The Hog1 Mitogen-Activated Protein Kinase Mediates a Hypoxic Response in Saccharomyces cerevisiae

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ABSTRACT

We have studied hypoxic induction of transcription by studying the seripauperin (PAU) genes of Saccharomyces cerevisiae. Previous studies showed that PAU induction requires the depletion of heme and is dependent upon the transcription factor Upc2. We have now identified additional factors required for PAU induction during hypoxia, including Hog1, a mitogen-activated protein kinase (MAPK) whose signaling pathway originates at the membrane. Our results have led to a model in which heme and ergosterol depletion alters membrane fluidity, thereby activating Hog1 for hypoxic induction. Hypoxic activation of Hog1 is distinct from its previously characterized response to osmotic stress, as the two conditions cause different transcriptional consequences. Furthermore, Hog1-dependent hypoxic activation is independent of the S. cerevisiae general stress response. In addition to Hog1, specific components of the SAGA coactivator complex, including Spt20 and Sgf73, are also required for PAU induction. Interestingly, the mammalian ortholog of Spt20, p38IP, has been previously shown to interact with the mammalian ortholog of Hog1, p38. Taken together, our results have uncovered a previously unknown hypoxic-response pathway that may be conserved throughout eukaryotes.

Changes in the environmental level of molecular oxygen can have profound effects on the growth of most organisms. Oxygen is required as an electron receptor in aerobic respiration, as well as for the biosynthesis of sterols, unsaturated fatty acids (UFAs), and heme, all of which are essential cellular components (Rosenfeld and Beauvoir 2003). In contrast, oxygen can also have negative consequences when it is metabolized into reactive oxygen species that can damage cellular components (JAMESON 1998). To adapt to altered levels of oxygen in the environment, most organisms, from bacteria to humans, respond to changes in oxygen levels by extensive changes in transcription (Bunn and Poyton 1996).

Several distinct mechanisms govern how cells respond to low levels of oxygen (hypoxia) in both metazoans and microorganisms, and four have been well described. First, in metazoans, many genes are induced during hypoxia by the transcription factor, hypoxia-inducible factor (HIF) (Kaelin 2005). In the presence of oxygen, HIF is inhibited by an oxygen-dependent hydroxylation that targets HIF for degradation; however, when oxygen levels are low, this hydroxylation cannot occur and HIF accumulates to activate transcription. Second, in Rhizobium and other bacterial species, the FixJ transcription factor promotes transcription during hypoxia (Rodgers 1999; Delgado-Nixon et al. 2000). Oxygen, when present, binds to a heme molecule attached to FixL, a histidine kinase, thereby preventing it from phosphorylating and activating FixJ. Third, in the yeast Schizosaccharomyces pombe, the transcription factor, sterol regulatory element-binding protein (SREBP) is activated during hypoxia (Hughes et al. 2005). This activation is caused by a decreased level of sterols, whose biosynthesis is oxygen dependent. Like its human ortholog, SREBP is tethered to a cellular membrane and is released to the nucleus when sterol levels are low (Brown and Goldstein 1997; Delgado-Nixon et al. 2000). While there is no SREBP ortholog in Saccharomyces cerevisiae, the Upc2 transcription factor is considered a functional homolog (Vik and Rine 2001) and is one focus of this work. Finally, S. cerevisiae contains an oxygen-responsive transcription factor, Hap1, that is described below.

In S. cerevisiae, ~400 genes respond to changes in oxygen levels. Several studies have shown that Hap1 plays a prominent role in this regulation, under both hypoxic and aerobic conditions (Zitomer and Lowry 1992; Ter Linde et al. 1999; Becerra et al. 2002; Kwast et al. 2002; Lai et al. 2005, 2006; Hickman and Winston 2007). Hap1 directly regulates many aerobic genes through activation in the presence of oxygen and repression in hypoxia (Zitomer and Lowry 1992; Hickman and Winston 2007). This switch between repression and activation is
regulated through the binding of heme, which, like sterols, requires oxygen for its biosynthesis and is thus absent from hypoxic cells (Hon et al. 2003; Hickman and Winston 2007). Hap1 also regulates several hypoxic genes and does so indirectly through aerobic induction of MOT3 and ROX1, which encode transcriptional repressors of these hypoxic genes (Sertil et al. 2003; Lai et al. 2006). However, there is likely at least one other oxygen-sensing pathway in S. cerevisiae since many genes respond to oxygen levels in the absence of Hap1 (Becerra et al. 2002; Hickman and Winston 2007).

In this work, we have studied hypoxic induction in S. cerevisiae by analysis of the hypoxia-induced seripauperin (PAU) genes. The PAU genes comprise a family of 24 genes that encode proteins related to fungal cell wall proteins and are thought to be important in remodeling the cell wall during certain types of stress, including hypoxic stress (Viswanathan et al. 1994; Rachidi et al. 2000; Abramova et al. 2001; Ai et al. 2002; Luo and van Vuuren 2009). Most PAU genes are subtelomeric and previous studies have shown that they are subject to regulation both by the Sir complex and by stresses that phosphorylate Sir3 (Ai et al. 2002; Radman-Livaja et al. 2011). With respect to hypoxic induction, the focus of our studies, one study has shown that adding heme to hypoxic cells prevents PAU induction (Rachidi et al. 2000), suggesting that the PAU genes are regulated through the known heme sensor, Hap1. However, the finding that PAU induction is Rox1 independent (Rachidi et al. 2000) and data from our previous microarray experiments (Hickman and Winston 2007) suggested that the hypoxic induction of PAU genes is Hap1 independent, and we confirm that in this study. Other work has shown that the PAU genes are induced by the transcription factor, Upc2, known to activate many other hypoxic genes through an unknown mechanism (Abramova et al. 2001; Kwast et al. 2002; Wilcox et al. 2002; Lai et al. 2006; Luo and van Vuuren 2009).

Here, we have identified additional factors and conditions required for the Upc2-dependent hypoxic induction of PAU genes, including the depletion of both sterols and heme, and the Hog1 mitogen-activated protein kinase (MAPK) pathway. Microarray analysis shows that, in addition to PAU genes, several other genes involved in maintaining the cell membrane or cell wall are induced by this pathway, demonstrating that the Hog1 pathway contributes to the maintenance of cell integrity during hypoxic growth. Extensive work has shown that Hog1 is required for the response to osmotic stress (Hohmann et al. 2007; de Nadal and Posas 2010); our results are the first demonstration that Hog1 is required as part of the response to hypoxic growth. Finally, we show that the SAGA transcriptional coactivator complex is also required for the expression of PAU genes. Our data, taken together, suggest the existence of a hypoxic-response regulatory system that may be conserved throughout eukaryotes.

