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A Role for Sterol Levels in Oxygen Sensing in *Saccharomyces cerevisiae*

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ABSTRACT

Upc2p and Ecm22p are a pair of transcription factors responsible for the basal and induced expression of genes encoding enzymes of ergosterol biosynthesis in yeast (*ERG* genes). Upc2p plays a second role as a regulator of hypoxically expressed genes. Both sterols and heme depend upon molecular oxygen for their synthesis, and thus the levels of both have the potential to act as indicators of the oxygen environment of cells. Hap1p is a heme-dependent transcription factor that both Upc2 and Ecm22p depend upon for basal level expression of *ERG* genes. However, induction of both *ERG* genes and the hypoxically expressed *DAN/TIR* genes by Upc2p and Ecm22p occurred in response to sterol depletion rather than to heme depletion. Indeed, upon sterol depletion, Upc2p no longer required Hap1p to activate *ERG* genes. Mot3p, a broadly acting repressor/activator protein, was previously shown to repress *ERG* gene expression, but the mechanism was unclear. We established that Mot3p bound directly to Ecm22p and repressed Ecm22p- but not Upc2p-mediated gene induction.

TRANSCRIPTIONAL regulation of cholesterol synthesis in mammalian cells involves regulated proteolysis and the liberation of transcription factors from membrane tethers (Goldstein *et al.* 2002). A similar regulatory scheme, including SREBP and SCAP orthologs, is found in fission yeast (Hughes *et al.* 2005). Budding yeast lacks orthologs of the mammalian regulators. Regulation of ergosterol in *Saccharomyces cerevisiae* has both intrinsic and practical interest as this pathway contains the targets of most antifungal drugs. Genes encoding enzymes of ergosterol biosynthesis are transcriptionally regulated in response to the need for ergosterol (Arthington-Skaggs *et al.* 1996; Dimster-Denk and Rine 1996; Smith *et al.* 1996; Kennedy *et al.* 1999) but in ways that differ markedly from the regulation of the corresponding genes in mammals (Dimster-Denk and Rine 1996; Davies *et al.* 2005).

Upc2p and Ecm22p, two transcription factors with similar sequences, bind a sequence motif known as the sterol regulatory element (SRE) and regulate the ergosterol biosynthetic genes *ERG1*, *ERG2*, *ERG3*, *ERG7*, *ERG25*, *ERG26*, and *ERG27* (Vik and Rine 2001; Germann *et al.* 2005). Although binding of Upc2p and Ecm22p at other *ERG* genes has not been tested directly, the SRE binding site is found in many of these genes, suggesting that these two proteins are major regulators of ergosterol biosynthesis.

In addition to binding the promoters of *ERG* genes, Upc2p binds a similar sequence in the regulatory region of hypoxically induced mannoprotein-encoding genes known as the *DAN/TIR* genes (Abramova *et al.* 2001a,b). These genes are responsible for changes in the cell wall of yeast grown hypoxically. Upc2p has also been implicated in the uptake of sterols under hypoxic conditions (Lewis *et al.* 1988; Crowley *et al.* 1998; Wilcox *et al.* 2002; Alimardani *et al.* 2004). Normally, yeast cells take up sterols from their environment only under hypoxic conditions (Andreasen and Stier 1953), but an overactive allele of *UPC2*, *UPC2-1*, allows aerobic uptake of sterols (Lewis *et al.* 1988). Moreover, nearly one-third of hypoxically induced genes contain at least one potential Upc2p/Ecm22p binding site (Kwast *et al.* 2002). These observations suggest that Upc2p, and perhaps Ecm22p, are major factors in the adaptation to hypoxia.

Upc2p and Ecm22p share similar binuclear cluster DNA binding domains (70% identical in sequence) and similar activation/regulatory domains (76% identical in sequence), but Upc2p and Ecm22p function at different times. Ecm22p is more abundant at *ERG* promoters under normal laboratory growth conditions. However, when sterols are depleted, Ecm22p levels drop and Upc2p replaces Ecm22p at the promoters of *ERG* genes (Davies *et al.* 2005). Such an activator switch implies that there must be other factors that regulate the activation, abundance, and localization of Upc2p and Ecm22p, as well as conditions that favor the use of one activator over the other.

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TABLE 1

Strains used in this study

Strain	Genotype	Source or reference
W303-1a	*MAT***a** *ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100*	R. Rothstein
BY4742	*MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.* (1998)
JRY7179	W303-1a *upc2Δ::HIS3*	Vik and Rine (2001)
JRY7180	W303-1a *ecm22Δ::TRP1*	Vik and Rine (2001)
JRY7181	W303-1a *ecm22Δ::TRP1 upc2Δ::HIS3*	Vik and Rine (2001)
JRY7865	W303 *UPC2-TAP (TRP1)*	Davies *et al.* (2005)
JRY7866	W303 *ECM22-TAP (TRP1)*	Davies *et al.* (2005)
JRY8037	Resulting diploid of W303-1a and BY4742 cross	
JRY8038	W303-1a *hap1Δ::NatMX4*	
JRY8040	W303-1a *hap1Δ::NatMX4 upc2Δ::HIS3*	
JRY8042	W303-1a *hap1Δ::NatMX4 ecm22Δ::TRP1*	
JRY8044	W303-1a *mot3Δ::HghMX4*	
JRY8048	W303-1a *mot3Δ::HghMX4 upc2Δ::HIS3*	
JRY8050	W303-1a *mot3Δ::HghMX4 ecm22Δ::TRP1*	
JRY8056	W303-1a *mot3Δ::HghMX4 upc2Δ::HIS3 ecm22Δ::TRP1*	
JRY8062	W303-1a *hap1Δ::NatMX4 UPC2-TAP (TRP1)*	
JRY8068	W303-1a *hap1Δ::NatMX4 ECM22-TAP (TRP1)*	
JRY8072	W303-1a *mot3Δ::HghMX4 UPC2-TAP (TRP1)*	
JRY8076	W303-1a *mot3Δ::HghMX4 ECM22-TAP (TRP1)*	
JRY8082	W303-1a *HAP1-flag (KanMX4)*	
JRY8084	W303-1a *HAP1-flag (KanMX4) ECM22-TAP (TRP1)*	
JRY8085	W303-1a *MOT3-flag (KanMX4)*	
JRY8087	W303-1a *MOT3-flag (KanMX4) ECM22-TAP (TRP1)*	
JRY8088	W303-1a *UPC2-flag (KanMX4)*	
JRY8089	W303-1a *UPC2-flag (KanMX4) ECM22-TAP (TRP1)*	
JRY8090	W303-1a *ECM22-flag (KanMX4)*	

Unless otherwise indicated, all strains were generated for this study.

Other transcription factors also participate in the regulation of ergosterol biosynthesis genes. Hap1p, a heme-activated transcriptional regulator, regulates *HMG1* (Thorsness *et al.* 1989), the structural gene for 3-hydroxy-3-methylglutaryl CoA reductase. A *HAP1* mutant allele can cause lower expression in several ergosterol biosynthetic genes with a corresponding reduction in ergosterol levels (Tamura *et al.* 2004). Yer064Cp, a nuclear protein of unknown function, is important for the activation of some *ERG* genes (Kennedy *et al.* 1999; Germann *et al.* 2005). Finally, deletion of *MOT3*, a zinc-finger protein that can act as both a transcriptional activator and a repressor, leads to increased expression of *ERG2*, *ERG6*, and *ERG9* (Hongay *et al.* 2002).

