

Heme Levels Switch the Function of Hap1 of *Saccharomyces cerevisiae* between Transcriptional Activator and Transcriptional Repressor^{∇†}

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Changes in oxygen levels cause widespread changes in gene expression in organisms ranging from bacteria to humans. In *Saccharomyces cerevisiae*, this response is mediated in part by Hap1, originally identified as a heme-dependent transcriptional activator that functions during aerobic growth. We show here that Hap1 also plays a significant and direct role under hypoxic conditions, not as an activator, but as a repressor. The repressive activity of Hap1 controls several genes, including three *ERG* genes required for ergosterol biosynthesis. Chromatin immunoprecipitation experiments showed that Hap1 binds to the *ERG* gene promoters, while additional experiments showed that the corepressor Tup1/Ssn6 is recruited by Hap1 and is also required for repression. Furthermore, mutational analysis demonstrated that conserved Hap1 binding sites in the *ERG5* 5' regulatory region are required for repression. The switch of Hap1 from acting as a hypoxic repressor to an aerobic activator is determined by heme, which is synthesized only in the presence of oxygen. The ability of Hap1 to function as a ligand-dependent repressor and activator is a property shared with mammalian nuclear hormone receptors and likely allows greater transcriptional control by Hap1 in response to changing oxygen levels.

Oxygen is essential for several important biological processes, including aerobic respiration and sterol synthesis. Oxygen can also be harmful, through the production of reactive oxygen species (26). As part of the response to changes in the level of this vital yet potentially toxic molecule, cells produce extensive changes in transcription (5, 32, 34, 53, 61). In many cases, cells sense and respond to changes in oxygen levels by oxygen-dependent pathways. For example, in human cells, the stability and activity of hypoxia-inducible factor 1 α , required for the transcription of hypoxic-specific genes, are regulated by the hydroxylation of proline residues, a modification that is catalyzed by a family of oxygen-dependent prolyl hydroxylases. In the absence of oxygen, hydroxylation does not occur, hypoxia-inducible factor 1 α protein is stabilized, and hypoxic-specific genes are induced (27). In *Saccharomyces cerevisiae*, oxygen is sensed, at least in part, by the level of heme, whose synthesis is dependent upon oxygen (23). Heme is an essential molecule for signaling oxygen levels in many organisms (42).

One of the most extensively studied roles for heme with respect to controlling oxygen-mediated transcription in *S. cerevisiae* is its ability to bind to and activate the transcriptional activator protein Hap1. Hap1 is required to activate the transcription of many aerobic genes (genes expressed only in the presence of oxygen), including those required for respiration and controlling oxidative damage (5, 31, 54, 65). Hap1 also activates the transcription of *ROX1*, which encodes a repressor of many hypoxic genes (genes expressed only in low-oxygen

conditions) (5, 32, 54). Thus, Hap1 directly activates aerobic genes and indirectly represses hypoxic genes. Under hypoxic conditions, when heme is not synthesized, Hap1 does not function as an activator and the aerobic genes, including *ROX1*, are not transcribed. Current evidence suggests that heme binding mediates the ability of Hap1 to activate transcription by controlling the interaction of Hap1 with particular Hsp proteins (24, 35, 65).

Along with its established role as a transcriptional activator, some previous studies have suggested that Hap1 can also repress transcription. One recent study showed that Hap1 directly represses its own transcription in a heme-independent fashion (25). In addition, older experiments suggest that a small number of genes are repressed by Hap1 in the absence of heme or oxygen, although the results did not determine whether Hap1 repression is direct (11, 12, 17, 45, 58, 59). These studies suggest that the prevailing view of Hap1 as an aerobic-specific and heme-dependent activator may be too narrow.

