The Yeast Anaerobic Response Element AR1b Regulates Aerobic Antifungal Drug-dependent Sterol Gene Expression*

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Background: Saccharomyces cerevisiae sterol gene expression is regulated by a consensus sterol-response promoter element (SRE/AR1).

Results: The anaerobic AR1b promoter element regulates global antifungal-dependent sterol gene expression.

Conclusion: Yeast sterol gene expression is regulated by multiple SRE-like elements.

Significance: Understanding sterol gene expression will yield valuable information concerning antifungal drug resistance.

Saccharomyces cerevisiae ergosterol biosynthesis, like cholesterol biosynthesis in mammals, is regulated at the transcriptional level by a sterol feedback mechanism. Yeast studies defined a 7-bp consensus sterol-response element (SRE) common to genes involved in sterol biosynthesis and two transcription factors, Upc2 and Ecm22, which direct transcription of sterol biosynthetic genes. The 7-bp consensus SRE is identical to the anaerobic response element, AR1. Data indicate that Upc2 and Ecm22 function through binding to this SRE site. We now show that it is two novel anaerobic AR1b elements in the UPC2 promoter that direct global ERG gene expression in response to a block in de novo ergosterol biosynthesis, brought about by antifungal drug treatment. The AR1b elements are absolutely required for auto-induction of UPC2 gene expression and protein and require Upc2 and Ecm22 for function. We further demonstrate the direct binding of recombinant expressed S. cerevisiae ScUpc2 and pathogenic Candida albicans CaUpc2 and Candida glabrata CgUpc2 to AR1b and SRE/AR1c elements. Recombinant endogenous promoter studies show that the UPC2 anaerobic AR1b elements act in trans to regulate ergosterol gene expression. Our results indicate that Upc2 must occupy UPC2 AR1b elements in order for ERG gene expression induction to take place. Thus, the two UPC2-AR1b elements drive expression of all ERG genes necessary for maintaining normal antifungal susceptibility, as wild type cells lacking these elements have increased susceptibility toazole antifungal drugs. Therefore, targeting these specific sites for antifungal therapy represents a novel approach to treat systemic fungal infections.

Mammalian cells regulate de novo cholesterol biosynthetic gene expression through the sterol-dependent cleavage of membrane-bound transcription factors (SREBPs) (1, 2). The SCAP and Insig proteins act antagonistically with one another in regulating proteolytic release of SREBPs and their subsequent translocation to the nucleus, where they bind to SRE sequences within the promoters of genes required for LDL receptor expression, and sterol and fatty acid biosynthesis (3, 4). SCAP senses sterol depletion through its sterol-sensing domain and initiates the translocation of SREBPs from the endoplasmic reticulum to the Golgi, where they are cleaved by site-1 and site-2 proteases (1, 5, 6). Proteolysis results in release of the soluble basic helix-loop-helix-zipper domain, which enters the nucleus (7). High sterol levels stabilize the binding of Insig to SCAP, retaining SCAP and SREBPs in the endoplasmic reticulum, thus inhibiting the translocation of SREBPs and sterol gene expression.

Saccharomyces cerevisiae sterol biosynthetic pathway is also transcriptionally regulated in response to changes in sterol levels (8) and contains functional orthologs of some of the mammalian components regulating sterol gene expression. Genes regulated include HMG-CoA reductase (HMG1), squalene synthase (ERG9), sterol C-5 desaturase (ERG3), and genes required for the conversion of 4,4-dimethylzymosterol tozymosterol (ERG25/26/27) (9–13). Many yeast genes contain an SRE similar to the core mammalian SRE-1 consensus sequence (11, 14). The sequence of this SRE is identical to the anaerobic response element AR1 characterized by Lowry and co-workers (15, 16); the same group also characterized other AR1 elements (AR1α, AR1β, and AR1γ).

S. cerevisiae contains the Insigs, ScNsg1/ScNsg2, which regulate proteolytic degradation of the HMG-CoA reductase, ScHmg2 (17). However, it lacks a functionally conserved ortholog of SCAP (1). In addition to responding to changes in sterol levels, ERG gene expression is regulated by glucose, heme, and oxygen levels (15, 16, 18–21).

2 The abbreviations used are: SREBP, sterol-response element-binding protein; SRE, sterol-response element; LDLR, low-density lipoprotein receptor; SCAP, sterol cleavage activating protein; Insig, insulin-induced protein; ERG, ergosterol; PA, phosphatidic acid; TEG, triethylene glycol; qRT, quantitative RT; NFDM, nonfat dry milk.
The ScUpc2 and ScEcM22 transcription factors are required for antifungal induced transcription (14, 22). They belong to the Zn(2)–Cys(6) binuclear cluster family of conserved fungal transcription factors (23). ScUpc2/ScEcM22 translocate to the nucleus in response to ergosterol depletion (24). Both ScUpc2 and ScEcM22 directly bind to yeast consensus SRE/AR1 elements in vivo to induce ERG gene expression upon antifungal drug assault (11, 14, 25).