In this study, we established the functions of Hap1p and Mot3p in regulating the gene targets of Upc2p and Ecm22p. In addition we tested the hypothesis that sterol, not heme, depletion was the signal responsible for hypoxic activation of Upc2p and Ecm22p and uncovered unanticipated complexity in how oxygen levels affect the expression of Upc2 and Ecm22p gene targets.

MATERIALS AND METHODS

Strains and media: Strains used in this study are listed in Table 1. All strains were isogenic with W303 except BY4741 and JR8037 (the W303/BY4741 diploid). Gene deletions were made by a PCR-based gene disruption method (Burke *et al.* 2000) such that the entire open reading frame (ORF) was replaced by the *NATMX4* (nourseothricin resistance) gene amplified from pAG25 or the *HPHMX4* (hygromycin B resistance) gene amplified from pAG32 (Goldstein and McCusker 1999). Sequences encoding the TAP-tag (Rigaut *et al.* 1999) or the FLAG-tag (Gelbart *et al.* 2001) were integrated in frame at the 3′ end of the ORFs using homologous recombination and one-step gene integration of PCR-amplified modules.

All yeast strains were grown in complete synthetic medium [0.67% Difco yeast nitrogen base without amino acids, complete supplemental mixture minus appropriate amino acids (Q-Biogene)] containing 2% glucose, except for the experiment presented in Figure 5, in which strains were grown in YPD. A 25 mg/ml stock solution of lovastatin (a generous gift from James Bergstrom) was prepared as previously described (Dimster-Denk *et al.* 1994). Lovastatin was added to liquid media to a final concentration of 30 μg/ml unless otherwise noted. Hemin (Sigma) was added from a 4 mg/ml stock (50% ethanol, 20 mm sodium hydroxide) to a final concentration of 40 μg/ml. Ketoconazole (Sigma) was added from a 10 mg/ml (DMSO) stock to a final concentration of 25 μg/ml. For hypoxic growth, cells were grown in tightly capped 50 ml flasks without shaking at 30°. Induction of *DAN1*, *TIR1*, *ANB1*, and *COX5b* in cells grown this way verified these conditions as hypoxic.

Plasmids: pJR2316 was a *pERG2:lacZ* reporter described previously (Vik and Rine 2001).

Genotyping of *HAP1* allele: Yeast spores were subjected to colony PCR with the primers bd194 (5′-GGAGCTGGAACTGCGAATAC-3′), bd195 (5′-CATTTGCGTCATCTTCTAACACCG-3′),

and bd196 (5′-CTCCCATATTGGAAAATCTGCTC-3′). In the presence of wild-type *HAP1*, bd194 and bd196 amplify a 250-bp product. In the presence of the S288C *HAP1* mutation, bd194 and bd195 amplify a 350-bp product.

β-Galactosidase assays: β-Galactosidase assays were performed essentially as previously described (Burke *et al.* 2000).

Analysis of protein levels: Strains were grown to midlog phase and whole-cell extracts were prepared as described previously (Foiani *et al.* 1994). Extracts were subjected to SDS–PAGE and immunoblotting. FLAG-tagged proteins were detected by immunoblotting with anti-flag (rabbit or mouse; Sigma). TAP-tagged proteins were immunoblotted with an anti-flag antibody (rabbit; Sigma) to detect the TAP tag. All immunoblots were also blotted with anti-3-phosphoglycerate kinase antibodies (Molecular Probes) as a loading control. Immunoblots were scanned using the Li-Cor Odyssey imaging system.

Chromatin immunoprecipitation: Chromatin immunoprecipitations were performed as described previously (Davies *et al.* 2005). For TAP-tagged proteins (Figures 2 and 4), immunoprecipitations were performed using 30 μl IgG sepharose (Amersham Biosciences). For Hap1-flag (Figure 2), immunoprecipitations were performed using 25 μl anti-flag M2-agarose (Sigma). Typically, IPs were performed overnight at 4°. DNA enrichment was analyzed by real-time PCR and Syber-Green fluorescence on a Stratagene MX3000 real-time PCR instrument. Real-time PCR was performed using primers to the *ERG3* promoter (5′-GACGCCTTTTGTTGCGATTGT CG-3′ and 5′-CAGCAACAACAATACCCGATCGC-3′) and to *ACT1* (5′-GGCATCATACCTTCTACAACGAATTG-3′ and 5′-CTACCGGAAGAGTACAAGGACAAAAC-3′) with DNA derived from whole-cell extracts as a standard.

Affinity purification using TAP-tagged proteins: Co-immunoprecipitations were performed essentially as described previously (Kobor *et al.* 2004), except that the DNAse-treated samples identified in Figure 5 were treated with DNAse as described previously (Watson *et al.* 2000). Samples were subjected to SDS–PAGE and immunoblotting with anti-flag antibody (Sigma). Immunoblots were scanned using the Li-Cor Odyssey imaging system.

Analysis of gene expression: Gene expression was measured using quantitative RT–PCR. RNA was prepared as described previously (Schmitt *et al.* 1990). RNA preps were digested with RNase-free DNase I (Roche and QIAGEN) and cDNA was synthesized using SuperScript III first-strand synthesis system for RT–PCR (Invitrogen) and oligo(dT). cDNA was analyzed by real-time PCR and Syber-Green fluorescence on a Stratagene MX3000 real-time PCR instrument. Samples were analyzed in triplicate for each of two independent RNA preparations. cDNA from *ACT1* was used as a loading control. Primer sequences are listed in supplemental Table 1 at http://www.genetics.org/supplemental/.

RESULTS

In this study we explored the mechanism by which the Upc2p and Ecm22p transcription factors regulate *ERG* genes. This issue has been complicated by the apparently conflicting results from different studies, by influences of additional regulators whose function is not known, and by ambiguity as to the physiological signal(s) that regulate the activity of Upc2 and Ecm22p. We first establish the genetic basis for some, if not most, of the conflicting data and then move on to clarify the remaining issues.

HAP1—the genetic basis of strain differences in *ERG2* expression: To find proteins that, in conjunction with Upc2p and Ecm22p, contribute to the regulation of *ERG* genes, a genetic screen was designed using a *pERG2::lacZ* reporter plasmid (pJR2316) and the yeast deletion collection (Research Genetics). The plan was to transform the knockout collection with the reporter plasmid and screen for either increased or decreased *ERG2* expression compared with the parent strain. However, when *ERG2* expression was tested in BY4742, the parent strain of the *MAT*α deletion collection, we found that expression was fivefold lower than what had been observed previously in W303-derived strains (Vik and Rine 2001; Davies *et al.* 2005). A hybrid diploid formed between the two strains exhibited an intermediate level of *ERG2::lacZ* expression (Figure 1A).

The difference in *ERG2* expression between W303 and BY4742 could have been due to variation at a single locus or the composite effect of strain differences at multiple loci. If the differences in *ERG2* expression were the result of a single Mendelian locus, high and low *ERG2* expression levels would be expected to segregate 2:2 when these two strains were crossed and sporulated. Therefore, the W303/BY4742 diploid was sporulated and the resulting tetrads tested for *ERG2* expression. For each of four tetrads, two spores had high *ERG2* expression, similar to that observed in W303, and two had low *ERG2* expression, similar to that observed in BY4742 (data not shown), indicating that the difference in *ERG2* expression mapped to a single locus.

