**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.

**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 parologue ScECM22, and inducibility of ScUPC2 may be affected by the presence of ScECM22. Studies showing that ScUPC2 expression is induced byazole 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 anaerobic, and investigates the hypothesis that CaUPC2 expression is self-regulated.

METHODS

Strains and growth conditions. C. albicans strain BW17 (ura3::λ434/ura3::λimm434 his1::hisG his1::hisG arg4::hisG arg4::hisG) and its derivative 6- (upr2::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 Wurzburg) 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 mg ml⁻¹ 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 BW17 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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'–3')</th>
</tr>
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<tr>
<td>SAT1Kpn</td>
<td>TACACGGTACCAGCGGCTAAAAGTAAGAATATAAAG</td>
</tr>
<tr>
<td>SAT1EcoRV</td>
<td>TACACGATATCATGTTTCTAGAAGGACCAC</td>
</tr>
<tr>
<td>UPC2Kpn</td>
<td>CTCTCGGTACCATGAGTGTGTTGATATCAGG</td>
</tr>
<tr>
<td>UPC2Sma</td>
<td>CTCTCCCGGGAAAATGGCTTTTGGTGAAAAA</td>
</tr>
<tr>
<td>RLUC</td>
<td>CACACCTGCGGAGACAGTTATCATCCGTTTCC</td>
</tr>
<tr>
<td>ADE2</td>
<td>CAGTTAAATAGTCTTCATATC</td>
</tr>
</tbody>
</table>
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 UPCI–RLUC activity. Drugs used for induction of the UPCI 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 UPCI–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 UPCI–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 OD\(_{500}\) 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 Upc2p (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 Upc2p self-activation in CaUPC2 transcriptional induction, it was necessary to express the CaUPC2–RLUC fusion in our Δupc2/Δupc2 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 UPCI 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.
transcriptionally self-regulated, CaUPC2-750D6, the To test the hypothesis that CaUPC2 of endogenous ergosterol biosynthesis inhibitors in the absence Ca inhibitors (approximately fivefold; Fig. 2, open squares). tional response, although the overall fold induction was 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 μg ml⁻¹ (●), TER at 100 μg ml⁻¹ (▲), FEN at 100 μg ml⁻¹ (■) or LOV at 20 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.

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 Erg1lp; FEN targets Erg2p, downstream of Erg1lp) 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 withazole exposure (Fig. 1), and this induction increased with prolonged growth in the presence of the drug. Incubation with 20 μg LOV ml⁻¹ 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 ∆upc2/∆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 μg 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 μg 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 μg 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. 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 μg ml⁻¹ (●), TER at 100 μg ml⁻¹ (▲), FEN at 100 μg ml⁻¹ (■) or LOV at 20 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. 3. Time-course of UPC2–RLUC induction in ∆upc2/∆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 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 drug relative to the luciferase activity in the absence of 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 anaerobiosis

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 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 μg 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 μg 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 μg 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).
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 paralleled the CaUPC2 transcriptional induction shown in Fig. 3. It is important to note that while the Δupc2/Δupc2 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 wild-type strain basal level in the absence of drug, respectively). Interestingly, the ergosterol level in the Δupc2/Δupc2 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 Δupc2/Δupc2 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 μg 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

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>CaUPC2-750WT</th>
<th>CaUPC2-750D6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ergosterol content</td>
<td>Induction level</td>
</tr>
<tr>
<td>No drug</td>
<td>100.00</td>
<td>NA</td>
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<tr>
<td>FLC (μg ml⁻¹)</td>
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<td>0.1</td>
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<tr>
<td>20 μg LOV ml⁻¹</td>
<td>60.32</td>
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<tr>
<td>100 μg TER ml⁻¹</td>
<td>55.20</td>
<td>31.97</td>
</tr>
<tr>
<td>100 μg FEN ml⁻¹</td>
<td>23.04</td>
<td>79.57</td>
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<td>Anaerobicty</td>
<td>21.72</td>
<td>23.49</td>
</tr>
</tbody>
</table>

*Expressed as percentage of wild-type content in the absence of drug.
†UPC2–RLUC induction level at 48 h expressed as fold induction.

NA, Not applicable.
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 CaUCP2–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 Δupc2/Δupc2 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 Δupc2/Δupc2
activation/regulatory domains but different response mechanisms. Mol Cell Biol 25, 7375–7385.


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