**MATERIALS AND METHODS**

**Yeast strains:** All S. cerevisiae strains are listed in Table 1 and are isogenic with a GAL2+ derivative of S288C containing a repaired \textit{HAP1} allele (Winston et al. 1993; Hickman and Winston 2007). Strains were constructed by standard methods, either by crosses or by transformation (Ausubel et al. 1991). The deletion alleles were created by replacing the respective ORF with the KanMX, URA3, LEU2, HPH (Brachmann et al. 1998), or NatMX marker (Goldstein and McCusker 1999). All markers used for strain construction are listed in supporting information, Table S1. Strains containing the \textit{hem1}$\Delta$::KanMX allele were grown on 200 \textmu g/ml \textit{δ}ala, except where indicated. The \textit{KanMX;GAL1-ERG25} allele was constructed by placing the KanMX marker and the \textit{GAL1} promoter upstream of the \textit{ERG25} ORF (Longtine et al. 1998); strains containing this allele of the essential \textit{ERG25} gene were maintained on \textit{YP} galactose medium, except where indicated. The \textit{HOCl-13X-myc::KanMX} allele was created by inserting 13 copies of the myc epitope tag at the C-terminal end while adding a \textit{KanMX} marker (Longtine et al. 1998); this allele did not affect hypoxic PAU induction (data not shown).

**Media and growth conditions:** Culture and hypoxic methods were as described (Hickman and Winston 2007), except where noted below. For all experiments, cells were grown at 30° in \textit{YP} (1% yeast extract and 2% peptone) supplemented with 2% glucose or, where indicated, 2% galactose. For RNA and protein analyses, overnight saturated cultures were diluted and grown aerobically for approximately four generations to mid-log (1–2 $\times 10^7$ cells/ml). For hypoxic growth, these aerobic mid-log cultures were diluted appropriately and grown again in 250-ml flasks continuously sparged with ultra-high-purity nitrogen gas at ~3 liters/min. For analysis of mRNA levels or proteins during hypoxic growth, cells were grown for 5 hr after the shift to nitrogen unless otherwise noted. Cells were chilled on ice for 5 min before removing from nitrogen gas. Heme, in the form of hemin (BioChemika), was made up at 50 mg/ml in 1:1 (ethanol):water with 0.1 M NaOH. Ergosterol (>95%) (Sigma) was made up at 2 mg/ml in 1:1 Tween 80:ethanol. \textit{δ}-Aminolevulinate (\textit{δ}-ala; Sigma) was dissolved in water at 20 mg/ml and added to the media at the indicated concentration. Importantly, except where indicated, ergosterol was not included in the media for our hypoxic experiments because it has an effect on expression of some genes, as described elsewhere in this report. For the short time points used, there was no decrease in cell viability even though the ergosterol solution is essential for long-term growth in hypoxia. The appropriate amount of the stock solution was added to cells to achieve the desired final concentration, and an equal volume of the solvent was used as a solvent-only control. For the heme and ergosterol depletion experiments in Figure 1, cells were grown for 12 hr.

**Expression microarray, Northern blot analysis, and real-time PCR:** The mRNA expression of all yeast genes was analyzed by hybridization to an Agilent yeast array as described (Brauer et al. 2008). The 24 PAU genes are >95% identical in nucleotide sequence (Luo and van Vuuren 2009), and hence probes designed for one PAU gene will cross-hybridize with all the other genes. Thus, we averaged all the expression values for PAU genes into one value that represents the average PAU expression. The microarray data (Table S2) have been deposited in the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) database and are accessible through GEO series accession no. GSE26593.

Northern blot analysis was performed as described (Ausubel et al. 1991), using PCR-amplified and random-primed $^{32}$P-
labeled probes. The primers for PCR are listed in Table S1. The Northern probe for PAU genes also detected a weak band and we showed that it was the homologous DAN1 gene by Northern analysis of a dan1Δ strain (data not shown). For all panels in all figures, a representative of at least three independent experiments is shown. SNR190 encodes a small nucleolar RNA and serves as a loading control.

For real-time PCR, RNA was isolated from cells as described above and then reverse transcribed using a poly(T) primer. The resulting cDNA was quantitated on the Stratagene (La Jolla, CA) MX3000P. Primers are listed in Table S1. Shown are the mean and standard deviation for at least three independent experiments. Detection of UPC2 mRNA by Northern analysis was hampered because the UPC2 mRNA overlaps with a ribosomal band. Thus, real-time PCR was used to quantify UPC2 mRNA levels.

### Western analysis
Whole-cell extracts were prepared by bead lysis. Protein concentrations were measured by Bradford assay and equal amounts of extract were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was incubated overnight at 4°C with primary antibodies to myc (1:1000; clone 9E10; BD Pharmingen no. 51-1485GR), total Hog1 (1:1000; polyclonal yG20; Santa Cruz no. sc-6815), phospho-Hog1 (1:1000; clone 3D7; Cell Signaling no. 9215S), and Mbp1 [1:5000; polyclonal; New England BioLabs (Beverly, MA) no. E8030S]. The membrane was then incubated for 1 hr at room temperature with HRP-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories) and treated with Lumigen HRP substrate (GE Healthcare).

### Deletion set screen for mutants unable to induce PAU genes
To screen for mutants with altered regulation of PAU

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### Table 1: Saccharomyces cerevisiae strains