Prior to initiating mapping of the locus responsible for this difference, we tested whether known differences between these two strains could account for the difference in *ERG2* expression. Strains derived from S288c (such as BY4742) have mutant forms of several genes including a mutant allele of *HAP1*, which encodes a heme-responsive transcriptional regulator (Gaisne *et al.* 1999). This mutation, caused by an insertion near the 3′ end of the *HAP1* ORF, has a detrimental effect on some, but not all, Hap1p targets (Gaisne *et al.* 1999). Several observations suggested that *HAP1* may be the basis of the difference in *ERG2* expression in these two strains: Deletion of both *UPC2* and *ECM22* results in lethality in S288C (Shianna *et al.* 2001) but not in W303 (Vik and Rine 2001). The lethality of the *upc2 ecm22* double null mutation in S288c appears to result from the *hap1* mutant allele, as restoration of the wild-type *HAP1* restores viability (M. Valachovic, personal communication). Moreover, deletion of *UPC2*, *ECM22*, and *HAP1* results in lethality in W303 (data not shown). The haploid progeny from the W303/BY4742 diploid were therefore tested for the S288c allele of *HAP1*. In every case tested, spores with low *ERG2* expression had the S288c-derived mutant allele of *HAP1* and spores with high expression had the W303-derived allele, indicating that low *ERG2* expression was a result of the mutant allele of *HAP1* (data not shown).

Upc2p and Ecm22p had different requirements for Hap1p: To examine the role of Hap1p in the regulation

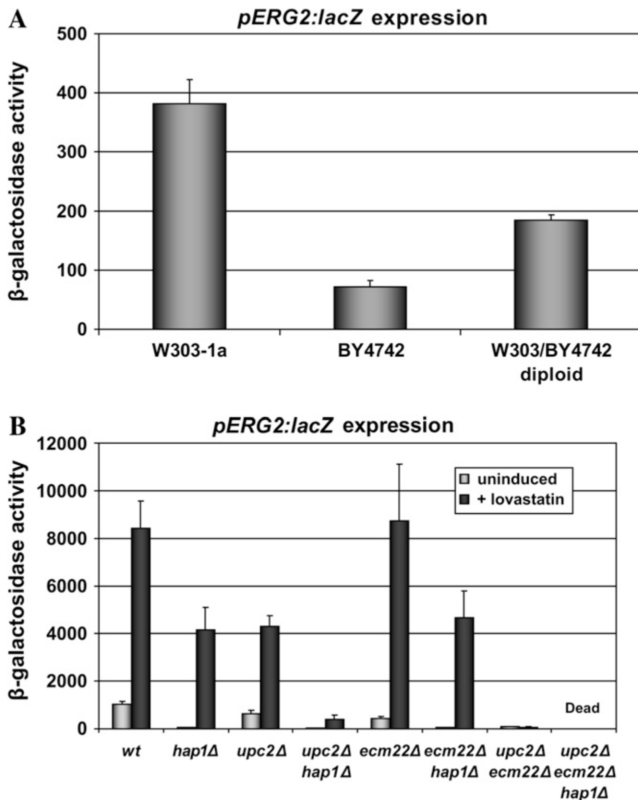

FIGURE 1.—(A) *ERG2* expression was impaired in BY4742, a parent strain for the deletion collection. W303-1a, BY4742, and the diploid strain resulting from the cross of the two (JRY8037) were transformed with a *pERG2::lacZ* reporter (pJR2316). Extracts from these strains were assayed for β-galactosidase activity as described in MATERIALS AND METHODS. (B) Uninduced *ERG2* expression depended on Hap1p, as did Ecm22p-mediated *ERG2* induction following sterol depletion. Wild-type W303, *hap1*Δ (JRY8038), *upc2*Δ (JRY7179), *upc2*Δ*hap1*Δ (JRY8040), *ecm22*Δ (JRY7180), *ecm22*Δ*hap1*Δ (JRY8042), and *upc2*Δ*ecm22*Δ (JRY7181) strains were transformed with a *pERG2::lacZ* reporter (pJR2316). Strains were grown in the presence or absence of 30 μg/ml lovastatin. Extracts prepared from these cultures were assayed for β-galactosidase activity as described in MATERIALS AND METHODS.

of *ERG* genes, especially with respect to Upc2p and Ecm22p, *hap1*Δ (JRY8038), *hap1*Δ*upc2*Δ (JRY8040), and *hap1*Δ*ecm22*Δ (JRY8042) derivatives of W303 were tested for *ERG2* expression in both the presence and the absence of lovastatin, an inhibitor of HMG-CoA reductase, which catalyzes an early step in sterol biosynthesis. Thus the presence or absence of lovastatin corresponds to inducing and noninducing conditions, respectively. Under noninducing conditions, *ERG2* expression was profoundly reduced in all strains lacking *HAP1* (Figure 1B, shaded bars). Thus, Hap1p was required for the basal expression of *ERG2*, regardless of whether that expression was activated by Ecm22p or Upc2p.

Unlike the *upc2*Δ*ecm22*Δ double mutant strain, however, a strain without *HAP1* was able to induce *ERG2* expression when sterols were depleted by growth in the presence of lovastatin, albeit to a lower level (Figure 1B, solid bars). Induction in the absence of *HAP1* depended mostly on Upc2p, as very little induction was observed in a strain when both *UPC2* and *HAP1* were deleted. Thus, upon inducing conditions, Upc2p could activate *ERG2* expression in a Hap1-independent manner, whereas Ecm22p was a Hap1p-dependent activator of *ERG2* expression.

Hap1p acted at *ERG* promoters: There are several possible ways that Hap1p could regulate Ecm22p and, to a lesser extent, Upc2p at *ERG* promoters. Hap1p might modulate *ERG* gene expression indirectly either by regulating transcription of *UPC2* and *ECM22* or by regulating the stability of Upc2p and Ecm22p. Alternatively, Hap1p might act more directly to influence either the binding of Upc2p and Ecm22p at *ERG* promoters or the activity of these two proteins once bound. To distinguish among these possibilities, the levels of Upc2p and Ecm22p were measured in a *hap1*Δ strain grown in the presence or absence of lovastatin. Neither Upc2p nor Ecm22p levels were affected by the deletion of *HAP1*, under inducing or noninducing conditions (Figure 2A). Similarly, Hap1p levels were unaffected by lovastatin treatment (data not shown). The increase in Upc2p and decrease in Ecm22p evident upon induction were described previously (DAVIES *et al.* 2005). These data argued for a direct mechanism of Hap1 function.

To determine if Hap1p acted directly on *ERG* gene promoters, a flag-tagged version of Hap1p was used for chromatin-immunoprecipitation experiments. The tagged version of Hap1p complemented the *hap1* null mutation with respect to *ERG2* expression (data not shown). As chromatin immunoprecipitation of *ERG2*, with its single SRE, has proven difficult (data not shown) and regulation of *ERG3* parallels regulation of *ERG2* (VIK and RINE 2001), Hap1p and Ecm22p were tested for their enrichment at the *ERG3* promoter, which contains five SREs. Hap1p was enriched at the *ERG3* promoter and to approximately the same extent under inducing and noninducing conditions (Figure 2B). Although Hap1p was at the *ERG3* promoter, deletion of *HAP1* had no effect on the presence of Ecm22p at the same promoter in cells grown in either the presence or the absence of lovastatin (Figure 2C). Thus, Hap1p influenced the activity, but not the presence, of Ecm22p at *ERG* promoters.