To gain a comprehensive understanding of the functions of Hap1 during both aerobic and hypoxic growth, we performed microarray studies and combined the results with existing *in vivo* Hap1 DNA binding data (22) to identify direct targets of Hap1 regulation. These analyses led to the conclusion that Hap1 is active under hypoxic conditions as a direct repressor of several genes, including *ERG2*, *ERG5*, and *ERG11*, required for the biosynthesis of ergosterol, the major sterol in *S. cerevisiae*. In addition, chromatin immunoprecipitation (ChIP) experiments demonstrated that Hap1 binds to the promoters of these genes under both aerobic and hypoxic conditions and that the corepressor Tup1/Ssn6 is recruited by Hap1 in a hypoxic-specific fashion. Furthermore, mutational analyses of conserved promoter elements led to the identification of Hap1 binding sites in the *ERG5* promoter that are required for repression. Finally, our data show that heme converts Hap1 from a hypoxic transcriptional repressor to an aerobic tran-

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
FY2607	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 hap1</i>
FY2608	<i>MATa arg4-12 ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 hap1</i>
FY2609	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1</i>
FY2610	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1</i>
FY2611	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 hap1Δ0::kanMX</i>
FY2612	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1</i>
FY2613	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1</i>
FY2618	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 erg5-3</i>
FY2619	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 erg5-4</i>
FY2620	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 erg5-5</i>
FY2621	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 erg5-6</i>
FY2622	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 hsp82Δ0::KanMX</i>
FY2623	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-2</i>
FY2624	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-2</i>
FY2625	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-1</i>
FY2626	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-1</i>
FY2627	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-1,2</i>
FY2628	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-1,2</i>
FY2631	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 ssa2Δ0::LEU2 ssa4Δ0::HIS3</i>
FY2635	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 hsp82Δ0::KanMX HIS3::GAL1_{pr}-HSC82</i>
FY2636	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 ssa2Δ0::LEU2 ssa4Δ0::HIS3 KanMX::GAL1_{pr}-SSA1</i>
FY2637	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 hem1Δ0::KanMX</i>
FY2638	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 hem1Δ0::KanMX hap1Δ0::KanMX</i>
FY2639	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-10</i>
FY2640	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-11</i>
FY2641	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-12</i>
FY2642	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-13</i>
FY2643	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-14</i>
FY2644	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-4</i>
FY2645	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-7</i>
FY2646	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-8</i>
FY2647	<i>MATα ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 myc-HAP1 erg5-9</i>
FY2673	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 tup1Δ0::KanMX</i>
FY2674	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 HAP1 TUP1-myc</i>
FY2675	<i>MATa ura3Δ0 his3Δ200 leu2Δ0 lys2-1288 hap1Δ0::KanMX TUP1-myc</i>
O807	<i>MATα ura3Δ0 his3Δ0::HIS3</i>

scriptional activator and provide evidence that this mechanism allows for differential regulation of target genes at intermediate oxygen levels. These results, taken together with previous studies, expand our understanding of Hap1, demonstrating key functions for this regulator during both aerobic and hypoxic growth and providing insight into the biological significance of each role.

MATERIALS AND METHODS

Strains. All *S. cerevisiae* strains (Table 1) are isogenic with a *GAL2*⁺ derivative of S288C (62). The strains were constructed by standard methods, either by crosses or by transformation (4). The mutant *hap1* allele of S288C (18) was repaired in two transformation steps. First, the Ty1 insertion at the 3' end of the open reading frame (ORF) was replaced with the *URA3* gene. Second, *URA3* was replaced with a wild-type *HAP1* sequence from the Σ1278b strain background (49) while selecting for loss of *URA3* on 5-fluoroorotic acid. The presence of the wild-type *HAP1* allele was verified by PCR of the *HAP1* locus, mRNA expression of a Hap1 target gene (*CYC1*), DNA sequencing, and Southern blot analysis (see Fig. S1 at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>; data not shown). The *hap1Δ::kanMX*, *hem1Δ::kanMX*, *hsp82Δ::kanMX*, *ssa2Δ::LEU2*, and *ssa4Δ::HIS3* alleles were created by replacing the respective ORF with the *kanMX*, *LEU2*, or *HIS3* marker (6). The *hem1Δ::kanMX* strain was grown on 200 μg/ml δ-aminolevulinic acid (δ-ala), except where indicated. The *kanMX::GAL1_{pr}-SSA1* and *HIS3::GAL1_{pr}-HSC82* alleles were constructed by placing the *kanMX* or *HIS3* marker and the *GAL1* promoter upstream of the *SSA1* or *HSC82* ORF (38). The *myc-HAP1* and *TUP1-myc* alleles were created by inserting three copies of the *myc* epitope tag at the N-terminal or C-terminal

