}$ (■) or LOV at $20 \mu\text{g ml}^{-1}$ (□). Luciferase activity was assayed at 6, 24 and 48 h. Data are presented as fold induction, i.e. the luciferase activity in the presence of the drug relative to the luciferase activity in the absence of the drug. The results are representative of three independent experiments.

activities between the two strains was highly reproducible, despite variation in absolute levels of luciferase activity between experiments. Additionally, this phenomenon was consistent in assays using other antifungal drugs (data not shown).

CaUPC2 expression is induced in response to anaerobicity

It has been shown that a *CaUPC2* homozygous deletion is impaired in anaerobic growth (MacPherson *et al.*, 2005). Additionally, it has been shown that *CaERG11*, which is under the control of *CaUpc2p*, is anaerobically induced (Song *et al.*, 2004), and it is known that *CaErg11p* requires

Fig. 5. Levels of *UPC2-RLUC* activity in the wild-type and $\Delta upc2/\Delta upc2$ over a range of FLC concentrations. CaUPC2-750WT (▲) and CaUPC2-750D6 (■) were grown for 48 h at various FLC concentrations. Luciferase activity is expressed as the specific activity of luciferase corrected for total protein content. The results are representative of three independent experiments.

oxygen to be functional. These data suggest that *CaUPC2* is transcriptionally activated in the absence of oxygen. To test this, CaUPC2-750WT and CaUPC2-750D6 were grown aerobically and anaerobically in the presence and absence of $100 \mu\text{g FLC ml}^{-1}$. Although the strain D6 is impaired for anaerobic growth compared with BWP17, it is able to undergo several generations of growth, presumably due to ergosterol stores. After 48 h of anaerobic growth, *CaUPC2-RLUC* activity was increased ~23-fold and 2.3-fold for the wild-type and deletion strains, respectively (Fig. 6). The lack of an additional effect when $100 \mu\text{g FLC ml}^{-1}$ was added during anaerobic growth indicates that the effects of FLC and anaerobicity are not additive (Fig. 6).

CaUPC2 transcriptional induction correlates with sterol depletion

Because *CaUPC2* transcription increases over time with drug exposure, it was of interest to test whether this response is linked to total cellular ergosterol levels. To measure ergosterol content in response to various inhibitors, cells were grown for 48 h (the time point when the transcriptional response is maximal) and total sterols were extracted and measured spectrophotometrically as described elsewhere (Arthington-Skaggs *et al.*, 1999). Sterol levels were shown to correlate well with the degree of *CaUPC2* induction over a range of FLC concentrations (Table 2), as increasing amounts of FLC resulted in a decrease in total sterols in CaUPC2-750WT, with the maximal degree of ergosterol depletion being seen with $100 \mu\text{g FLC ml}^{-1}$ (5.85 % of total ergosterol in cells grown in the absence of FLC). Similarly, with other ergosterol biosynthesis inhibitors as well as anaerobicity, a decrease in ergosterol production was seen (Table 2).

Fig. 6. Anaerobic inducibility of *UPC2-RLUC* activity in the wild-type and $\Delta upc2/\Delta upc2$. Data were collected after 48 h of growth. *UPC2-RLUC* induction in $100 \mu\text{g FLC ml}^{-1}$ is indicated by black bars, induction during anaerobic growth is indicated by grey bars, and induction in the presence of $100 \mu\text{g FLC ml}^{-1}$ during anaerobicity by hatched bars. Data are presented as fold induction, i.e. the luciferase activity in the presence of drug relative to the luciferase activity in the absence of drug. The results are representative of three independent experiments.

Table 2. Ergosterol content of cells grown under sterol-depleting conditions

NA, Not applicable.