Mot3p inhibited Ecm22p: Mot3p, a dose-dependent repressor/activator, represses expression of several *ERG* genes including *ERG2* (HONGAY *et al.* 2002), although the mechanism was unknown. To determine whether Mot3p acted through Upc2p, Ecm22p, or by some independent mechanism, *mot3*Δ (JRY8044), *mot3*Δ*upc2*Δ (JRY8048), *mot3*Δ*ecm22*Δ (JRY8050), and *mot3*Δ*upc2*Δ-*ecm22*Δ (JRY8056) deletion strains were constructed and tested for *ERG2* expression. As reported by HONGAY *et al.* (2002), the deletion of *MOT3* led to an increase in

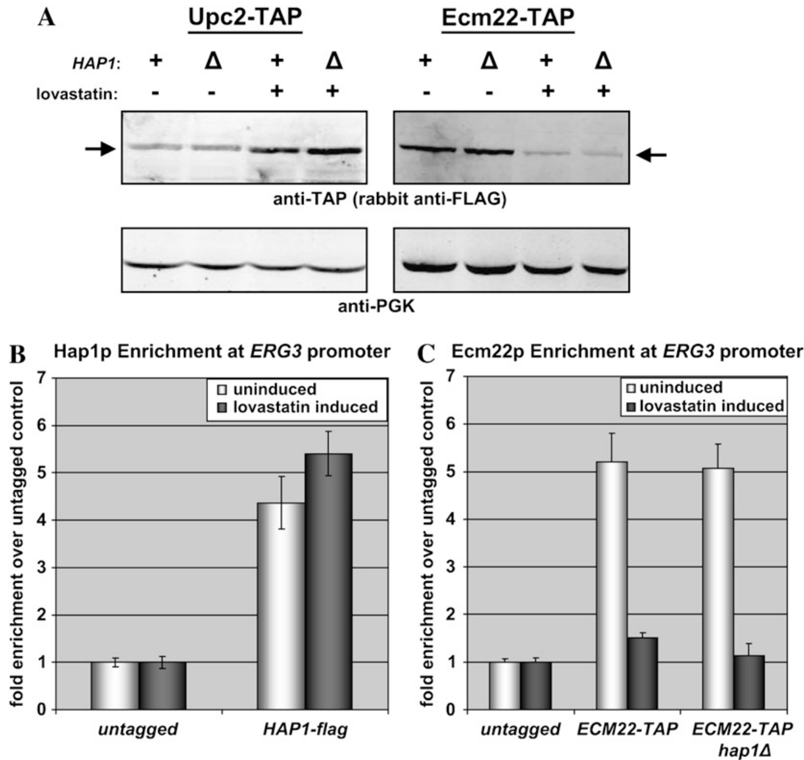

FIGURE 2.—(A) Levels of Upc2p and Ecm22p were unaffected by deletion of *HAP1*. Whole-cell extracts were prepared from JRY7865 (*UPC2-TAP*), JRY8062 (*UPC2-TAP hap1*Δ), JRY7866 (*ECM22-TAP*), and JRY8068 (*ECM22-TAP hap1*Δ) grown in the presence or absence of 30 μg/ml lovastatin. Protein levels were analyzed by immunoblotting as described in MATERIALS AND METHODS. Arrows indicate tagged proteins. (B). Hap1 was enriched at the *ERG3* promoter. Chromatin immunoprecipitations were performed using untagged and *HAP1*-flag tagged strains as described in MATERIALS AND METHODS. Real-time PCR was used to quantify the levels of *ERG3* promoter DNA and *ACT1* control DNA. The ratio of *ERG3* promoter DNA to *ACT1* DNA was calculated for each sample. Bars are the average of three reactions and indicated the fold enrichment of Hap1-flag experiments over the untagged control. (C) Deletion of *HAP1* has no affect on the levels of Ecm22p at the *ERG3* promoter. Chromatin immunoprecipitations were performed using untagged and *ECM22-TAP* tagged strains as described in MATERIALS AND METHODS. Experiments were analyzed using real-time PCR as described in B.

basal *ERG2* expression (Figure 3, first two shaded bars). The increase in *ERG2* expression depended primarily on Ecm22p, as the deletion of *MOT3* had no significant effect on *ERG2* activation by Upc2p (Figure 3). The activation of *ERG2* by Ecm22p was increased in a *mot3*Δ strain in both inducing and noninducing conditions (Figure 3, compare *upc2*Δ to *upc2*Δ*mot3*Δ). Thus, Mot3p repressed Ecm22p-dependent expression of *ERG2* but not Upc2-dependent expression. It is important to note that induction of *ERG2* by Ecm22p in response to sterol depletion was not due simply to relief of Mot3p repression, as considerable *ERG2* induction was still evident in a *mot3* mutant.

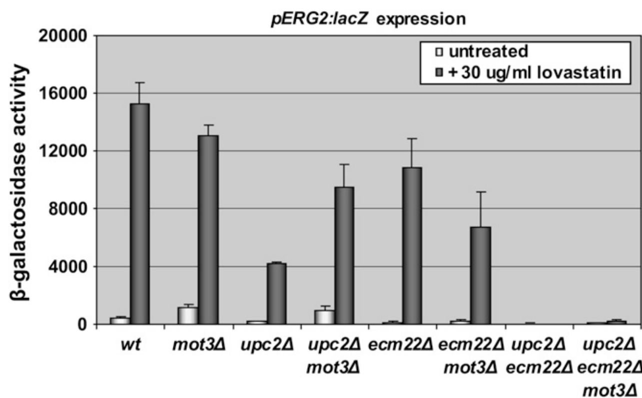

FIGURE 3.—Mot3p inhibited Ecm22p. Wild-type W303, *mot3*Δ (JRY8044), *upc2*Δ (JRY7179), *upc2*Δ*mot3*Δ (JRY8048), *ecm22*Δ (JRY7180), *ecm22*Δ*mot3*Δ (JRY8050), *upc2*Δ*ecm22*Δ (JRY7181), and *upc2*Δ*ecm22*Δ*mot3*Δ (JRY8056) strains were transformed with a *pERG2::lacZ* reporter (pJR2316). Strains were grown in the presence or absence of 30 μg/ml lovastatin. Extracts prepared from these cultures were assayed for β-galactosidase activity as described in MATERIALS AND METHODS.

Upon sterol depletion, reduction of Ecm22p levels was partially blocked in a *mot3*Δ strain: In cells treated with lovastatin to deplete sterols, the level of Ecm22p decreases to about one-third the level observed in noninduced cells, whereas the level of Upc2p increases about fourfold (DAVIES *et al.* 2005). To determine whether the deletion of *MOT3* affected Ecm22p or Upc2p levels, Ecm22p-TAP and Upc2p-TAP levels were measured in a *mot3*Δ strain. Deletion of *MOT3* had little, if any, effect on Upc2p levels. However, upon sterol depletion, the level of Ecm22p was approximately twofold higher in *mot3*Δ strains (Figure 4A), which corresponded quantitatively to the twofold greater *ERG2* induction by Ecm22p in *mot3*Δ strains (Figure 3). A similar pattern was observed when the enrichment of Ecm22p at the *ERG3* promoter was tested using chromatin immunoprecipitation in a *mot3*Δ strain (Figure 4B). Thus, Mot3p caused at least part of the reduction in overall Ecm22p levels under inducing conditions, and at least part of the reduction in SRE occupancy at the *ERG3* promoter.

Mot3p interacted directly with Ecm22p: To test whether Mot3p affects Ecm22p by direct physical interaction, TAP-tagged Ecm22p was immunoprecipitated from cells with FLAG-tagged Hap1p, Mot3p, or Upc2p and then immunoblotted with an anti-FLAG antibody. These experiments revealed a strong and specific interaction between Ecm22p and Mot3p (Figure 5A).