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genes, we constructed a strain in which the PAU5 ORF was replaced with that of *URA3*, and a NatMX cassette was integrated 152 nucleotides downstream of the PAU5 stop codon to provide a closely linked selectable marker. This strain has a URA− phenotype during aerobic growth and a URA+ phenotype during hypoxic growth; it also showed the expected *URA3* expression by Northern analysis (data not shown). The strain also contains the *com1Δ::MFA1−HIS3* marker, to allow a screen of the *S. cerevisiae* deletion set (Tong et al. 2001). After crossing by the deletion set on YPD plates, diploids were selected on YPD plates containing the drugs nourseothricin (to select for the NatMX marker) and G418 (to select for the KanMX marker). The diploids were sporulated and the MATα haploids were selected on synthetic complete media lacking histidine and arginine and containing the drug canavanine. The haploids were then tested for hypoxic growth, *GAL1−ERG25* (FY2871, lane 4) controls, an equal volume of the solvent was added as described in materials and methods. (D) Upc2 is required for *PAU* induction during heme or ergosterol depletion. To test whether Upc2 is required during heme depletion, *hem1Δ* (FY2637, lane 1) and *hem1Δ upc2Δ* (FY2872, lane 2) strains were grown in the absence of δ-ala for 12 hr and *PAU* mRNA levels were measured. To test whether Upc2 is required during ergosterol depletion, *GAL1−ERG25* (FY2870, lane 3) and *GAL1−ERG25 upc2Δ* (FY2871, lane 4) were each grown in glucose for 12 hr and *PAU* mRNA levels were measured. (E) Manipulating membrane fluidity with DMSO or glycerol affects hypoxic *PAU* induction. Northern analysis is shown of wild-type cells (FY2609) grown in the presence (+O₂) or absence (−O₂) of oxygen for 4 hr. DMSO (5%, 10%, or 15% v/v; lanes 3–5) or glycerol (5%, 10%, or 15% v/v; lanes 7–9) was added to the medium immediately before shifting cells to hypoxic growth.

**RESULTS**

*PAU* genes are strongly regulated by Upc2 but not by Hap1 or Hap2: Previous studies have demonstrated that *PAU* genes are tightly regulated by oxygen levels. During aerobic growth, *PAU* transcription is undetectable; however, upon a shift to hypoxic conditions, *PAU* transcription is strongly induced (Rachidi et al. 2000; Abramova et al. 2001). Our previous microarray experiments (Hickman and Winston 2007) suggested that hypoxic induction of *PAU* genes can occur independently of Hap1 and other studies have shown that the induction is dependent upon Upc2 (Abramova et al. 2001; Kwast et al. 2002). To test these results in our strains, we performed Northern analysis, also testing the requirement for Hap2, another heme-responsive transcription factor that regulates many of the same genes as Hap1 (Pinkham and Guarente 1985; Lai et al. 2006). Our results show a modest requirement for Hap1 and no requirement for Hap2 for *PAU* activation.
during hypoxic growth (Figure 1A). The modest requirement for Hap1 is likely due to its requirement as a repressor of heme and ergosterol biosynthetic genes under hypoxic conditions (Hickman and Winston 2007 and see below). Further, Hap1 and Hap2 play no role in the aerobic repression of PAU genes (Figure 1A). In contrast to Hap1 and Hap2 independence, our results show a strong dependence upon Upc2 for the hypoxic induction of PAU (Figure 1A). This induction is likely direct, as there is a Upc2 DNA-binding site found in almost all PAU gene promoters (Cohen et al. 2001; Kwast et al. 2002; Luo and van Vuren 2009). Thus, hypoxic induction of PAU expression is controlled by a Upc2-dependent pathway that is independent of Hap1 and Hap2.

**PAU transcription is regulated by heme and ergosterol levels:** A previous study suggested that aerobic repression of PAU transcription requires heme (Rachidi et al. 2000). As heme is required for ergosterol biosynthesis in *S. cerevisiae* (Gollub et al. 1977; Lorenz and Parks 1991), we tested the roles of both heme and ergosterol in PAU regulation. First, we depleted either heme or ergosterol during aerobic growth to test the effect on aerobic repression of PAU transcription. To deplete heme during aerobic growth, we used a strain with a deletion of *HEM1*, required for the production of δ-ala in the first committed step of heme biosynthesis (Gollub et al. 1977). When *hemΔ* cells were grown without δ-ala (and thus could not produce heme), the PAU genes were induced (Figure 1B). To deplete cells of ergosterol, we constructed a strain containing the essential *ERG25* gene, required for ergosterol biosynthesis, under the control of the *GAL1* promoter and shifted it from galactose, which induces the gene, to glucose, which represses the gene. Similar to heme depletion, when *ERG25* is repressed, PAU transcription is induced (Figure 1B). Thus, depletion of either heme or ergosterol during aerobic growth abolishes repression of PAU transcription. As heme is required for ergosterol biosynthesis (Gollub et al. 1977; Lorenz and Parks 1991), heme depletion might induce PAU genes because it leads to ergosterol depletion. To address this possibility, we added heme to ergosterol-depleted cells during aerobic growth and found that the addition of heme blocked PAU induction (Figure S1A). Similarly, adding ergosterol to heme-depleted cells dramatically reduces PAU induction (Figure S1B). These results suggest that heme and ergosterol each are required to regulate PAU transcription. As the depletion experiments suggest that decreased levels of heme and ergosterol are necessary during hypoxic growth for PAU induction, we added exogenous heme or ergosterol to hypoxic cells. In both cases, we found that they repressed PAU induction (Figure 1C). Finally, to determine the relationship of heme or ergosterol depletion to activation by Upc2, we repeated each depletion in a *upc2Δ* mutant background. Our results show that neither heme nor ergosterol depletion leads to PAU induction in a *upc2Δ* mutant (Figure 1D), suggesting that Upc2 activation occurs downstream of the signal from heme or ergosterol depletion. Taken together, these results show that heme and ergosterol levels each play critical roles downstream of oxygen levels and upstream of Upc2 in mediating the regulation of PAU.

**Membrane fluidity, but not sterol sensing, is important for PAU regulation:** Our finding that ergosterol depletion mimics hypoxic induction of PAU genes suggested that *S. cerevisiae* might employ a sterol-sensing system, similar to what has been described in *S. pombe* and mammals (Yang et al. 2002; Hughes et al. 2005). However, the results described in the previous section suggest that this is not the case as heme itself plays a role. Second, we did not find a significant role for putative sterol sensors in hypoxic PAU induction. While *S. cerevisiae* does not have an SREBP ortholog, it does contain two orthologs of the *S. pombe* and human INSIG protein, required for sterol-sensing and SREBP regulation (Yang et al. 2002; Hughes et al. 2005). We found that neither of the *S. cerevisiae* orthologs, Nsg1 and Nsg2 (Flury et al. 2005), plays a significant role in PAU regulation (Figure S2). In addition, Ncr1, predicted to be a sterol sensor (Malathi et al. 2004), is not required for PAU regulation (data not shown). These results suggest that direct sterol sensing, as occurs in *S. pombe* and humans, does not play a role in PAU induction in *S. cerevisiae*.