Condition	CaUPC2-750WT		CaUPC2-750D6	
	Ergosterol content*	Induction level†	Ergosterol content*	Induction level†
No drug	100.00	NA	74.66	NA
FLC ($\mu\text{g ml}^{-1}$)				
0.1	80.36	2.02	65.21	3.09
1	52.06	86.58	27.49	14.97
10	4.74	95.72	12.19	14.44
100	5.85	99.66	3.07	17.02
20 $\mu\text{g LOV ml}^{-1}$	60.32	7.08	43.51	2.48
100 $\mu\text{g TER ml}^{-1}$	55.20	31.97	85.77	4.78
100 $\mu\text{g FEN ml}^{-1}$	23.04	79.57	39.07	3.06
Anaerobicity	21.72	23.49	44.81	2.31

*Expressed as percentage of wild-type content in the absence of drug.

†*UPC2-RLUC* induction level at 48 h expressed as fold induction.

Similar decreases in total ergosterol levels were seen with the CaUPC2-750D6 strain, in that exposure to ergosterol biosynthesis inhibitors resulted in reduced ergosterol production (Table 2). The CaUPC2-750D6 strain exhibited ergosterol depletion over a range of FLC concentrations, which parallels the *CaUPC2* transcriptional induction shown in Fig. 3. It is important to note that while the $\Delta upc2/\Delta upc2$ strain had lower total ergosterol than the wild-type strain following FLC treatment, the intrinsic level of ergosterol in the deletion strain was lower even in the absence of drug treatment. The deletion strain showed a decrease in total ergosterol following exposure to FEN, LOV or anaerobicity (39.07, 43.51, and 44.81 % of the ergosterol level in BWP17 in the absence of the drug, respectively). Interestingly, the ergosterol level in the $\Delta upc2/\Delta upc2$ strain did not decrease in response to TER. As reported previously (Silver *et al.*, 2004), the deletion strain exhibited a lower level of ergosterol than the wild-type (74.66 %, Table 2) in the absence of the drug, which correlates with the difference between wild-type and deletion strain basal *CaUPC2* reporter activity in the absence of drug, as shown in Fig. 5.

Chemical inhibition of cell wall biosynthesis does not affect *CaUPC2* induction

Based on altered susceptibility of *C. albicans* $\Delta upc2/\Delta upc2$ to the chitin synthase inhibitor NKZ (Silver *et al.*, 2004), as well as the role of *ScUPC2* in regulation of cell wall genes (Abramova *et al.*, 2001), it was hypothesized that *CaUPC2* responds transcriptionally to NKZ treatment. Growth of CaUPC2-750WT or CaUPC2-750D in the presence of 10 or 100 $\mu\text{g NKZ ml}^{-1}$ did not alter *CaUPC2* expression (data not shown). In parallel, NKZ exposure did not alter total ergosterol levels in either strain (data not shown).

DISCUSSION

The goal of this study was to characterize the role of transcriptional inducibility of *CaUPC2* in response to antifungal drugs that target ergosterol biosynthesis. Positive auto-regulation has been suggested to be an important component of *ScUPC2* transcriptional regulation (Abramova *et al.*, 2001; Wilcox *et al.*, 2002). Therefore, this study also addressed whether *CaUPC2* regulates its own transcription, by using a *CaUPC2* homozygous deletion strain and a *CaUPC2* luciferase reporter construct.

Initial characterization of the *CaUPC2* transcriptional response to ergosterol depletion showed that in the wild-type strain CaUPC2-750WT, *CaUPC2* is highly regulated at the transcriptional level. With prolonged exposure to many ergosterol biosynthesis inhibitors, *CaUPC2-RLUC* activity increased up to 100-fold. This suggests that, in addition to other potential mechanisms of regulation of the ergosterol biosynthetic pathway, transcriptional control of the major sterol regulator *CaUPC2* plays an important role in the response to antifungal drugs. Previous work has suggested that a post-translational control mechanism regulates *ScUpc2p* in *S. cerevisiae* (Davies *et al.*, 2005; Vik & Rine, 2001). Data suggest a model in which *ScUpc2p* is present in the nucleus and is bound by a repressor under sterol-rich conditions. Upon sterol depletion, however, the repression is released and *ScUpc2p* binds to and activates sterol biosynthesis genes (Davies *et al.*, 2005). An alternative model is based on an analogy to the mammalian sterol regulator SREBP and was proposed in a recent review (White & Silver, 2005). In this model, the N-terminal *CaUpc2p* DNA-binding domain (DBD) is anchored to a membrane via four predicted transmembrane spans found in the C-terminal portion of the protein. Upon sterol depletion, it is proposed that a cleavage event liberates the