CaUpc2 gain-of-function mutations have been associated with azole resistance in Candida albicans clinical isolates (26–31). Mutations in CaUpc2 result in the constitutive induction of ERG11 gene expression, which is the target of the common azole drugs. Increased ERG11 gene expression has also been seen in azole-resistant isolates of Candida glabrata (32–35). C. glabrata resistance is on the rise, and it is the second most common cause of disseminated candidiasis (36–38). As C. glabrata also contains Upc2 orthologs (39), it is only a matter of time before gain-of-function mutations are reported in clinical resistance isolates. Thus, a greater understanding of how Upc2 functions to regulate gene expression is required to fully comprehend how pathogenic yeast gain resistance.

In this study, we determined the in vivo promoter activities of SRE/AR1 elements in several ERG genes. The data show for the first time that two anaerobic AR1b elements in the UPC2 promoter are required for inducing global sterol gene expression in response to antifungal-induced blocks in sterol biosynthesis. These two elements are the nucleus for global ERG expression in response to antifungal assault, as strains lacking these promoter elements show increased susceptibility to antifungal drug treatment. The data have further increased the knowledge of how multiple pathogenic yeast gain resistance to antifungal drug therapies.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Miscellaneous Methods**—The yeast strains used in this study are isogenic to W303 (MATa leu2-3, 112 trp1-1 ura3-1 his3-11, 15 can1-100) or BY4741 (MATa his3 leu2 met15 ura3). Strains were grown in YEPD (1% yeast extract, 2% bacto-pectone, 2% glucose) or synthetic minimal media containing 0.67% yeast nitrogen base (Difco), supplemented with the appropriate amino acids, adenine, and uracil. Lovastatin (50 μg/ml) was added directly to liquid YEPD or synthetic media and was incubated with cells for 16 h. Lovastatin was solubilized as follows: 25 mg of lovastatin (Sigma) was dissolved in 250 μl of 0.2 M NaOH/EtOH (0.8 g of NaOH per 100 ml of 100% ethanol) and incubated in a 65 °C water bath for 40 min. 600 μl of 0.2 M Tris-HCl, pH 8.0, and 150 μl of 1 M Tris-HCl were then added. Yeast transformation was performed as described by Ito (40). Escherichia coli XL1Blue cells were used for plasmid propagation and grown in LB medium supplemented with ampicillin (150 μg/ml).

**Plasmid Construction**—The yeast Ylp353 integrating plasmid was used to construct promoter-lacZ constructs. Plasmids were integrated at the endogenous URA3 locus. The ERG25 promoter contained 1500 bp, ERG3 contained 1000 bp, ERG1 contained 750 bp, and UPC2 contained 750 bp. The URA3 centromeric plasmid, pRS416, was used to construct low copy ERG vectors. pRS416 constructs contained the promoter sequence, the entire coding sequence, and 500 bp of the 3‘-untranslated region. pRS416-ERG25 was transformed into an ERG25/erg25::psre/ar1c-ERG25 diploid strain. This strain contains an endogenous HIS3-generated ERG25 promoter disruption allele. ERG25/erg25::psre/ar1c-ERG25 haploids were obtained by sporulation and selecting for His + Ura +. Haploids were FOA + (41), demonstrating the need for plasmid-driven ERG25 activity. pRS416-ERG3 was transformed into an erg3::kan’ null strain. This strain was generated in W303 by PCR amplification using template DNA from an erg3::kan’ haploid strain (Open Biosystems, Huntsville, AL). All SRE mutant constructs were generated using the QuikChange® multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) and were verified by DNA sequencing.

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<th>TABLE 1</th>
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<td>Various yeast strains used in this work</td>
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<td><strong>Promoter</strong></td>
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<td>ERG25::HIS3::par1b-ar1b-ERG25</td>
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<td>ERG25/erg25::psre/ar1c-ERG25</td>
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<td>ERG3/erg1::psre/ar1c-ar1b-ERG3, ERG1::HIS3::par1b-ar1b-ERG1, and UPC2::HIS3::par1b-ar1b-UPC2</td>
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<td>UPC2::HIS3::par1b-ar1b-UPC2</td>
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*NA means not applicable.

**Regulation of Ergosterol Gene Expression**

**IC<sub>50** Microdilution Assay—IC<sub>50** assays were performed as follows. Yeast cultures were diluted to 5 × 10<sup>3</sup> cells/ml in YEPD. Cell aliquots of 100 μl were distributed to wells of a 96-well flat-bottom plate, except for row A, which received 200 μl. Drug was added to row A at the desired concentration and then serially 2-fold diluted to rows B–G; row H served as a drug-free control. Plates were incubated at 30 °C for 24 and 48 h. Absorbance at 620 nm was read with a microplate reader (Beckman Coulter, Inc., Fullerton, CA); background due to medium was subtracted from all readings. IC<sub>50** values were defined as the lowest concentration inhibiting growth at least 50% relative to the drug-free control.