On the basis of these results, we speculated that the PAU genes may be induced in response to a more general effect on membranes, as both heme and sterols are known to decrease membrane fluidity (Lees et al. 1979; Shyiro et al. 1982; Ginsburg and Demel 1984; Schmitt et al. 1993; Berg et al. 2002; Abe et al. 2009). To test directly whether changing membrane fluidity contributes to PAU induction, we employed dimethyl sulfoxide (DMSO) and glycerol, both of which are known to decrease membrane fluidity (Surewicz 1984; Lewis et al. 1994; Gurtovenko and Anwar 2007). Indeed, we found that increased levels of DMSO or glycerol repressed PAU transcription during hypoxia (Figure 1E) or sterol depletion (data not shown). This result suggests that during the hypoxic response, the increased membrane fluidity that occurs as a consequence of decreased heme and ergosterol levels is required for PAU induction.

**The Hog1 MAP kinase is activated by hypoxia and is required for PAU induction:** The Hog1 MAP kinase is important for the response to osmotic stress, as well as other stresses that may affect membranes (Hohmann et al. 2007). We wondered whether Hog1 is also involved in the response to hypoxia, given our evidence that membrane changes are a key aspect of PAU induction. Indeed, we found that Hog1 plays a major role in the hypoxic induction of PAU expression, as a *hog1Δ*
mutant exhibited little induction compared to wild type (Figure 2A). Hog1 is activated in response to osmotic stress by phosphorylation of two threonine residues (Brewster et al. 1993; Saito and Tatebayashi 2004). Using a phospho-specific antiserum and Western blot analysis, we found that Hog1 is also phosphorylated under hypoxic conditions (Figure 2B). We verified the identity of Hog1 in our Western blots by showing that a myc epitope tag added to Hog1 caused a band shift to a higher molecular weight (Figure 2B).

The UPC2 gene is transcriptionally induced under hypoxia (Abramova et al. 2001) and we wanted to test whether this induction is Hog1 dependent. To do this, we measured UPC2 mRNA levels by real-time PCR. While we did confirm that UPC2 is induced by hypoxia, we found that Hog1 is not required for the induction (Figure 2C). Thus, Hog1 does not induce the PAU genes through regulation of UPC2 mRNA levels.

Other members of the Hog1 MAPK pathway are required for the hypoxic response: The Hog1 pathway contains two independent branches upstream of Hog1, both of which are used during osmotic stress (Saito and Tatebayashi 2004; de Nadal and Posas 2010). One pathway uses two redundant MAPKKs, Ssk2 and Ssk22, while the other pathway uses a different MAPKK, Ste11. Both of these pathways activate the MAPKK, Pbs2, which then phosphorylates Hog1 (Brewster et al. 1993; Saito and Tatebayashi 2004), although some evidence suggests that the Ssk2/Ssk22 pathway plays a more prominent role (O’Rourke and Herskowitz 2004). To determine whether either of these pathways is required during the hypoxic response, we measured PAU induction in mutants that are defective for each pathway. To test the Ssk2/Ssk22 pathway, we used an ssk1Δ mutant. Ssk1 is an upstream kinase that phosphorylates and activates both Ssk2 and Ssk22; an ssk1Δ allele thus abrogates activation of both of these MAPKKs (Maeda et al. 1994). Our results show that an ssk1Δ mutant exhibited the same low level of PAU induction as a hog1Δ mutant (Figure 3A), suggesting that this pathway is activated during hypoxia. In contrast, a ste11Δ mutation did not have any effect on hypoxic PAU induction (Figure 3A and Figure S3), suggesting that this pathway does not play a role in this hypoxic response. As Ste11 is also the MAPKK for the filamentous/invasive growth and pheromone response pathways (Harris et al. 2001), our results suggest that these other pathways that signal from the cell membrane do not participate in hypoxic PAU induction. In addition, an ssk1Δ ste11Δ double mutant had the same defect as an ssk1Δ single mutant, again suggesting that Ste11 plays no role in the hypoxic activation of Hog1 (Figure 3A). As expected, a pbs2Δ mutant had the same defect in PAU induction as a hog1Δ strain (Figure 3B). These data show that the Ssk2/Ssk22 branch of the Hog1 pathway, but not the Ste11 branch, is required for PAU induction.

The Hog1-mediated hypoxic response does not require the general stress response factors Msn2 and Msn4: Previous studies have suggested that the Hog1 pathway interacts with the general stress response pathway (Martinez-Pastor et al. 1996; Gasch et al. 2000; Rep et al. 2000; Capaldi et al. 2008). The general stress response pathway is activated by a number of environmental changes, including osmotic shock, oxidative stress, heat shock, and nutritional starvation (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996; Gorner et al. 1998). Upon any one of these events, the transcription factors Msn2/Msn4 are activated and induce a transcriptional response. We tested whether Msn2 and Msn4 are required for the hypoxic induction of PAU transcription and found that in msn2Δ, msn4Δ, and msn2Δ msn4Δ mutants the hypoxic induction of PAU transcription was normal (Figure 3C). Thus, the Hog1-mediated hypoxic response is independent of the general stress response.

The ER-membrane protein, Mga2, is required for PAU induction: The transcription factor Mga2 has been previously shown to be required for the hypoxic induction of the OLE1 gene (Zhang et al. 1999; Jiang et al. 2001, 2002). Additional experiments have shown

![Figure 2](https://academic.oup.com/genetics/article/188/2/325/6063412)
that Mga2 is normally associated with the endoplasmic reticulum, but can be activated by ubiquitin-dependent proteolysis, under a set of conditions that includes low levels of unsaturated fatty acids (UFAs) and hypoxia (Hoppel et al. 2000; Jiang et al. 2002). UFA biosynthesis, like that of heme and sterols, is dependent on oxygen and is therefore impaired in hypoxic cells. We wanted to test whether the Mga2 pathway plays a role in hypoxic induction of PAU genes. Indeed, we have found that in an mga2Δ mutant, PAU induction is defective, equivalent to the defect in a hog1Δ mutant (Figure 3D).

As MGA2 is partially redundant with the SPT23 gene, we also tested an spt23Δ mutant, but found that it did not impair PAU induction (Figure 3D). These results are similar to induction of OLE1, which requires Mga2 but not Spt23 (Jiang et al. 2001).

Hog1 distinguishes between the hypoxic and osmotic stress signals: The demonstration that Hog1 is required for a normal hypoxic response and that this pathway requires Pbs2 and Ssk1 raised the question of whether the hypoxic pathway is distinct from that for osmotic stress, the pathway best characterized for Hog1. To examine this, we assayed the regulation of GRE2, a gene that responds to osmotic stress (Garay-Arroyo and Covarrubias 1999), and PAU genes, which respond to hypoxic conditions. Our results show that each response is specific, as GRE2 is induced only upon osmotic stress, and PAU genes are induced only upon hypoxic induction, all occurring in a Hog1-dependent manner (Figure 4A).