DBD, which can then translocate to the nucleus and activate target genes. This model is consistent with *S. cerevisiae* localization experiments that show that the C-terminally tagged Upc2p is not nuclear-localized (Marie *et al.*, 2008). The current evidence cannot determine which model is correct, but either model suggests an important post-translational regulatory mechanism. This work demonstrates that, in addition to these proposed mechanisms, transcriptional activation of *CaUPC2* probably plays an important role in regulating downstream genes via a large increase in abundance of *CaUPC2* mRNA and, putatively, its subsequent protein product.

It was also shown that transcriptional induction became maximal after 48 h of growth in the presence of drugs. This observation is consistent with the hypothesis that it is depletion of sterols that triggers *CaUPC2* upregulation, as sterol depletion will only become severe after prolonged exposure to inhibitors.

The observation that inhibition of multiple steps in the ergosterol biosynthetic pathway results in an increase in *CaUPC2-RLUC* activity is consistent with evidence that *CaUpc2p* acts as a global regulator of sterol biosynthesis genes. These data are consistent with previous results from this laboratory showing that *CaERG11-RLUC* activity is responsive to inhibition of multiple steps in the ergosterol biosynthetic pathway (Song *et al.*, 2004). It is important to note that all of the genes for enzymes that were inhibited in this study (*HMG1*, *ERG1*, *ERG11* and *ERG2*) contain putative *CaUpc2p*-binding sites within their promoters (MacPherson *et al.*, 2005; Silver *et al.*, 2004). In addition, microarray analysis suggests that transcriptional activation of each of these drug targets in response to FLC is *CaUpc2p*-dependent (P. Silver and T. White, unpublished results). When these data are taken together with the level of sterol depletion caused by these inhibitors, it seems likely that the signal that induces *CaUPC2* expression and subsequent upregulation of *ERG* genes may be the lack of ergosterol or a late sterol pathway intermediate. Indeed, this work has shown that inhibition of multiple steps in the biosynthetic pathway results in a decrease in the end product ergosterol, and therefore it seems likely that this decrease serves as a signal to induce expression of the transcriptional activator of the pathway, *CaUpc2p*. This hypothesis is supported by the observation that ergosterol depletion (Table 2) correlates with induction of *CaUPC2-RLUC* activity (Figs 1–4).

The response of *CaUPC2* to anaerobicity is also consistent with previous data. In *S. cerevisiae*, anaerobic growth is not possible in the absence of exogenous ergosterol, and this appears to be largely due to the dependence of Erg11p on molecular oxygen as a cofactor, as well as the haem requirement of this enzyme (Setiadi *et al.*, 2006; White *et al.*, 1998). *C. albicans*, however, can grow anaerobically in the absence of exogenous ergosterol, and this growth is accompanied by an increase in *ERG11* expression (Setiadi *et al.*, 2006; Song *et al.*, 2004). The anaerobic induction of

ERG11 is likely due to the oxygen dependence of the sterol pathway, which utilizes 12 molecules of O₂ for every molecule of ergosterol synthesized (Hughes *et al.*, 2005). This study shows that the amount of ergosterol biosynthesis under anaerobic conditions is clearly decreased when compared with aerobically grown cells, although not to the same degree as seen with drug inhibition. This intermediate degree of anaerobic sterol depletion is paralleled by an increase in *CaUPC2-RLUC* activity that is somewhat lower than that seen with direct chemical inhibition of ergosterol biosynthesis. Earlier studies have also shown that a *CaUPC2* deletion strain is deficient in anaerobic growth (MacPherson *et al.*, 2005), suggesting that transcriptional activation of the ergosterol biosynthetic pathway by *CaUpc2p* is essential in anaerobicity, which is consistent with the data presented in this study. It is important to note that the anaerobic induction experiment in this study was performed using aerobically grown inocula, so that luciferase activity reflects the adaptation to anaerobicity, not true anaerobic growth.