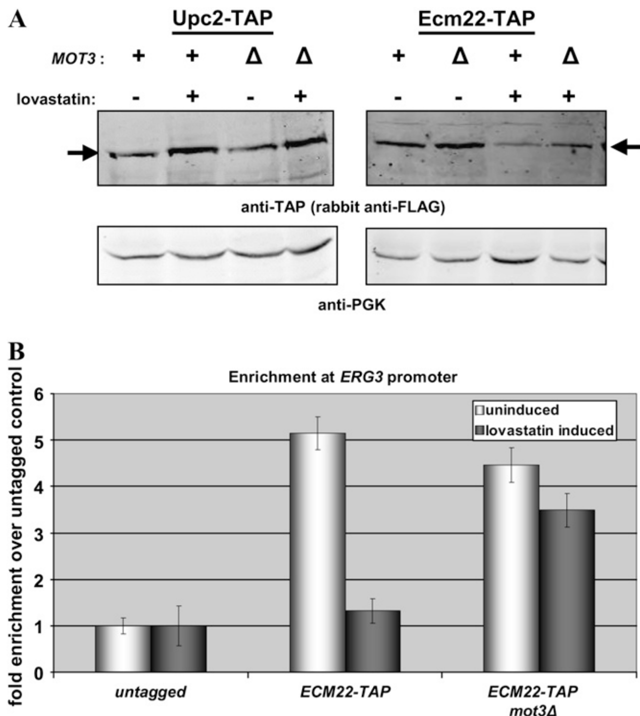

Figure 4.—(A) Deletion of *MOT3* does not significantly affect Upc2p levels, but does result in higher Ecm22p levels following sterol depletion. Whole-cell extracts were prepared from JRY7865 (*UPC2-TAP*), JRY8072 (*UPC2-TAP mot3*Δ), JRY7866 (*ECM22-TAP*), and JRY8076 (*ECM22-TAP mot3*Δ) grown in the presence or absence of 30 μg/ml lovastatin. Protein levels were analyzed by immunoblotting as described in materials and methods. Ecm22p levels were quantified using the Li-Cor Odyssey imaging system and are normalized to the level of Ecm22p in uninduced wild type. Arrows indicate tagged proteins. (B) Deletion of *MOT3* led to increased promoter occupancy of Ecm22p following sterol depletion. Chromatin immunoprecipitations were performed using untagged, *ECM22-TAP* tagged, and *ECM22-TAP mot3*Δ strains as described in materials and methods. Real-time PCR was used to quantify the levels of *ERG3* promoter DNA and *ACT1* control DNA. The ratio of *ERG3* promoter DNA to *ACT1* DNA was calculated for each sample. Bars are the average of three reactions and indicate the fold enrichment of Ecm22p-TAP experiments over the untagged control.

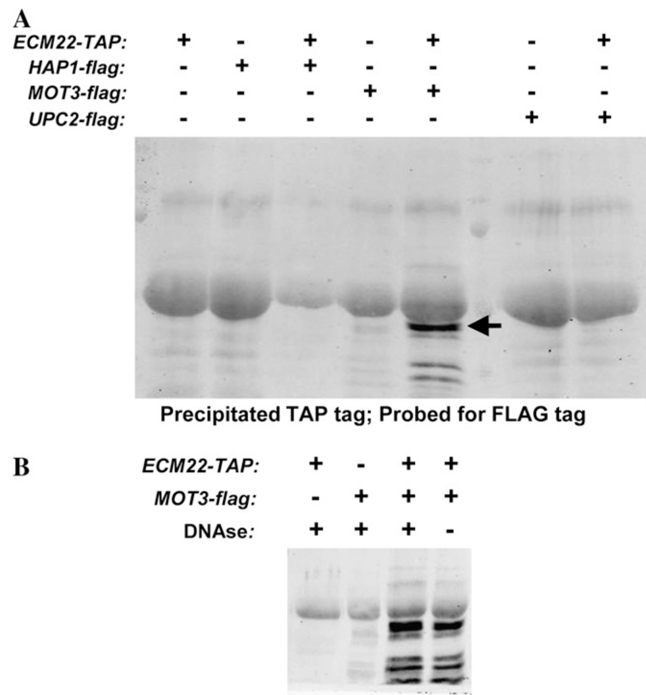

Figure 5.—Mot3p physically interacted with Ecm22p. Strains containing the indicated tagged proteins were precipitated with IgG beads to pull down TAP-tagged Ecm22p as outlined in materials and methods. The precipitate was then subjected to immunoblotting with anti-flag antibody. (A) Arrow indicated MOT3-flag specific band. (B) The indicated samples were treated with DNAse prior to precipitation with IgG.

Adding a DNAse digestion prior to precipitation had no effect on this interaction, thus precluding a DNA bridge between two proteins binding at the same promoter as an explanation (Figure 5B). It should be noted that the observed band did not correspond to full-length Mot3p, but did correspond to the major *MOT3-flag*-specific band observed in whole-cell extracts (data not shown). Thus a major mechanism for the repression of *ERG* gene expression by Mot3p was by direct binding to the Ecm22p activator.

Testing the role of heme A in *ERG* gene expression: As lovastatin inhibits an early step in ergosterol biosynthesis, it reduces not only the synthesis of ergosterol, but also the synthesis of certain nonsterol products, such as heme A. Heme A, a farnesylated version of heme, is an essential prosthetic group of cytochrome C oxidase (Moraes *et al.* 2004). Because cytochrome C oxidase is thought to play a role in oxygen sensing and the activation of some hypoxically expressed genes (Kwast *et al.* 1999), in principle, depletion of heme A by lovastatin might result in a hypoxic response. Thus, depletion of heme A, rather than sterol depletion *per se*, might be responsible for the switch from Ecm22p to Upc2p at *ERG* promoters under inducing conditions. Such a model would be consistent with Upc2p's role in activating the hypoxically expressed *DAN/TIR* genes if they too are keyed into hypoxia through a cytochrome C oxidase-mediated signal (Abramova *et al.* 2001a,b). The importance of Hap1p, a heme-activated transcription factor, in the activation of *ERG* genes again suggested that heme levels may have a prominent role in the regulation of Upc2p and Ecm22p.

If the activation of either Upc2p or Ecm22p were the result of reduced heme A, rather than the result of sterol depletion, depleting sterols without lowering heme A levels would not be expected to induce *ERG* gene expression through that transcription factor. To test this hypothesis directly, ketoconazole was used to deplete sterols. Ketoconazole inhibits Erg11p, an ergosterol biosynthetic enzyme that acts in the sterol-specific part of the pathway, downstream of the point from which nonsterol products of the pathway, such as heme A, are derived. By treating with ketoconazole, sterols can