We then compared the kinetics of Hog1 phosphorylation during osmotic stress and hypoxic induction. We found that osmotic stress causes a dramatic peak of Hog1 phosphorylation within minutes of treatment (Figure 4B, lanes 1–5), as previously reported (Maeda et al. 1995). We also found a second peak of Hog1 that reproducibly appeared 2 hr after treatment (Figure 4B). In contrast, hypoxia led to a gradual increase in Hog1 phosphorylation, reaching a peak at 5 hr (Figure 4A).
Figure 5.—Several hypoxic genes are dependent upon Hog1. (A) Expression microarray analysis showing hypoxic induction of genes in wild-type (FY2609, xayis) and hog1Δ (FY2873, yayis) strains. Only the genes hypoxically induced more than twofold in two of two experiments are shown on the plot. The solid line represents the same induction in wild-type and hog1Δ strains, while the dotted lines represent a twofold difference in induction. Genes shown in red have mRNA levels decreased by twofold or more in the hog1Δ mutant. The data point labeled “PAU” represents the average expression of all 24 PAU genes. (B) Genes in the sterol/heme/Hog1/Upc2 pathway are listed with their function and conserved Upc2 binding site(s). The list of genes includes only those that meet three criteria: (1) regulated by O2 and Hog1 (Figure 5A), (2) induced by depletion of sterols and depletion of heme (Figure S4), and (3) contain a conserved Upc2 binding site in the promoter, on the basis of the previously determined consensus (TCGTATA or TCGTTYAG) (Cohen et al. 2001; Znaidi et al. 2008).

4B, lanes 6–12 and data not shown). The kinetics of Hog1 phosphorylation in response to each treatment are consistent with the kinetics of gene induction seen in Figure 4A, strongly supporting the idea that Hog1 responds differently to hypoxia and osmotic stress.

Hog1 plays a role in the induction of several hypoxic genes: As Hog1 regulates the transcription of 50–200 genes in response to osmotic stress (Posas et al. 2000; Rep et al. 2000), we wanted to determine how many genes are regulated by Hog1 in response to hypoxia. To test this, we used expression microarrays to compare the hypoxic induction of genes in wild-type and hog1Δ strains. In addition to PAU genes, there were 9 other hypoxic genes that exhibited a more than twofold decrease in mRNA levels in a hog1Δ mutant compared to wild type under hypoxic conditions (Figure 5A, gene names in red). There were also three hypoxic genes (DAN1, INO1, and TDH1) that had increased mRNA levels in a hog1Δ mutant under hypoxic conditions; these have not yet been analyzed further.

To test if the nine Hog1-dependent, hypoxia-induced genes that we identified are also regulated by ergosterol and heme levels, we examined a previous data set of strains. In addition to Hog1-dependent, hypoxia-induced genes, we identified several components of the SAGA transcriptional coactivator complex, including Ada2, Gcn5, and Sgf29. We then directly tested the requirement for most nonessential SAGA components in response to osmotic stress (Pammentes et al. 2011), perhaps to maintain proper membrane fluidity during this different form of membrane stress.

The SAGA coactivator complex is required for PAU and UPC2 induction: To identify other regulators of PAU expression, we screened the S. cerevisiae deletion set for mutants unable to express a PAU reporter under hypoxic conditions. This screen identified several components of the SAGA transcriptional coactivator complex, including Ada2, Gcn5, and Sgf29. We then directly tested the requirement for most nonessential SAGA genes by Northern analysis and found that the SAGA mutants with the strongest defects in PAU induction occurred in four classes of SAGA genes (Figure 6A and data not shown): those encoding core components (ADA1, SP77, and SP720), those required for histone acetyltransferase activity (GCN5, ADA2, and ADA3), a component required for TBP recruitment (SPT3), and a component required for the assembly of the histone deubiquitination (DUB) module of SAGA (SGF73) (see Koutelou et al. 2010 for a review).

We were particularly interested in the strong requirement for Sgf73 in PAU induction. While Sgf73 is clearly important in humans (Helmlinger et al. 2004), only a few studies have identified phenotypes for sgf73 mutants in S. cerevisiae (Shukla et al. 2006; Jordan et al. 2007; Gresham et al. 2008). Sgf73 was recently found to anchor the DUB module of SAGA within the complex (Lee et al. 2009; Rodriguez-Navarro 2009; Kohler et al. 2010; Samara et al. 2010). The DUB module contains three proteins that are required for histone deubiquitination: (1) Ubp8, the deubiquitinase; (2) Sus1, also involved in SAGA-mediated mRNA export; and (3) Sgf11, a possible structural protein. To determine
whether Sgf73 plays a role in PAU expression via the DUB module, we deleted the genes encoding each of the DUB components and compared their effects, singly and in combination, to sgf73Δ's. Our results show that even in a sus1Δ sgf11Δ ubp8Δ triple mutant, there is still significant PAU induction, albeit reduced (Figure 6B, lanes 11 and 12). In contrast, in the sgf73Δ single mutant, or when sgf73Δ is combined with the other three mutations, there is no detectable PAU induction. These results show that Sgf73 plays a prominent role in the hypoxic induction of PAU genes and does so, at least partly, in a DUB-independent manner.

To further define the role of SAGA in the hypoxic response, we tested whether SAGA is required for UPC2 transcription. Our results show that most of the SAGA mutants tested that impair the hypoxic expression of PAU also impair the hypoxic expression of UPC2 (Figure 6C). One notable exception is gcn5Δ, which significantly reduces PAU mRNA levels but not those for UPC2. Thus, SAGA may contribute to PAU expression in two ways: (1) by regulating transcription of UPC2 in an Sgf73- and Spt3-dependent fashion and (2) by directly regulating PAU expression via Gcn5.

**DISCUSSION**

In this work, we have identified several factors that control the hypoxic induction of the PAU genes of *S. cerevisiae*. Combined with previous results concerning PAU regulation (Rachidi et al. 2000; Abramova et al. 2001; Kwast et al. 2002), our studies suggest the existence of a previously unknown hypoxic-response regulatory system that is distinct from the well-studied Hap1-dependent pathway and that requires the Hog1 MAPK pathway. Our microarray analysis has shown that Hog1 is required for the hypoxic induction of several genes in addition to the PAU genes, showing that it plays a significant role in the *S. cerevisiae* hypoxic response. As the mammalian Hog1 ortholog, p38, is also activated by hypoxia (Seko et al. 1997; Jin et al. 2000; Kacimi et al. 2000; Blaschke et al. 2002; Kulisz et al. 2002; Zhu et al. 2002; Emerling et al. 2005), this role may be conserved throughout eukaryotes.