**Liquid β-Galactosidase Assays—β-Galactosidase activity assays were performed as described using the substrate, chloroform-red β-β-galactopyranoside (42).**

**Total RNA Isolation and Northern Analysis—Cells were grown to exponential phase at 30 °C in synthetic medium.**
RNA was extracted from 1 × 10^7 cells using acid-washed glass beads, SDS-lysis buffer (200 mM Tris-HCl, pH 7.5, containing 500 mM NaCl, 10 mM EDTA, 1% SDS), and phenol/chloroform/isomyl alcohol. Total RNA was denatured in formaldehyde/MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM Na2EDTA, 7% formaldehyde) for 20 min at 65 °C. 20 μg of denatured RNA was resolved using 1% agarose gels containing 6% formaldehyde. Total RNA was blotted onto Hybond-N membranes (Amer sham Biosciences) overnight using capillary action. Membranes were then washed in 1× SSC (300 mM NaCl, 30 mM sodium citrate) for 5 min and UV cross-linked. They were then hybridized to gel-purified radiolabeled PCR-amplified probes in phosphate buffer (250 mM NaH2PO4, 250 mM Na2HPO4, 1 mM EDTA, 7% SDS, 1% BSA). Post-hybridization, membranes were washed several times with both 2× and 0.1× SSC containing 0.5% SDS. Hybridization and all washes were performed at 65 °C. The Megaprime labeling kit (Amer sham Biosciences) was used to 32P, radiolabel all PCR-amplified probes. The level of total rRNA was used as a loading control. Gene expression levels were determined by autoradiography (Kodak XAR5) followed by densitometry using a Bio-Rad Gene Scan System and Software, version 4.0.

qRT-PCR Analysis—Cells were grown to exponential phase at 30 °C. RNA was resuspended in diethyl pyrocarbonate-treated water. 50 ng of RNA, 11.5 μl of the SYBR Green Master Mix (Quanta), and 5 μM primer sets were loaded in triplicate onto a 96-well plate. qRT-PCR amplification and analysis were completed using the Stratagene Max-Pro (Mx3000P) software version 4.0.

Western Analysis—Strains were grown to exponential phase at 30 °C in YEPD medium. Cells were disrupted with cold glass beads and lysis buffer (Tris base, pH 7.9, containing (NH4)2SO4, MgSO4, glycerol, and EDTA). Lysed cells were centrifuged at 3000 rpm for 5 min to obtain a total cell-free extract. 100 μg of cell extract were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Western blot analysis was performed using the following: anti-Pah1 polyclonal antibodies, 1:500 dilution in buffer A + 1% NFDM, 1% goat serum; anti-Erg25 polyclonal antibodies, 1:1000 dilution in buffer A + 1% NFDM, 1% goat serum; anti-Upc2 polyclonal antibodies, 1:500 dilution in buffer A + 1% NFDM, 1% goat serum; and anti-Pgk1 polyclonal antibodies, 1:1000 dilution in buffer A, 1% NFDM, 1% goat serum. Horseradish peroxidase-conjugated secondary antibodies were anti-IgG antibodies. For Pah1, a 1:1000 dilution was used in buffer A + 1% NFDM, 1% goat serum. For Erg25, a 1:2000 dilution in buffer A + 1% NFDM and 1% goat serum was used. For Upc2, a 1:1000 dilution in buffer A + 1% NFDM, 1% goat serum was used. For Pgk1, a 1:10000 dilution in buffer A + 1% NFDM, 1% goat serum was used. Pah1 migrates at 125 kDa, Erg25 at 100 kDa, and Pgk1 at 45 kDa (loading standard).

Phosphatidic Acid Phosphatase Enzymatic Assay—PA phosphatase activity was measured by following the release of water-soluble 32P, from chloroform-soluble [32P]PA for 20 min at 30 °C in a total reaction volume of 0.1 ml. For measurement of PA phosphatase activity, the reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 0.2 mM [32P]PA (10,000–12,000 cpm/nmol), 2 mM Triton X-100, and 5 mM MgCl2 (43–49), and 10 μg of cell extract. A unit of PA phosphatase activity was defined as the amount of enzyme that catalyzed the dephosphorylation of 1 nmol of PA/min. Specific activity was defined as units/mg of protein.

Expression and Purification of Glutathione S-transferase-Upc2 DNA Binding Domain Fusion Proteins—The UPC2 gene fragment encoding the DNA binding domains from S. cerevisiae (amino acids 1–148), C. albicans (amino acids 1–170), or C. glabrata (amino acids 1–186) were amplified by PCR using chromosomal DNA. The oligonucleotides used as primers were as follows: 5’-CGGGATCCTATGATGACAGTAAAAC-A-3’ and 5’-GGATTTCTAAAATACCCCGCTAGGTATTTTA-3’; 5’-GGGGATCCTATGATGACAGTAAAAC-A-3’ and 5’-GGATTTCTAAAATACCCGGCTAGGTATTTTA-3’; and 5’-CCGTGATCAATGACTACGCTAGCTGTC-3’ and 5’-GAATTCTTAAATACCGGCTAGGTATTTTA-3’. The amplified probes in phosphate buffer (250 mM NaH2PO4, 250 mM Na2HPO4, 1 mM EDTA, 7% SDS, 1% BSA). Post-hybridization, membranes were washed several times with both 2× and 0.1× SSC containing 0.5% SDS. Hybridization and all washes were performed at 65 °C. The Megaprime labeling kit (Amer sham Biosciences) was used to 32P, radiolabel all PCR-amplified probes. The level of total rRNA was used as a loading control. Gene expression levels were determined by autoradiography (Kodak XAR5) followed by densitometry using a Bio-Rad Gene Scan System and Software, version 4.0.