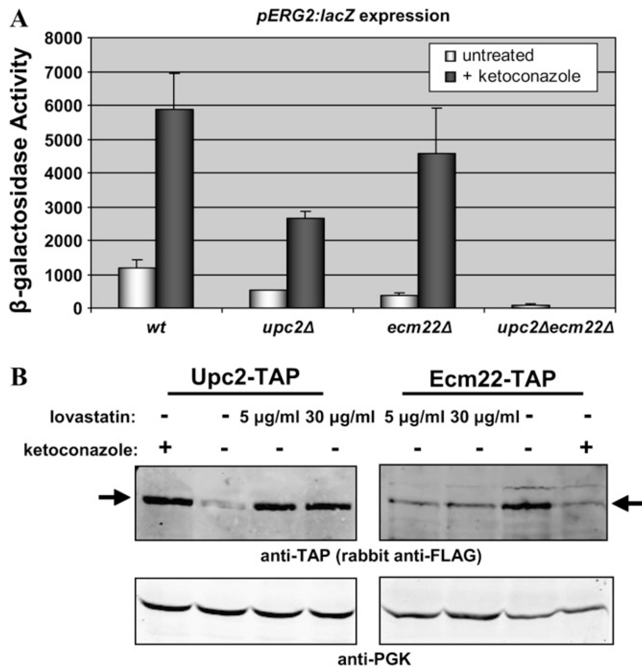

FIGURE 6.—(A) Ketoconazole induced *ERG2* expression in wild-type and *upc2*Δ and *ecm22*Δ single deletion strains. Wild-type W303, *upc2*Δ (JRY7179), *ecm22*Δ (JRY7180), and *upc2*Δ*ecm22*Δ (JRY7181) strains were transformed with a *pERG2::lacZ* reporter (pJR2316). Strains were grown in the presence or absence of 25 μg/ml ketoconazole. Extracts prepared from these cultures were assayed for β-galactosidase activity as described in MATERIALS AND METHODS. (B) Upon sterol depletion, the level of Upc2p increased and the level of Ecm22p decreased. Whole-cell extracts were prepared from JRY7865 (*UPC2-TAP*) and JRY7866 (*ECM22-TAP*) grown in the presence of lovastatin (5 μg/ml or 30 μg/ml), ketoconazole (25 μg/ml), or no drugs. Protein levels were analyzed by immunoblotting as described in MATERIALS AND METHODS. Arrows indicate tagged proteins.

be depleted without depleting the nonsterol products of the pathway. If changes in Upc2p and Ecm22p activity or levels were indeed the result of heme depletion and not sterol depletion, following ketoconazole treatment neither *ERG2* expression nor Upc2p or Ecm22p levels would be expected to change as they do following lovastatin treatment. In wild-type, *upc2*Δ (JRY7179), *ecm22*Δ (JRY7180), and *upc2*Δ*ecm22*Δ (JRY7181) strains treated with ketoconazole, induction of *ERG2* was similar to that seen after lovastatin treatment (Figure 6A). More tellingly, Upc2p and Ecm22p levels following ketoconazole treatment changed just as with lovastatin treatment (Figure 6B). Thus, induction of *ERG* genes and changes in Upc2p and Ecm22p levels resulted from sterol depletion and not from depletion of heme A or any other early product from the sterol pathway.

Hypoxic sterol depletion activates Upc2p and Ecm22p targets: A number of Upc2p targets, including the *DAN/TIR* genes, are induced under hypoxic conditions (ABRAMOVA *et al.* 2001a,b; TER LINDE *et al.* 2003). This observation led to the view that Upc2p is activated in response to low heme levels (ABRAMOVA *et al.* 2001b; KWAST *et al.* 2002), as heme biosynthesis is tightly controlled by oxygen, and heme levels often serve as a surrogate for oxygen levels (ZHANG and HACH 1999; HON *et al.* 2003). However, as ergosterol biosynthesis also requires oxygen (PARKS 1978), hypoxic conditions would also lead to reduced sterol biosynthesis and might activate Upc2p by reducing sterol levels rather than heme levels.

To test whether hypoxic targets of Upc2p were induced in response to sterol depletion or heme depletion, the expression of several Upc2p/Ecm22p target genes was measured by quantitative RT–PCR in wild-type, *upc2*Δ, *ecm22*Δ, and *upc2*Δ*ecm22*Δ strains grown aerobically with or without ketoconazole or hypoxically with or without hemin (Table 2). The logic behind this experiment is as follows: genes that are induced by sterol depletion should be induced both by ketoconazole treatment and by hypoxic growth, and addition of hemin should not reduce hypoxic induction. Genes induced only by low heme should not be induced by ketoconazole, but should be induced under hypoxic growth. When hemin is added to hypoxically growing strains, hypoxic induction should be reduced or eliminated, as heme is no longer limiting. If a gene is activated by low heme and low sterols in combination, ketoconazole might induce the gene to some degree, while hypoxia will result in even greater induction. Adding hemin should reduce, but not eliminate, hypoxic induction of such genes, as sterols will still be limiting.

COX5b and *ANB1* are known to be induced by hypoxia due to low heme. Neither contains the SRE promoter element to which Upc2p and Ecm22p bind. As expected, the expression of both *COX5b* and *ANB1* was induced under hypoxic conditions, but not by ketoconazole. Hypoxic induction was greatly reduced by the addition of heme (Table 2).

ERG2, *ERG3*, *ERG10*, and *IDI1* all encode enzymes in the sterol biosynthesis pathway. *ERG2* and *ERG3* encode late enzymes in the sterol-specific branch of the pathway and are known targets of Upc2p and Ecm22p (VIK and RINE 2001; DAVIES *et al.* 2005). *ERG10* encodes the enzyme that catalyzes the first step in the pathway and contains a potential SRE in its promoter. *IDI1* also encodes an early gene in the sterol pathway, but does not contain an SRE in its promoter. Expression of *ERG2*, *ERG3*, and *ERG10* was induced by ketoconazole, but this induction required either Upc2p or Ecm22p. Hypoxia also induced these three genes, and when either Upc2p or Ecm22p is present hypoxic induction is not affected by the addition of heme. Interestingly, in the absence of both Upc2p and Ecm22p, hypoxic induction of *ERG2*, *ERG3*, and *ERG10* is observed. This Upc2/Ecm22p-independent induction appeared to be a response to low heme, as the addition of heme greatly reduced the hypoxic response. In contrast, *IDI1* was not induced by sterol depletion, but, as with the other three *ERG* genes,