Our results suggest a regulatory framework for most of the factors now known to be required for the hypoxic induction of PAU genes (Figure 7). In this regulatory pathway, the direct consequence of decreased oxygen levels is the depletion of heme and sterols, which causes an increase in membrane fluidity. This step is supported by our result that chemicals that decrease membrane fluidity (DMSO and glycerol) block the hypoxic induction of PAU transcription and that the levels of both ergosterol (Lees et al. 1979; Berg et al. 2002; Abe et al. 2009) and heme (Kirschner-Zilber et al. 1982; Shviro et al. 1982; Ginsburg and Demel 1984; Shaklai et al. 1985; Light and Olson 1990; Balla et al. 1991; Schmitt et al. 1993) affect membrane fluidity.

Our results showing that PAU activation requires Ssk1 and Pbs2 suggest that the change in membrane fluidity then activates the Hog1 pathway via Shn1, a transmembrane protein that responds to osmotic stress and that is part of the Shn1-Pdi1-Ssk1 phospho-relay complex that activates the Hog1 pathway (Maeda et al. 1994). Shn1 has been proposed to be a sensor of membrane turgor pressure (Reiser et al. 2003), but Shn1 may more directly be a sensor of membrane fluidity, since membrane turgor influences membrane fluidity (Yamazaki et al. 1989; Laroche et al. 2001; Hayashi and Maeda 2006; Panadero et al. 2006). Indeed, studies have shown that treatments that affect membrane fluidity, such as DMSO and low temperature, influence Hog1 signaling via the Shn1 pathway (Hayashi and Maeda 2006; Panadero et al. 2006). Once activated by changing membrane fluidity, Shn1 would initiate the MAPK cascade that leads to activation...
of Pbs2 and Hog1. Our results have also shown that another pathway to activate Hog1, via Sho1 and Ste11, is not involved in the hypoxic response. Past results have suggested that the Shl1 branch plays a more prominent role in Hog1 activation under certain conditions, including low-temperature activation, and the Sho1/Ste11 branch plays a distinct role in pseudohyphal growth (O’Rourke and Herskowitz 1998, 2004; Maeta et al. 2005; Hayashi and Maeda 2006; Panadero et al. 2006). Our results have provided a further distinction between these two pathways.

Once activated, Hog1 may have multiple functions in hypoxic induction and our results have not yet distinguished among these possibilities. In the osmotic stress response, Hog1 functions in several ways, with both transcriptional and cytoplasmic roles (Proft and Struhl 2004; Mettetal et al. 2008; Westfall et al. 2008; de Nadal and Posas 2010). Interestingly, the cytoplasmic roles of Hog1 are sufficient for the osmotic stress response (Westfall et al. 2008). In the hypoxic response, one obvious possibility is that Hog1 activates Upc2. While we have shown that Hog1 is not required for UPC2 transcriptional induction under hypoxic conditions, previous studies have suggested that Upc2 requires an activation step beyond transcriptional induction (Kwast et al. 2002; Davies and Rine 2006) and that it relocates from intracellular membranes to the nucleus uponsterol depletion (Marie et al. 2008). Thus, Hog1 may control Upc2 localization or activity by phosphorylation, the latter possibility similar to its role in activation of Sko1 during osmotic stress (Proft et al. 2001). Chromatin immunoprecipitation experiments have not yet been able to detect either Hog1 or Upc2 association with PAU promoters. However, all PAU promoters have consensus Upc2 binding sites (Kwast et al. 2002; Luo and van Vuuren 2009), suggesting that Upc2 acts directly on PAU induction and that the lack of a ChIP signal is due to the technical complication of multiple copies of PAU promoters. Future experiments will address the levels at which Hog1 controls the hypoxic response.

The role of the SAGA transcriptional coactivator in hypoxic induction may involve both Hog1-independent and Hog1-dependent activities. One role of SAGA must be independent of Hog1 activity, as SAGA is required for UPC2 transcriptional induction, while Hog1 is not. However, SAGA may also function directly at PAU promoters, possibly recruited by Upc2, where SAGA components could be regulated more directly by Hog1 in response to changing membrane fluidity. Indeed, there is strong evidence for connections between stress, Hog1, and SAGA: (1) SAGA-dependent genes are mainly those regulated by stress (Huisinga and Pugh 2004), (2) SAGA is required for Hog1-dependent gene expression in response to osmotic stress (Proft and Struhl 2002; Zapater et al. 2007), and (3) during mouse development, the SAGA subunit Spt20 interacts with p38, the mammalian Hog1 ortholog (Zohn et al. 2006; Wang et al. 2008; Nagy et al. 2009). Whatever the exact role of SAGA in hypoxic induction, it is of interest that its activity during hypoxic induction is strongly dependent upon the SAGA component, Sgf73. This role of Sgf73 is at least partially independent of its well-characterized requirement for the assembly and activity of the histone deubiquitylase activity of SAGA (Lee et al. 2009; Rodriguez-Navarro 2009; Kohler et al. 2010; Samara et al. 2010). Other studies have shown that Sgf73 is also required for preinitiation complex formation at some SAGA-dependent promoters (Shukla et al. 2006) and that Sgf73 can bind to nucleosomes (Bonnet et al. 2010). Further analysis of Sgf73 in the hypoxic response may reveal whether these or additional Sgf73 functions are important during the hypoxic response.

While both osmotic stress and hypoxic induction require Hog1 activation, there are clear differences in these responses. There is a large difference in the kinetics of Hog1 activation with respect to osmotic stress and hypoxic induction. This is likely due to the distinct ways that these environmental changes affect membrane fluidity. Osmotic stress rapidly changes the membrane turgor pressure and therefore likely changes the
fluidity almost immediately. In contrast, hypoxia causes sterol and heme depletion, with a change in membrane fluidity probably occurring more slowly, as the dilution of heme and ergosterol is required to change membrane fluidity. The kinetics of Hog1 activation correlate with the induction of the respective transcriptional programs. In addition, although both pathways depend upon Hog1, they have distinct transcriptional outputs, as the PAU genes respond solely to hypoxia while GRE2 responds solely to osmotic stress. This result strongly suggests that there are additional components that are distinctly required for each response.