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and anti-GST-coated AlphaScreen acceptor beads, which bind to the GST-Upc2 fusion protein. DNA binding by the fusion protein brings the two types of beads into close proximity, allowing the generation of an AlphaScreen signal. The DNA sequences of probes used for the screening assay are as follows: S. cerevisiae novel AR1p, 5′-biotin-TEG-CTGTATTTTCGTT- TAAAAGTGG-3′ and 5′-CCACTTTTTAACAAGCATACAG- G-3′; C. albicans ERG2 consensus SRE/AR1c, 5′-biotin-TEG- CTGTATTTTCGTTATTAAAGTGG-3′ and 5′-CCACTTTT- ATACGACAATACAG-3′; C. albicans ERG2 mutant SRE/ AR1c, 5′-biotin-TEG-CTGTATTTCGATAAAGTGG-3′ and 5′-CCACTTTTTATACGACAATACAG-3′. TEG is a triethylene glycol spacer. The two probes were hybridized to form a biotinylated, double-stranded oligonucleotide by combining them at a concentration of 50 μM each in a buffer consisting of 100 mM KCl, 30 mM HEPES, pH 7.4. The mixture was heated to 91–95 °C for 2 min and allowed to slowly cool.

To perform the assay, fusion protein was diluted to 8 nM (0.34 μg/ml) in an assay buffer consisting of 25 mM HEPES, 200 mM NaCl, 0.1% Tween 20, and 3 mM ZnSO4. Protein was then combined with an equal volume of acceptor beads, which had been diluted to 80 μg/ml in assay buffer, and 4 μl/well of the mixture was transferred to a white 1536-well assay plate. The plate was incubated for 30 min at room temperature. Biotinylated and double-stranded DNA was diluted to 40 nM in assay buffer and combined with an equal volume of 80 μg/ml donor beads in assay buffer. 4 μl of this mixture was added to each well of the assay plate, and the plate was incubated for a further 1 h at room temperature in the dark. The results were then read using an Envision microplate reader (PerkinElmer Life Sciences).

**Chromatin Immunoprecipitation Assay**—Cells were treated with 1% formaldehyde for 15 min at room temperature. Cross-linking was stopped by addition of 125 mM glycine. Cells were pelleted, washed with PBS, and again pelleted. Cells were then lysed using lysis buffer (50 mM HEPES, pH 7.5, containing 140 mM NaCl, 0.1% Triton X-100, 0.1% sodium deoxycholate, and a protease mixture) and spun down to remove cellular debris. 1 mg of the resulting supernatant was used for immunoprecipitating ScUpc2-bound DNA for 1 day at 4°C, using 2 μg of anti-Myc monoclonal antibody. Immunoprecipitated complexes were isolated after 1 h at 4°C using protein A-Sepharose/agarose beads. The beads were washed with lysis buffer containing 500 mM NaCl, and finally with 10 mM Tris-HCl, pH 8.0, containing 0.25 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate. ScUpc2-DNA complexes were eluted from beads using 50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA, and 1% SDS. Cross-linking was reversed using 5 mM NaCl at 65 °C for 6 h. Samples were then diluted using 500 mM Tris-HCl, pH 8.0, containing 10 mM EDTA and 0.67% SDS. Samples were treated with 250 μg of proteinase K for 1 h at 37 °C. DNA was eluted from samples using the DNA Easy Kit (Qiagen). The degree of binding was determined using qRT-PCR.

**RESULTS**

**ERG25 Promoter-lacZ Fusion Lacking Consensus SRE/AR1c Elements Has Lovastatin-induced Activity**—We previously demonstrated that ERG25 gene expression was induced in response to blocking several steps in ergosterol biosynthesis and that induction required Upc2 and Ecm22 (9). The ERG25 promoter contains three consensus SRE/AR1c sites (Table 2) (11, 14–16). A β-galactosidase promoter-lacZ fusion assay was used to determine whether these elements harbored activity. All combinations of ERG25-lacZ SRE/AR1c promoter deletion/mutation constructs were tested in response to treatment with the HMG-CoA reductase inhibitor lovastatin. The upc2Δ ecm22Δ null cells harboring all constructs were also tested to determine whether ScUpc2/ScEcm22 functions were required for activity.

Of all the combinations tested, only an ERG25-lacZ fusion lacking SRE/AR1c sites 1 and 3 together had reduced lovastatin-induced activity (Fig. 1, A and B, SRE/AR1c,2 versus SRE/ AR1c1,2,3). In some cases, the loss of a single or double SRE/ AR1c site removed due to insertion of HIS3.

**TABLE 2**

The promoter positions for SRE/AR1c sites alone or together reduced, but did not abolish, lovastatin-induced activity (Fig. 1, A and B, SRE/AR1c1,2,3 versus SRE/AR1c1,2). This suggested that novel promoter elements regulated ERG25 promoter activity in response to a block in ergosterol biosynthesis.

To determine whether the sre−/ar1c− active phenotype was common to other ERG promoters, ERG1 and ERG3 AR1c promoter sites were tested using ERG1-lacZ and ERG3-lacZ fusions. ERG1 and ERG3 contain 1 and 2 SRE/AR1c sites, respectively (Table 2). Sites were deleted/mutated, and cells were assayed for promoter-lacZ activity in the absence or presence of lovastatin.