TABLE 2

Induction of gene expression

Gene	Condition	W303	*upc2*Δ	*ecm22*Δ	*upc2*Δ*ecm22*Δ
ANB1	Ketoconazole	1.23 (±0.01)	0.90 (±0.03)	1.64 (±0.54)	0.64 (±0.21)
	Hypoxia	139 (±22.0)	148 (±10.8)	165 (±6.34)	67.5 (±1.89)
	Hypoxia + hemin	8.43 (±1.02)	37.0 (±1.24)	17.3 (±2.05)	4.87 (±0.45)
COX5b	Ketoconazole	0.80 (±0.09)	0.67 (±0.09)	0.62 (±0.01)	0.86 (±0.41)
	Hypoxia	2.63 (±0.50)	2.63 (±0.14)	3.6 (±0.10)	2.80 (±0.12)
	Hypoxia + hemin	0.99 (±0.21)	1.34 (±0.01)	0.98 (±0.20)	0.74 (±0.14)
ERG2	Ketoconazole	2.99 (±0.40)	1.51 (±0.13)	2.58 (±0.35)	0.39 (±0.17)
	Hypoxia	2.98 (±0.17)	5.11 (±0.48)	3.34 (±0.01)	2.67 (±0.28)
	Hypoxia + hemin	2.60 (±0.61)	4.32 (±0.70)	2.13 (±0.27)	0.79 (±0.09)
ERG3	Ketoconazole	3.46 (±0.97)	2.47 (±0.42)	2.09 (±0.23)	0.43 (±0.03)
	Hypoxia	5.3 (±0.27)	7.27 (±0.78)	3.21 (±0.18)	12.4 (±0.48)
	Hypoxia + hemin	6.0 (±1.52)	8.33 (±0.28)	2.79 (±0.13)	3.38 (±0.06)
ERG10	Ketoconazole	1.95 (±0.12)	1.40 (±0.01)	2.02 (±0.44)	0.51 (±0.31)
	Hypoxia	2.72 (±0.44)	3.65 (±0.51)	3.94 (±0.56)	4.69 (±2.59)
	Hypoxia + hemin	2.09 (±0.95)	3.44 (±0.29)	3.13 (±0.83)	0.81 (±0.23)
IDI1	Ketoconazole	1.14 (±0.14)	0.88 (±0.20)	0.74 (±0.13)	0.52 (±0.13)
	Hypoxia	7.46 (±0.33)	7.11 (±0.93)	5.76 (±0.02)	4.81 (±1.12)
	Hypoxia + hemin	2.63 (±0.18)	4.95 (±1.33)	2.91 (±0.04)	1.98 (±0.18)
TIR1	Ketoconazole	6.99 (±1.21)	1.26 (±0.06)	6.66 (±1.56)	1.61 (±0.40)
	Hypoxia	70.7 (±6.44)	1457 (±6.78)	295 (±13.5)	33.5 (±0.31)
	Hypoxia + hemin	11.8 (±2.17)	176 (±25.4)	52.5 (±5.50)	2.34 (0.04)
DAN1	Ketoconazole	12.2 (±4.4)	1.59 (±0.12)	27.7 (±12.0)	2.17 (±0.11)
	Hypoxia	1474 (±136)	1800 (±643)	3259 (±191)	35.8 (±2.08)
	Hypoxia + hemin	287 (±59.7)	145 (±39.3)	485 (±72.0)	11.7 (±0.85)
DAN2	Ketoconazole	2.06 (±0.03)	1.22 (±0.15)	2.93 (±0.28)	1.39 (±0.84)
	Hypoxia	5.29 (±1.05)	2.11 (±0.15)	18.6 (±1.70)	1.79 (±0.47)
	Hypoxia + hemin	3.99 (±1.11)	3.06 (±1.79)	7.3 (±1.27)	1.33 (±0.19)
DAN4	Ketoconazole	2.32 (±0.22)	1.25 (±0.26)	1.83 (±0.18)	0.76 (±0.27)
	Hypoxia	5.32 (±0.30)	1.97 (±0.33)	2.90 (±0.08)	1.15 (±0.17)
	Hypoxia + hemin	2.38 (±0.17)	1.63 (±0.62)	2.52 (±0.35)	0.90 (±0.11)
UPC2	Ketoconazole	3.50 (±1.12)	NA	6.31 (±2.44)	NA
	Hypoxia	2.03 (±0.23)	NA	2.05 (±0.08)	NA
	Hypoxia + hemin	3.47 (±1.27)	NA	3.11 (±0.54)	NA
ECM22	Ketoconazole	1.64 (±0.18)	0.92 (±0.12)	NA	NA
	Hypoxia	3.67 (±0.42)	2.09 (±0.05)	NA	NA
	Hypoxia + hemin	1.63 (±0.37)	1.61 (±0.40)	NA	NA

Induction is shown as fold-change over aerobic gene expression (without ketoconazole and without heme). NA, not applicable.

was induced by hypoxia in a Upc2p- and Ecm22p-independent manner. Thus it appeared that hypoxia created two induction signals, one mimicked by ketoconazole that could not be suppressed by hemin and a second that could be. The hemin suppression was robust only in the *upc2*Δ*ecm22*Δ strain, which lacked the proteins that respond to sterol depletion.

The *DAN/TIR* genes encode hypoxically expressed mannoproteins and are known targets of Upc2p induction (Abramova *et al.* 2001a,b). *DAN1* and *TIR1* appeared to be induced both by low sterols and by low heme. Ketoconazole treatment resulted in some induction, but hypoxic induction was 10- to 100-fold higher than ketoconazole induction. Much, but not all, of the hypoxic induction could be abated by adding back heme. This result is consistent with previous reports that *DAN1* and *TIR1* expression are regulated both by Upc2p and by Rox1p, a transcriptional repressor that is expressed when heme levels are high (Abramova *et al.* 2001b; Kwast *et al.* 2002; Lai *et al.* 2005). *DAN2* and *DAN4*, which are not regulated by Rox1p (Abramova *et al.* 2001b), appeared to be induced primarily by low sterols. Ketoconazole and hypoxia (with or without hemin) induced both genes in a Upc2p/Ecm22p-dependent manner. Interestingly, unlike previous reports, under hypoxic conditions Ecm22p was quite capable of inducing all of the *DAN/TIR* genes tested.

UPC2 itself was induced by ketoconazole and by hypoxia, consistent with previous reports (Abramova *et al.* 2001b; Agarwal *et al.* 2003; Davies *et al.* 2005). The addition of heme to hypoxically growing cells did not reduce induction of *UPC2*, indicating that this induction was a result of low sterols rather than of low heme.

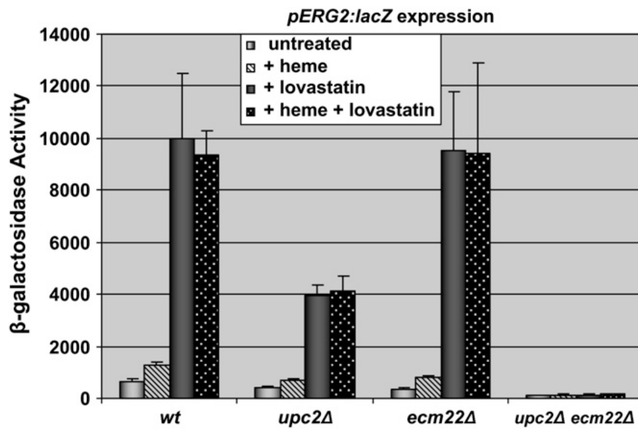

FIGURE 7.—Additional heme did not affect lovastatin induction of *ERG2* expression. The indicated strains were transformed with a *pERG2::lacZ* reporter (pJR2316). Strains were grown untreated or in the presence of 30 μg/ml lovastatin or 30 μg/ml lovastatin and 40 μg/ml hemin. Extracts prepared from these cultures were assayed for β-galactosidase activity as described in MATERIALS AND METHODS.

Thus, as reported previously, Upc2p appears to be autoregulated, most likely through the SRE elements in its promoter (ABRAMOVA *et al.* 2001b).

ECM22 expression was modestly induced by hypoxia. This induction could be suppressed by additional heme, indicating that this induction was the result of heme depletion. As reported above, activation of target genes by Ecm22p was the result of low sterols, suggesting a certain disconnect between *ECM22* expression and Ecm22p activity.

To control for the possibility that sterol depletion might create an unanticipated hypoxic response through heme levels, *ERG2* expression was monitored in the presence and absence of lovastatin and in the presence and absence of exogenous heme. No effects of heme levels on induction by sterol depletion were evident under these conditions (Figure 7). Thus, although hypoxic conditions created depletion of both heme and sterol levels, the responses to heme and sterol depletion were distinct and resolvable.