To conclude, our results have shown that the Hog1 MAPK pathway, SAGA, and other factors are required for the hypoxic induction of several genes in S. cerevisiae. These genes likely help cells adapt to an environment with little or no oxygen, where they are unable to make heme and sterols and hence unable to properly maintain the membrane. Most of these genes were previously shown to be induced by different stresses, such as changing temperature, that cause damage to membranes and are all involved in protecting and maintaining cell membrane and cell wall components (Viswanathan et al. 1994; Mazur et al. 1995; Mao et al. 1997; Mandala et al. 1998; Rachidi et al. 2000; Sales et al. 2000; Abramova et al. 2001; Swain et al. 2002; Caesar and Blomberg 2004; Serrano et al. 2006; Pacheco et al. 2009). In addition to conservation of heme, sterols, the Hog1 pathway, and SAGA, at least three of the induced genes (YNR3, ERG26, and TPS1) are conserved in humans and in most eukaryotes. We thus speculate that parts of this pathway also function in metazoans and play important roles in the adaptation of cells and tissues to changing oxygen levels.

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The Hog1 Mitogen-Activated Protein Kinase Mediates a Hypoxic Response in *Saccharomyces cerevisiae*

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FIGURE S1.—Heme suppresses ergosterol depletion, and ergosterol suppresses heme depletion. A. Northern analysis of a GAL1PR-ERG25 strain (FY2870) grown for 12 hours under aerobic conditions with 2% glucose, in the absence (lanes 1-3) or presence (lanes 4-6) of heme. Lane 7 contains a sample grown under hypoxic conditions as a positive control for PAU expression. B. Northern analysis of hem1Δ strain (FY2657) grown for 12 hours under aerobic conditions without δ-ala (lanes 2-10), in the absence (lanes 3-6) or presence (lanes 7-10) of ergosterol. Lane 1 contains a sample with δ-ala added and lane 2 contains a heme-depleted sample without solvent or ergosterol.
**Figure S2.**—Nsg1 and Nsg2 do not play a major role in PAU oxygen regulation. The reduction of hypoxic PAU expression in the nsg1Δ single and the nsg1Δnsg2Δ double mutants is consistently modest in comparison to that seen in other mutants, like hog1Δ.

Northern analysis of wild-type (FY2609), nsg1Δ (FY2892), nsg2Δ (FY2893) or nsg1Δ nsg2Δ (FY2894) strains grown in the presence (+) or absence (-) of oxygen for 5 hours.
Figure S3.—The MAPKKK, Ste11, is not required for hypoxic induction of PAUs or osmostress induction of GRE2, even in the absence of Hog1. O’Rourke and Herskowitz (Genes Dev 12: 2874-2886 (1998)) reported that there is signaling cross-talk in a hog1Δ mutant that can be eliminated by simultaneously deleting STE11. We found that this is not the case for PAUs or GRE2. A. Northern analysis was used to monitor PAU expression in cells grown in the presence (+) or absence (-) of oxygen for 5 hours. Wild-type (FY2609), hog1Δ (FY2873), ste11Δ (FY2875), and hog1Δ ste11Δ (FY2876) strains were used in both panels. B. Northern analysis was used to monitor GRE2 expression in cells grown in the presence of 1M sorbitol (+) or an equal volume of solvent (-) for 20 minutes.
FIGURE S4.—Heme and ergosterol depletion induces a subset of the Hog1-dependent hypoxic genes. Genes identified in Figure 5A as regulated by Hog1 that also contain consensus Upc2 binding sites in their promoters, were analyzed for their fold-induction when heme or ergosterol biosynthetic genes are repressed. The data shown here were obtained from a previous study that employed microarrays to monitor expression of all yeast genes 12 hours after turning off individual essential genes (Mnaimneh S, Davierwala AP, Haynes J, Moffat J, Peng WT, et al. Cell 118: 31-44 (2004)). Fold-induction was determined by comparing the expression of each gene to a wild-type strain grown under the same conditions. The bar labeled “average of all genes” represents the average induction of all ~6300 genes for that particular essential gene shutoff, and the standard deviation. The symbol labeled “PAUs” represents the average expression of all 24 PAU genes.
**TABLE S1**

*Primer#*   *Location*   *Purpose*   *Sequence (5' to 3')*
---   ---   ---   ---
FO7527   *UPC2* +690  Northern probe, forward  GCAGCAAATCTCAACCACAGAG
FO7528   *UPC2* +1381 Northern probe, reverse  CTGCAAGCTGATGAGCTCCT
FO2934   *PAU13* +13  Northern probe, forward  AATTTGAAGAGCTACATCTG
FO2935   *PAU13* +356  Northern probe, reverse  GTGATAGTGAGATACCGTC
FO1324   *SNR190* +1  Northern probe, forward  GCCCTGATGATAATG
FO1325   *SNR190* +190 Northern probe, reverse  GCCCTGATGATAATG
FO6549   *GRE2* +17  Northern probe, forward  AATTTGAAGAGCTACATCTG
FO6550   *GRE2* +903 Northern probe, reverse  ATGATATGAGATACCGTC
FO7977   *UPC2* +754 Real-time PCR, forward  CATTGGATGATGGAGGTAG
FO7978   *UPC2* +900 Real-time PCR, reverse  GCCCTGATGATAATG
FO961    *ACT1* +532 Real-time PCR, forward  TGTCACAACTTGAGGACGTA
FO962    *ACT1* +721 Real-time PCR, reverse  GCCCTGATGATAATG
FO3137   *PAU5* -40 Replace *PAU5* ORF with *URA3* ORF, forward  AAAAAATCAATATGCGAAAGCTACATATA
FO3138   *PAU5* +409 Replace *PAU5* ORF with *URA3* ORF, reverse  TATTTTCTATAATCGGAGCTCCTGAGTAT
FO3225   *PAU5* +521 Insert NatMX marker downstream of *PAU5*, forward  TTTGAAAGCTGTTAATGCGAAATTTGCTG
FO3226   *PAU5* +599 Insert NatMX marker downstream of *PAU5*, reverse  TTTGAAAGCTGTTAATGCGAAATTTGCTG
FO2647   *HAP2* -40 Replace *HAP2* ORF with *LEU2* marker, forward  TGGAAAAAGCTTCTTGTAGTTGAGGAA
FO2648   *HAP2* +839 Replace *HAP2* ORF with *LEU2* marker, reverse  TTTTTTTTGAGTAGATATGCGAAATTTGCTG
FO2965   *UPC2* -40 Replace *UPC2* ORF with *HIS3* marker, forward  TCAAAAAAGTTAAGCTAAGAATATATACAG
FO2966   *UPC2* +2782 Replace *UPC2* ORF with *HIS3* marker, reverse  TCAAAAAAGTTAAGCTAAGAATATATACAG
FO5707   *ERG25* -90 Insert KanMX marker and *GAL1* upstream of *ERG25* ORF, forward  AATCTTTTATATTAGTTGTAACTTTTTCTC
FO5708   *ERG25* +4 Insert KanMX marker and *GAL1* upstream of *ERG25* ORF, forward  AATCTTTTATATTAGTTGTAACTTTTTCTC
FO6385  
*HOG1* -40  
Replace *HOG1* ORF with *LEU2* marker, forward