The deletion of the single SRE/AR1c in the ERG1-lacZ promoter resulted in a total loss of lovastatin-induced activity (Fig. 1D, sre−/ar1c− versus SRE/AR1c1), whereas loss of each ERG3 SRE/AR1c site alone or together reduced, but did not abolish, lovastatin-induced ERG3 promoter activity (Fig. 1E, sre−/ ar1c−, SRE/AR1c1, SRE/AR1c2 versus SRE/AR1c1,2). A 40% reduction was observed using the ERG3 sre−/ar1c− construct.
when compared with the wild type ERG3 promoter-lacZ fusion. Importantly, promoter activities required the presence of Upc2/Ecm22, as induction was lost in upc2Δ ecms22Δ cells expressing various promoter-lacZ constructs (Fig. 1F). So, some, but not all, SRE/AR1c sites are functional.

To mimic the DNA topology of the chromosomal locus in the context of in vivo SRE/AR1c function, plasmids pRS416-ERG25 and pRS416-ERG3 were constructed, which contained a wild type or sre′/ar1c– promoter driving expression of its coding sequence. Plasmids were transformed into an ERG25 promoter-disrupted haploid strain (ERG25::erg25::HIS3) or an erg3::kan′ null strain, respectively. The loss of ERG25 is lethal (50). The erg25::erg25::HIS3 haploid strain lacks endogenous ERG25 promoter activity but is viable due to plasmid-driven ERG25 expression. Lovastatin-induced mRNA expression was quantitated using Northern analysis.

Lovastatin-induced ERG25 promoter activity was seen in cells containing or lacking all three SRE/AR1c sites (Fig. 2A). The loss of ERG25 promoter activity required the presence of all SRE/AR1c elements. The plasmid constructs were also transformed into an upc2Δ ecms22Δ null strain. Promoter activity was assayed in the absence (−) or presence of lovastatin (+). B, pRS-ERG3 plasmid construct that includes the ERG3 cds was transformed into an erg3::kan′ haploid strain. The pRS-ERG3 plasmid contained (+) or lacked (−) all three SRE/AR1c elements. The plasmid constructs were also transformed into an erg3::kan′ haploid strain. Promoter activity was assayed for in the absence (−) or presence of lovastatin (+). rRNA was used as a loading control. Numbers represent relative densitometry units divided by the densitometry of rRNA. The numbers are compared with the cells carrying the plasmid in the absence of lovastatin (Lov). The values are the averages of three independent experiments. Adjacent lanes are from the same gel (example, lanes 1 and 2, same gel; lanes 3 and 4, same gel, etc.).

Further strengthen the idea that not all consenus SRE/AR1c sites are functional, as a plasmid-driven promoter lacking these elements still drives lovastatin-induced gene expression. The results also indicate that novel promoter elements function to regulate ERG gene expression.

ERG3 and ERG25 Promoters Contain AR1b Sites—ERG3 and ERG25 promoter searches revealed the presence of novel variant SRE sites, all containing an identical single nucleotide change; the variant SRE sequence is TAAACGA rather than the consenss SRE/AR1c site sequence, TATAACG, and is identical to the anaerobic response element AR1b (15, 16). We refer to this element as AR1b, for the remainder of this study.

To determine whether these AR1b sites were functional, promoter lacZ-fusion assays were used. AR1b sites were assayed in the absence or presence of SRE/AR1c sites.
However, deleting all six resulted in an almost total loss of activity (Fig. 3A, sre’/ar1c ar1b versus SRE/AR1c AR1b). In the case of ERG3, deletion of the two SRE/AR1c or three AR1b elements alone significantly reduced promoter activity (Fig. 3B, SRE/AR1c ar1b, sre’/ar1c ar1b versus SRE/AR1c AR1b). Residual activity was abolished when both elements were deleted together (Fig. 3B, sre’/ar1c ar1b versus SRE/AR1c AR1b). Importantly, promoter activities required ScUpc2/ScEcm22 function (data not shown). Thus, AR1b sites in the ERG3/25 promoters harbor lovastatin-induced activity.

Not All SRE/AR1 Elements Drive Lovastatin-induced Expression—Pah1 catalyzes the conversion of phosphatidic acid to diacylglycerol (48, 49). The PAH1 gene promoter contains a single AR1b site that was tested for lovastatin-induced activity. PAH1 mRNA expression was determined using qRT-PCR, and protein level was determined by Western analysis, and phosphatidic acid phosphatase activity was determined using radiolabeled enzymatic assays. Studies were performed in wild type cells grown in the absence or presence of lovastatin.

No change in PAH1 mRNA expression was observed in the presence of lovastatin when compared with untreated cells (Fig. 4A, black bars versus white bars). This was in contrast to an increase in ERG25 expression upon drug treatment. Erg25 protein level also increased (data not shown), whereas the Pah1 protein level and phosphatidic acid phosphatase enzymatic activity remained constant (Fig. 4, B and C). Under our assay conditions, we cannot tell if the App1, Dpp1, and Lpp1 phosphatases contribute to the phosphatidic acid phosphatase activity seen (43, 45, 51). However, their contribution would be minimal, as no statistical differences were seen in mRNA expression, protein level, or activity, in the absence or presence of lovastatin.