The *CaUPC2* deletion has been shown previously to exhibit hypersensitivity to cell wall-perturbing agents such as NKZ (Silver *et al.*, 2004), but it is unclear whether this sensitivity is a direct result of *CaUpc2p* activation of cell-wall-associated genes, or a pleiotropic effect resulting from altered membrane sterol composition. *ScUpc2p* activates some cell wall-associated proteins transcriptionally in response to anaerobicity, such as those in the *DAN/TIR* family (Abramova *et al.*, 2001). This evidence suggests that the effect of *ScUPC2* deletion on cell wall sensitivity is due to a direct effect of *ScUpc2p* on cell wall gene expression. If the effect was the result of transcriptional activation by *CaUpc2p*, *CaUPC2* would be expected to respond transcriptionally to treatment with NKZ. When this was tested, however, there was no change in *CaUPC2-RLUC* activity in the presence of NKZ. Additionally, NKZ did not alter total ergosterol levels. These data suggest that *CaUPC2* transcriptional activation is specific to alterations in the sterol biosynthetic pathway. The NKZ susceptibility of the *CaUPC2* deletion mutant may be due to pleiotropic effects of the lower sterol level of the mutant rather than direct control of expression of cell wall-associated genes by *CaUpc2p*.

The comparison of *CaUPC2-RLUC* activity between the wild-type and the *CaUPC2* deletion mutant suggests either direct autoregulation by *CaUpc2p* or an indirect consequence of the lower basal level of total sterols in the deletion strain. The difference in fold induction between the two strains demonstrates clearly that the *CaUPC2* mutant has an altered regulation of *CaUPC2* promoter activity (Figs 1–4). The lower fold inducibility of *CaUPC2-RLUC* in $\Delta upc2/\Delta upc2$ suggests an important component of transcriptional self-regulation, which is consistent with the limited evidence reported for *S. cerevisiae* (Abramova *et al.*, 2001; Davies *et al.*, 2005) as well as the presence of a putative *CaUpc2p*-binding site within the *CaUPC2* promoter. The increase in basal activity of the $\Delta upc2/\Delta upc2$

strain when compared with the wild-type, however, suggests more than one possibility. It is possible that *CaUpc2p* acts as a transcriptional repressor at its own promoter, but no evidence has yet been collected to support this. Alternatively, the intrinsically lower level of ergosterol in the $\Delta upc2/\Delta upc2$ mutant (Silver *et al.*, 2004) may account for the increased *CaUPC2-RLUC* activity in the absence of drug. This indirect effect may mask direct consequences of *CaUPC2* deletion on *CaUPC2-RLUC* activity, and further study, including nested deletions and direct binding to the putative SRE, is needed to address autoregulation definitively. These studies are currently under way in this laboratory. While the intrinsically lower level of sterol in the deletion strain may explain the higher level of *UPC2-RLUC* activity in the absence of drugs, recent work has demonstrated that *CaUpc2p* does indeed bind to the *CaUPC2* promoter (Znaidi *et al.*, 2008). This evidence, along with the data presented in the present study, suggests that at least some component of *UPC2* inducibility is transcriptionally self-regulated. The *CaUPC2-RLUC* inducibility that remains in the $\Delta upc2/\Delta upc2$ strain suggests strongly that in addition to *CaUpc2p*, a novel sterol-responsive transcription factor also controls *CaUPC2* expression. This is consistent with earlier work in which deletion of the *ScUpc2p*-binding site within the *ScUPC2* promoter reduced, but did not eliminate, *ScUPC2* inducibility (Abramova *et al.*, 2001). Identification of additional transcription factors that control *CaUPC2* expression is currently being addressed in this laboratory and will contribute to our understanding of how *C. albicans* cells respond to antifungal drugs.