DISCUSSION

In addition to respiration, molecular oxygen is required for several important yeast cell processes, including the biosynthesis of heme and the biosynthesis of ergosterol (PARKS 1978; HON *et al.* 2003). Therefore, it is important for the cell to monitor oxygen levels and regulate these processes accordingly. In *S. cerevisiae* heme has been thought to be the primary oxygen sensor in the cell. Because heme biosynthesis is tightly regulated by oxygen, heme levels reflect oxygen availability (ZHANG and HACH 1999; HON *et al.* 2003). Sterol levels may also play a role in oxygen sensing. In *Schizosaccharomyces pombe*, the SREBP ortholog Sre1 activates both those genes required for adaptation to hypoxia and the ergosterol biosynthetic genes (HUGHES *et al.* 2005). Although *S. cerevisiae* lacks orthologs to the SREBP and SCAP, the results presented here suggest an analogous, but not homologous, oxygen-sensing mechanism in budding yeast.

Upc2p and Ecm22p regulate the expression of many of the ergosterol biosynthesis (*ERG*) genes (VIK and RINE 2001; GERMANN *et al.* 2005) as well as the transcription of several hypoxically expressed genes, including genes involved in anaerobic cell wall reorganization and anaerobic sterol uptake (ABRAMOVA *et al.* 2001a,b; WILCOX *et al.* 2002). As expression of these genes responds to oxygen availability, it makes sense that the activity of Upc2p and Ecm22p must also be regulated by oxygen. Because low oxygen limits biosynthesis of both heme and sterols, regulation of Upc2p and Ecm22p activity may respond to either or to both of these signals.

One important result of this study was the clear identification of sterol levels as the primary regulator of Upc2p (see Figure 8). The activation of target genes by Upc2p occurred in response to low sterols, whether caused by early blocks in ergosterol biosynthesis (lovastatin), by late blocks (ketoconazole), or by hypoxia. *ERG2*, *ERG3*, *ERG10*, *DAN2*, and *DAN4* were activated by Upc2p solely in response to sterol depletion rather than to heme depletion, whereas *DAN1* and *TIR1* responded to both sterols and heme (Table 2), consistent with previously reported repression of these genes by Rox1p and Mot3p and activation by Upc2p (ABRAMOVA *et al.* 2001b).

Like Upc2p, Ecm22p was able to induce SRE-containing *ERG* genes in response to sterol depletion (See Figure 6 and Table 2). Contrary to previous reports, Ecm22p also activated *DAN/TIR* genes in response to hypoxia. Previous studies used strains derived from S288C, with its mutant version of *HAP1* (ABRAMOVA *et al.* 2001a,b), whereas this study used W303. However if that is the explanation for the discrepancy, then Hap1p must aid activation by Ecm22p despite decreasing heme levels under hypoxic conditions. No large-scale study comparing the hypoxic responses of S288c and W303 has been reported.

Clearly, both heme levels and sterol levels contribute to oxygen sensing in yeast. Although the role of heme in oxygen sensing has been well studied (ZHANG and HACH 1999; HON *et al.* 2003), the broader role of Upc2p and its activation in response to oxygen through sterol depletion is just beginning to emerge. Several targets have been identified (ABRAMOVA *et al.* 2001b; VIK and RINE 2001; AGARWAL *et al.* 2003; TER LINDE *et al.* 2003; GERMANN *et al.* 2005), but if, as indicated by KWAST *et al.* (2002), Upc2p regulates nearly one-third of all anaerobically expressed genes, activation of Upc2p targets following sterol depletion represents a significant contribution to the adaptation to hypoxia.

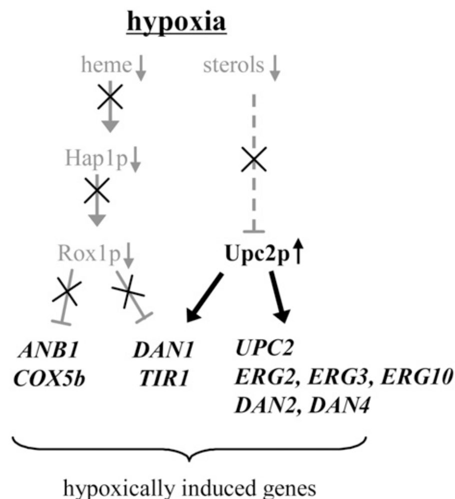

Figure 8.—A simplified model of hypoxic induction of gene expression.

Certainly, response to low heme and response to low sterols are not completely separable. Even within a specific pathway, such as ergosterol biosynthesis or the *DAN/TIR* gene family, mechanisms responding to both low heme and low sterols are at work. The involvement of Hap1p in ergosterol biosynthesis serves as one such example. Both Upc2p and Ecm22p required a functional version of Hap1p for basal expression of *ERG2* (Figure 1). In contrast, when sterols were depleted with lovastatin, Upc2p's dependence on Hap1p was largely abolished, as robust induction of *ERG2* was observed, whereas Ecm22p still depended upon Hap1p for *ERG* gene activation. Upc2p's ability to induce *ERG2* expression in the absence of Hap1p may be the consequence of several factors. Both Upc2p levels and the amount of Upc2p at *ERG* promoters increase upon sterol depletion (Davies *et al.* 2005), whereas Ecm22p levels and their occupancy at SREs decreased. The derepression of Upc2p activity upon sterol depletion (Davies *et al.* 2005) and the resulting increases in Upc2p levels may enable Upc2p to induce *ERG2* expression in a *HAP1*-independent manner.

It is unlikely that the need for both Hap1p and Upc2p or Hap1p and Ecm22p for basal gene expression is limited to *ERG2*. Full expression of *HMG1* requires both the Hap1p binding site and an additional 55-bp region in its promoter (Tamura *et al.* 2004). This region contains the SRE binding site for Upc2p and Ecm22p (Vik and Rine 2001). Indeed, conserved core consensus binding sites are found for both Hap1p and Upc2p/Ecm22p in several *ERG* genes (Chiang *et al.* 2003).

The involvement of Mot3p in *ERG* gene expression revealed that *ERG* gene expression involved both induction and repression. Mot3p modulates the transcription of a diverse set of genes in a dosage-dependent manner (Grishin *et al.* 1998; Abramova *et al.* 2001a). Mot3p acts in combination with Rox1p to recruit the Tup1-Ssn6 repressor complex to anaerobically expressed genes under aerobic conditions (Kastaniotis and Zitomer 2000; Mennella *et al.* 2003; Sertil *et al.* 2003; Klinkenberg *et al.* 2005). Mot3p inhibited Ecm22p, but not Upc2p (Figure 3), and the direct physical interaction of Mot3p with Ecm22p (Figure 5) was likely an important part of the inhibitory mechanism. Mot3p inhibited Ecm22p-mediated *ERG2* activation both before and after sterol depletion, so it remains unclear what signal or influence Mot3p responds to. Mot3p seemed to play some role in the decreased Ecm22p levels under inducing conditions, but not under non-inducing conditions (Figure 4A).

One unexpected result from this study was the component of the hypoxic induction of *ERG* genes that was Upc2p/Ecm22p independent. In the absence of Upc2p and Ecm22p, all the *ERG* genes tested, including *IDI1*, were hypoxically induced (Table 2). Unlike Upc2p- or Ecm22p-dependent induction, this induction responded to heme levels rather than to sterol levels. None of the *DAN/TIR* genes exhibited this type of induction, suggesting that this type of gene activation was specific to the ergosterol pathway. It is unlikely that this activation results from depression by Rox1p or Mot3p, as deletion of *ROX1* does not change expression of any *ERG* genes apart from *ERG26* and *HMG2* (Ter Linde and Steensma 2002), and, in the absence of Upc2p or Ecm22p, deletion of *MOT3* does not result in induction of *ERG2* (Figure 3).

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