The LCB1 promoter contains a single consensus SRE/AR1c, although the LCB2 promoter contains two. These elements were found to be nonfunctional based on LCB1/LCB2-lacZ.
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promoter fusion assays and Northern analysis (data not shown). Thus, not all SRE/AR1c/AR1b elements drive lovastatin-induced promoter activity.

Two UPC2 AR1b Promoter Sites Drive Lovastatin-induced UPC2 Expression—UPC2 gene expression is induced when sterol biosynthesis is blocked (25). Both ScUpc2 and ScEcm22 are required for lovastatin-induced expression. The UPC2 promoter contains two previously uncharacterized AR1b sites (Table 2). These sites were assayed for promoter activity using UPC2 promoter-lacZ assays. Cells were treated with lovastatin.

Lovastatin-induced activity was severely reduced by deletion of either AR1b site alone when compared with the wild type UPC2 promoter (Fig. 3C, ar1b−/−585 to −579, ar1b−/−359 to −353 versus AR1b). All residual activity was lost when both AR1b sites were deleted together (Fig. 3C, ar1b−/−585 to −579 and ar1b−/−359 to −353 versus AR1b).

Deletion of Endogenous SRE/AR1b, or AR1b Sites Causes Defects in in Vivo Lovastatin-induced Aerobic Gene Expression—To definitively show that AR1b sites drive in vivo gene expression, strains were constructed harboring endogenous ERG promoters lacking either SRE/AR1c/AR1b sites alone or in combination. qRT-PCR analysis was used to quantify gene expression in the absence and presence of lovastatin.

The single consensus SRE/AR1b site in the ERG1 promoter was first tested. When this site was endogenously deleted, >75% of the lovastatin-induced activity was lost, when compared with the endogenous wild type ERG1 promoter (Fig. 5A, sre−/−ar1c− versus AR1b).

The endogenous ERG3 promoter SRE/AR1c and AR1b sites were next assayed. Promoter activity was induced in the presence of lovastatin when both SRE/AR1c and AR1b sites were present (Fig. 5B, SRE/AR1c/AR1b). Loss of the AR1b sites resulted in the near total loss of induction, although loss of SRE/AR1c sites reduced activity by ~50% (SRE/AR1c/AR1b versus sre−/−ar1c−/−AR1b). This result correlates well with the Northern analysis data (Fig. 2). The loss of both endogenous elements mimicked loss of AR1b alone (SRE/AR1c/AR1b versus sre−/−ar1c−/−ar1b−/−).

Finally, the activities of the three ERG25 SRE/AR1c and AR1b promoter sites were determined. Deleting the three SRE/AR1c or AR1b sites alone almost completely abolished lovastatin-induced activity (Fig. 5C, SRE/AR1c/AR1b versus sre−/−ar1c−/−AR1b). Induced gene expression due to drug treatment required ScUpc2/ScEcm22 (data not shown). Thus, SRE/AR1c and AR1b sites function within the context of their endogenous promoters.

Deletion of the AR1b Sites in the UPC2 Promoter Results in Defects in Lovastatin-induced Aerobic Gene Expression—If the AR1b sites in the UPC2 promoter harbor endogenous activity, then they should drive lovastatin-induced expression of ERG genes in trans, through cis up-regulation of UPC2 expression and protein. To test this hypothesis, lovastatin-induced UPC2 and ERG1/3/25 expressions were determined in ecm22 null cells harboring a UPC2 locus-specific integrated wild type or mutant UPC2 promoter lacking AR1b sites. These strains lack Ecm22 protein and contain only the level of Upc2 expressed due to endogenous UPC2 promoter activity.

The wild type UPC2 promoter induced UPC2 expression in the presence of lovastatin, although the mutant promoter could not (Fig. 6A, AR1μ1/AR1μ2 versus ar1μ1/1 ar1μ2). The mutant promoter lacked the ability to induce UPC2 protein levels in the presence of the drug, when compared with the UPC2 wild type promoter, which is a critical event required for maintaining normal antifungal drug sensitivity (Fig. 6B, AR1μ1/AR1μ2 versus ar1μ1/1 ar1μ2). Moreover, lovastatin-induced ERG1/3/25 expression remained at basal levels in cells harboring a mutant promoter, although UPC2-dependent activity was observed in cells carrying a wild type promoter (Fig. 6C, WT versus mutant). Moreover, the loss of Upc2-driven ERG25 expression resulted in the loss of Erg25. Thus, the two AR1b sites in the UPC2 promoter were absolutely required for cis induction of UPC2.

3 C. Gallo-Ebert and J. T. Nickels, unpublished data.
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TABLE 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amphotericin</th>
<th>Terbinafine</th>
<th>Itraconazole</th>
<th>Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPC2 ECM22</td>
<td>0.13 ± 0.02*</td>
<td>100 ± 7.5</td>
<td>&gt;64 ± 8.3</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>upc2 ecm22</td>
<td>0.03 ± 0.01</td>
<td>6.3 ± 1.2</td>
<td>0.25 ± 0.04</td>
<td>8.6 ± 1.1</td>
</tr>
<tr>
<td>upc2&lt;sup&gt;ar1b−&lt;/sup&gt; ecm22</td>
<td>0.08 ± 0.01</td>
<td>12.5 ± 0.7</td>
<td>1.4 ± 0.4</td>
<td>12.7 ± 1.3</td>
</tr>
</tbody>
</table>

* Measured in micrograms/ml.

sense, as the upc2<sup>ar1b−</sup> ecm22 strain does have a basal level of Upc2. The results indicate that the two AR1<sub>b</sub> elements have a critical role in mounting a response to antifungal drug treatment, as strains lacking these elements show a higher susceptibility to several antifungals.