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REFERENCES

- Abramova, N. E., Cohen, B. D., Sertil, O., Kapoor, R., Davies, K. J. & Lowry, C. V. (2001). Regulatory mechanisms controlling expression of the *DAN/TIR* mannoprotein genes during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*. *Genetics* **157**, 1169–1177.
- Arthington-Skaggs, B. A., Crowell, D. N., Yang, H., Sturley, S. L. & Bard, M. (1996). Positive and negative regulation of a sterol biosynthetic gene (*ERG3*) in the post-squalene portion of the yeast ergosterol pathway. *FEBS Lett* **392**, 161–165.
- Arthington-Skaggs, B. A., Jradi, H., Desai, T. & Morrison, C. J. (1999). Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J Clin Microbiol* **37**, 3332–3337.
- Davies, B. S., Wang, H. S. & Rine, J. (2005). Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: similar activation/regulatory domains but different response mechanisms. *Mol Cell Biol* **25**, 7375–7385.
- Dimster-Denk, D. & Rine, J. (1996). Transcriptional regulation of a sterol-biosynthetic enzyme by sterol levels in *Saccharomyces cerevisiae*. *Mol Cell Biol* **16**, 3981–3989.
- Henry, K. W., Nickels, J. T. & Edlind, T. D. (2000). Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother* **44**, 2693–2700.
- Hoffman, C. S. & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267–272.
- Hughes, A. L., Todd, B. L. & Espenshade, P. J. (2005). SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. *Cell* **120**, 831–842.
- MacPherson, S., Akache, B., Weber, S., De Deken, X., Raymond, M. & Turcotte, B. (2005). *Candida albicans* zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. *Antimicrob Agents Chemother* **49**, 1745–1752.
- Marie, C., Leyde, S. & White, T. C. (2008). Cytoplasmic localization of sterol transcription factors Upc2p and Ecm22p in *S. cerevisiae*. *Fung Genet Biol* (in press). <http://dx.doi.org/10.1016/j.fgb.2008.07.004>
- Oliver, B. G., Song, J. L., Choiniere, J. H. & White, T. C. (2007). *cis*-Acting elements within the *Candida albicans* *ERG11* promoter mediate the azole response through the transcription factor Upc2p. *Eukaryot Cell* **6**, 2231–2239.
- Pfaller, M. A. & Diekema, D. J. (2004). Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J Clin Microbiol* **42**, 4419–4431.
- Reuss, O., Vik, A., Kolter, R. & Morschhauser, J. (2004). The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341**, 119–127.
- Sanglard, D., Ischer, F., Monod, M. & Bille, J. (1996). Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob Agents Chemother* **40**, 2300–2305.
- Setiadi, E. R., Doedt, T., Cottier, F., Noffz, C. & Ernst, J. F. (2006). Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. *J Mol Biol* **361**, 399–411.
- Silver, P. M., Oliver, B. G. & White, T. C. (2004). Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot Cell* **3**, 1391–1397.
- Song, J. L., Harry, J. B., Eastman, R. T., Oliver, B. G. & White, T. C. (2004). The *Candida albicans* lanosterol 14- α -demethylase (*ERG11*) gene promoter is maximally induced after prolonged growth with antifungal drugs. *Antimicrob Agents Chemother* **48**, 1136–1144.
- Srikantha, T., Klapach, A., Lorenz, W. W., Tsai, L. K., Laughlin, L. A., Gorman, J. A. & Soll, D. R. (1996). The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. *J Bacteriol* **178**, 121–129.
- Vik, A. & Rine, J. (2001). Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **21**, 6395–6405.
- White, T. C. & Silver, P. M. (2005). Regulation of sterol metabolism in *Candida albicans* by the *UPC2* gene. *Biochem Soc Trans* **33**, 1215–1218.
- White, T. C., Marr, K. A. & Bowden, R. A. (1998). Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin Microbiol Rev* **11**, 382–402.

Wilcox, L. J., Balderes, D. A., Wharton, B., Tinkelenberg, A. H., Rao, G. & Sturley, S. L. (2002). Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. *J Biol Chem* **277**, 32466–32472.

Znaidi, S., Weber, S., Al-Abdin, O. Z., Bomme, P., Saidane, S., Drouin, S., Lemieux, S., De Deken, X., Robert, F. & Raymond, M.

(2008). Genomewide location analysis of *Candida albicans* Upc2p, a regulator of sterol metabolism and azole drug resistance. *Eukaryot Cell* **7**, 836–847.

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