GAL1<sub>pr</sub> upstream of *ERG25* ORF, reverse

GAAAACGGCAGACATTTTGAGATCCGGGT

TTT

AAAGGGAAAAACAGGGAAAAACTACAACAT

CGTATATAATAAGATTGTACTGAGAGTCG

AC

FO6386  
*HOG1* +1348  
Replace *HOG1* ORF with *LEU2* marker, reverse

FO6672  
*HOG1* +1266  
Insert 13 copies of myc and KanMX marker downstream of *HOG1*, forward

CGGTAACCAGGCCATACAGTACGCTAATG

ACGAAATATACAGATTGTACTGAGAGTCG

AC

FO6673  
*HOG1* +1348  
Insert 13 copies of myc and KanMX marker downstream of *HOG1*, reverse

FO6531  
*STE11* -40  
Replace *STE11* ORF with KanMX marker, forward

TAAGCTAGTATAGATAAGATCACCGGTAG

ACGAAATATACAGATTGTACTGAGAGTCG

AC

FO6532  
*STE11* +2194  
Replace *STE11* ORF with KanMX marker, reverse

CACTTTAGTGCATAAAAAGAATTAATAA

GTAGCCCTTTTCTGTGCGGTATTTCACAC

CG

FO6624  
*PBS2* -40  
Replace *PBS2* ORF with *HIS3* marker, forward

ATTATTATATTAAGCAGATCGAGACGT

TTGGCTACGAAAGATTGTACTGAGAGTCG

AC

FO6625  
*PBS2* +2047  
Replace *PBS2* ORF with *HIS3* marker, reverse

TATATTCACGTGCCTGTTTGCTTTTATTT

GGATATTAACGCTGTGCGGTATTTCACAC

CG

FO6682  
*SSK1* -40  
Replace all but the last 40 nucleotides of the *SSK1* ORF with *HIS3* marker, forward

ATGCTCAATTCTGCGTTACTGTGGAAGGT

TTGGCTACGAAAGATTGTACTGAGAGTCG

AC

FO6683  
*SSK1* +2139  
Replace all but the last 40 nucleotides of the *SSK1* ORF with *HIS3* marker, reverse

TCACAATTCTATTTGAGTGGGCGAGAGGT

TTGAATTTTTTCTGTGCGGTATTTCACAC

CG

FO3125  
*MGA2* -40  
Replace *MGA2* ORF with NatMX marker, forward

ACACACATATATATATATATATACGTAAAA

AAGCAGAGCATAGGCCACTAGTTGGAACAGT

CCFGTACAAAAACAGTATCATACACAGAACTAGTA

CACCACCTGAAAGATTTGTACTGAGAGTCG

AC

FO3126  
*MGA2* +3382  
Replace *MGA2* ORF with NatMX marker, reverse

FO7979  
*SPT23* -40  
Replace *SPT23* ORF with *URA3* marker, forward

ATCTATATAGTGAAGATTATAGTACGT

GAAAATGTCTCTGTGCGGTATTTCACAC

FO7980  
*SPT23* +3289  
Replace *SPT23* ORF with *URA3* marker, reverse
FO3998  *SGF73* -101  Replace *SGF73* ORF with KanMX marker, PCR of *sgf73D0::KanMX* allele from FY2475 in (Martens et al, 2005), forward

FO4000  *SGF73* +2062  Replace *SGF73* ORF with KanMX marker, PCR of *sgf73D0::KanMX* allele from FY2475 in (Martens et al, 2005), reverse

FO4785  *SUS1* -40  Replace *SUS1* ORF with *HIS3* marker, forward

FO4786  *SUS1* +481  Replace *SUS1* ORF with *HIS3* marker, reverse

FO4434  *SGF11* -514  Replace *SGF11* ORF with KanMX marker, PCR of *sgf11D0::KanMX* allele from deletion set (Giaever et al, 2002), forward

FO4435  *SGF11* +848  Replace *SGF11* ORF with KanMX marker, PCR of *sgf11D0::KanMX* allele from deletion set (Giaever et al, 2002), reverse

FO2201  *UBP8* -40  Replace *UBP8* ORF with *URA3* marker, forward

FO2202  *UBP8* +1456  Replace *UBP8* ORF with *URA3* marker, reverse

FO6530  *NSG1* -40  Replace *NSG1* ORF with *HIS3* marker, forward

FO6531  *NSG1* +916  Replace *NSG1* ORF with *HIS3* marker, reverse

FO6534  *NSG2* -40  Replace *NSG2* ORF with *LEU2* marker, forward

FO6535  *NSG2* +940  Replace *NSG2* ORF with *LEU2* marker, reverse

FO7014  *MNX2* -40  Replace *MNX2* ORF with *URA3* marker, forward
Replace MSV2 ORF with URA3 marker, reverse

TTATGAAGAAAGATCTATCGAATTAAAA
AATGGGCTCTACTGTGCGGTATTTCACAC
CG

Replace MSY4 ORF with LEU2 marker, forward

TTCCGCTTTTTTTTTTTTCTTCTTTATTT
AAAACAATATAAGATTGTACTGAGAGTGC
AC

Replace MSY4 ORF with LEU2 marker, reverse

TAGCTTTGCTCTTGGTATTGGCTTTTGA
CCTTATTTTTCTGTGCGGTATTTCACAC
CG

1 Location shown relative to the ATG start codon.

References

**TABLE S2**

**Microarray Data**

Table S2 is available for download as an Excel file at [http://www.genetics.org/cgi/content/full/genetics.111.128322/DC1](http://www.genetics.org/cgi/content/full/genetics.111.128322/DC1).