Pathogen and Nonpathogenic Upc2 Directly Bind to SRE/AR1<sub>c</sub> and AR1<sub>b</sub> Sites—To determine whether there was a direct physical interaction between Upc2 and SRE/AR1<sub>c</sub>/AR1<sub>b</sub> sites, an AlphaScreen<sup>®</sup> assay (see “Experimental Procedures”) was developed to examine the direct interaction between recombinant expressed Upc2 and SRE/AR1<sub>c</sub> or AR1<sub>b</sub> sites. Upc2s from pathogenic fungi (C. albicans and C. glabrata) as well as S. cerevisiae Upc2 were tested for binding. All Upc2 orthologs were highly conserved, and their binding to these elements strengthened the argument that Upc2 from multiple common pathogenic fungal species can bind to SRE/AR1<sub>c</sub>/AR1<sub>b</sub> sites (23).

Upc2 protein binding to SRE/AR1<sub>c</sub> and AR1<sub>b</sub> sites was first tested. A set concentration of 50 nM of each element was used, and Upc2 protein concentration was varied. A dose-dependent increase in binding to each element was observed with increasing concentrations of each Upc2 tested (Fig. 7, A–C, open and filled circles). Relative K<sub>m</sub> values were determined to be 1, 0.5, and 25 nM for ScUpc2, CaUpc2, and CgUpc2, respectively. In contrast, no binding was observed to a mutated promoter (TCAGATAA) (Fig. 7, A–C, filled diamonds).

Competition studies using “cold” SRE/AR1<sub>c</sub> or AR1<sub>b</sub> sites were next performed (no beads attached). In all cases, the respective cold element competed with its identically labeled SRE (Fig. 7, D–F, open and filled circles). Relative IC<sub>50</sub> values were determined for ScUpc2, CaUpc2, and CgUpc2, and all were in the low nanomolar range. In all cases, the mutated SRE was incapable of competing with the SRE/AR1<sub>c</sub> or AR1<sub>b</sub> sites for Upc2 binding (Fig. 7, D–F, filled diamonds). Thus, pathogenic and nonpathogenic Upc2 have similar binding affinities for both elements. These results indicate that fungal Upc2 proteins can directly bind to either element. The binding is highly specific for specific nucleotide sequences, as no binding was observed to a mutated SRE.

Endogenous Upc2 Binds AR1<sub>b</sub> Elements—Finally, the extent of ScUpc2 binding to endogenous UPC2/ERG3/ERG25 AR1<sub>b</sub> elements was determined using ChIP (Fig. 8). A wild type strain expressing an endogenous C-terminal Myc-tagged Upc2 was used to perform the ChIP analysis. Upc2-Myc binding was determined in the absence and presence of lovastatin.

UPC2 AR1<sub>b</sub> Sites Are Required for Maintaining Normal Susceptibility to Antifungal Drugs—If the UPC2 AR1<sub>b</sub> elements are critical for responding normally to antifungal assault, their loss should increase drug susceptibility. To test this hypothesis, a upc2<sup>Δ</sup> ecm22 strain harboring an endogenous mutated UPC2 promoter (upc2<sup>ar1b−</sup>) was tested for susceptibility to several antifungal drugs; the mutated strain contains a basal level of Upc2, but it cannot induce Upc2 upon drug treatment. Wild type, upc2 ecm22, and upc2<sup>ar1b−</sup> strains were tested for growth against amphotericin B (ergosterol-binding agent), terbinafine, itraconazole (targets the Erg11, lanosterol 14α-demethylase), and lovastatin (targets the Hmg1, HMG-CoA reductase). IC<sub>50</sub> values were determined after 24 h of treatment.

The upc2<sup>ar1b−</sup> ecm22 cells had increased susceptibility to all antifungals tested, when compared with values obtained for wild type cells (Table 3). The strain was 1.6-fold more sensitive to amphotericin B, 8-fold more sensitive to terbinafine, >45-fold more sensitive to itraconazole, and ~5.0-fold more sensitive to lovastatin. These values were somewhere in between those seen for wild type and upc2 ecm22 strains. This makes
the ERG25 promoter (Fig. 9, ERG25, AR1b, 1, 2, and 3; black bar versus white bar). The highest degree of binding was seen for the ERG25 AR1b,1 element. Based on the results as a whole, the direct binding of Upc2 to AR1b elements in the UPC2 promoter results in the cis up-regulation of UPC2 and the trans induction of multiple ERG genes, events that are required for inducing sterol biosynthesis in response to blocks in sterol metabolism caused by antifungal treatment.

**DISCUSSION**

The work presented establishes a novel function for the anaerobic AR1b promoter element (11, 15, 16, 25, 53); it regulates ERG gene expression in response to blocks in ergosterol biosynthesis caused by antifungals, through regulating UPC2 gene expression. Evidence is presented for the first time showing that SRE/AR1c elements function in vivo to regulate ERG gene expression. Moreover, this is the first report thoroughly examining SRE/AR1c/AR1b elements in the context of their endogenous promoters. We demonstrated that not all SRE/AR1c/AR1b elements function in vivo, so only a subset of these promoter sites are critical for mounting an antifungal resistance response. Finally, we have shown that pathogenic CaUpc2 and CgUpc2 bind to AR1b elements in vitro and that ScUpc2 binds to Ar1b elements in vivo. Our results indicate that the UPC2 AR1b elements are critical for initiating a normal response to antifungal assault.

In mammals, SREBPs bind promoters containing sequences other than the consensus SRE (54–57). Alternative binding sites have been found in the rat farnesyl-diphosphate synthase promoter (58), the 3β-hydroxyysterol Δ(14)-reductase promoter (59), and the HMG-CoA reductase promoter (60), suggesting the existence of additional SRE-like elements. SREBPs also bind some E-box elements (61). We now show that the AR1b element is a functional binding site for Upc2 in response to antifungus-dependent blocks in ergosterol biosynthesis.

There are cases where promoter heterogeneity differentially regulates gene expression (62–64). Our data indicates AR1b elements work in concert with certain SRE/AR1c sequences to
Regulate ERG gene expression, all through directing Upc2/Ecm22 binding. Our AlphaScreen and ChIP data substantiate this hypothesis and have demonstrated similar ScUpc2 binding affinities for either element, which extends to pathogenic CaUpc2 and CgUpc2. This sets up the opportunity to use the AlphaScreen assay as a high throughput screening tool aimed at developing novel antifungals, as Upc2 is a fungus-specific transcription factor (23).

The 7-bp SRE/AR1c element found in many ERG genes has been shown to regulate sterol gene expression (11, 14–16, 25), and it was later found to be required for regulating DAN/TIR gene expression under anaerobic conditions (15, 16). In vitro studies have demonstrated a role for this element in lovastatin-induced transcriptional induction, through its acting as a binding site for Upc2 in the ERG2/3/10 promoters (11, 25). Our ERG3-lacZ and pRS416-ERG3 studies demonstrated that ERG3 SRE/AR1c sites were functional. A reduction in endogenous promoter activity was seen upon loss of these sites. However, most endogenous ERG3 promoter activity came from AR1b elements.

Leber et al. (13) identified an ERG1 promoter element consisting of two 6-bp direct repeats separated by 4 bp (AGCTCG-GCGAGCTCG). Its deletion eliminated sterol-dependent ERG1-lacZ activity (13). Our in vivo studies demonstrated that a SRE/AR1c promoter site upstream of this element was required for most but not all activity (~25% remaining). Thus, residual activity most likely comes from the repeat sequence, as it was present in the endogenous ERG1 promoter designed.

Kennedy et al. (10) showed that the heme activator protein transcription factor complex Hap1/2/3/4 (66, 67), the yeast activator protein transcription factor Yap1, and the phospholipid transcription factor complex Ino2/4 regulate ERG9 gene expression. Hap2/3/4 is a heterotrimeric complex that functions as a transcriptional activator (68). A single putative E-box (CANNTG) was also identified (10), which is the binding element for the INO2/4 heterodimeric trans-activator complex (69). Thus, ERG9 expression is regulated by multiple diverse factors, consistent with the idea that yeast sterol biosynthesis as a whole is highly regulated by multiple promoter elements that respond to different environmental signals.

Microarray studies have shown that lovastatin treatment caused an increase in the level of UPC2 transcription but not ECM22 (52). Davies et al. (25) demonstrated that ScUpc2 binding to trans SRE/AR1c promoter elements significantly increased following lovastatin treatment. Higher levels of ScUpc2 binding were seen at the ERG3 promoter upon a block in sterol biosynthesis, although ScEcm22 levels decreased with a concomitant decrease in ScEcm22 at the promoter. The binding of ScUpc2 to ERG3 AR1c sites was not tested. In addition, determining whether this had any effect on expression was not determined.

Here, we show that increased UPC2 gene expression is exclusively controlled by two AR1c elements. These elements drive the critical trans activation of ERG gene expression in response to antifungal treatment, where overexpression gives rise to antifungal resistance. The C. albicans UPC2 promoter contains two elements that are similar but not identical to the AR1b element (65). Whether these elements function in vivo to regulate ERG gene expression in response to antifungal assault still requires investigation. Studies investigating their function in the context of the endogenous promoter are needed before any significance can be attributed to these elements. Our results using S. cerevisiae suggest this will be the case, further validating the use of S. cerevisiae as an excellent model to study mechanisms leading to fungal pathogenicity (15, 16).

We have discovered that S. cerevisiae anaerobic AR1b elements in the UPC2 promoter act as binding sites for Upc2. We have demonstrated that an endogenous UPC2 promoter strain lacking these sites is unable to induce ERG gene expression in response to blocks in sterol biosynthesis, leading to hypersusceptibility to antifungal drug treatment. Moreover, we have shown that pathogenic Upc2 proteins can bind to the ScAR1b element. Based on our results and those of others, we conclude that the AR1c sites in the UPC2 promoter regulate global ERG gene expression. These elements regulate in vivo ERG gene expression in trans, an event that is critical for fungi to respond to blocks in sterol biosynthesis caused by antifungal agents.

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