end, respectively, while retaining the endogenous promoter (48). The *myc-HAP1* allele is fully functional, as aerobic and hypoxic expression of both *ERG5* and *CYC1* is not affected (data not shown) (see Fig. 5B). The *TUP1-myc* allele is functional, as *ERG5* repression is not affected (data not shown). The *ERG5* promoter mutations were created as follows, with positions indicated relative to the *ERG5* ATG. The two Hap1 binding site mutations (*erg5-1* and *erg5-2*) were constructed by in vivo site-directed mutagenesis (50), replacing 12 bp from -426 to -415 or -571 to -560, respectively, with 5'-TACGGATCCACA-3' (a BamHI site flanked by random sequences) or 5'-AGTAGAATTCTC-3' (an EcoRI site flanked by random sequences), respectively. The 100-bp mutations (*erg5-3*, -144 to -243; *erg5-4*, -244 to -343; *erg5-5*, -344 to -443; and *erg5-6*, -444 to -543) were constructed by replacing the designated sequence with DNA encoding three copies of the *myc* epitope tag, a sequence that is 192 bp in length (48). The 50-bp mutations (*erg5-9*, -244 to -293; *erg5-8*, -269 to -318; and *erg5-7*, -294 to -343) were constructed by in vivo site-directed mutagenesis, replacing the designated sequence with 5'-ATCAAGATACCTGAAGTGTATGGATCCGCGCCGTAAGCCATCTGGAGT-3' (a BamHI site flanked by random sequences). The 20-bp mutations (*erg5-14*, -244 to -263; *erg5-13*, -264 to -283; *erg5-12*, -284 to -303; *erg5-11*, -304 to -323; and *erg5-10*, -324 to -343) were constructed by in vivo site-directed mutagenesis, replacing the designated sequence with 5'-GTAACATGGATCCCGTGCTA-3' (a BamHI site flanked by random sequences). Hsp70 and Hsp90 are essential, so conditional strains were constructed. Hsp90 is encoded by two genes, *HSP82* and *HSC82*; *HSP82* was deleted while *HSC82* was placed under control of the *GAL1* promoter. Hsp70 is encoded by four genes, *SSA1-4*, although *SSA3* is not normally expressed. *SSA2* and *SSA4* were deleted, and *SSA1* was placed under *GAL1* control. Strains containing only *ssa2Δ ssa4Δ* or *hsp82Δ* were used as controls for the carbon source.

Media and growth conditions. Cells were grown at 30°C in YPD (1% yeast extract and 2% peptone supplemented with 2% glucose) except where indicated. YP galactose medium contained 1% yeast extract and 2% peptone supplemented with 2% galactose. For all experiments, mid-log-phase cells (approximately 1 × 10⁷ to 2 × 10⁷ cells/ml) were used for analysis. For hypoxic growth, 25- to 250-ml cultures growing in 250-ml or 500-ml flasks were continuously sparged with ultra-high-purity nitrogen at approximately 3 liters/min. A critical aspect of observing hypoxic effects after the growth of cultures in nitrogen was chilling the cultures by surrounding the flasks with ice for 5 min per 50 ml of culture, with continued exposure to nitrogen prior to the preparation of RNA. Aerobic cultures were chilled in an identical manner. A previous study (9) did not observe hypoxic repression of *ERG* genes, and the different results may be due to this difference in treatment of the cells. For hypoxic growth and certain other experiments described in the text, the media were supplemented with 20 μg/ml ergosterol (Sigma), 0.5% (vol/vol) Tween 80 (Sigma) as a source of unsaturated fatty acids, and 0.5% (vol/vol) ethanol. For supplementing growth media with heme, a 6.5 mg/ml heme (hemin; Sigma/BioChemika) solution in 0.1 M NaOH was prepared, incubated at 37°C for 1 h, and adjusted to pH 7.0 with HCl. The solution was added to the media within 3 days of preparation, 40 min before exposing the cells to hypoxic conditions. δ-Aminolevulinic acid (δ-ala; Sigma) was dissolved in water at 20 mg/ml and added to the media at the indicated concentration. Strains containing mutations in the *HSP* or *SSA* genes were maintained on YP galactose medium, shifted to YP galactose or YPD for 4 h, and then grown under aerobic or hypoxic conditions for an additional 4 h.

Expression microarray analysis. Cells were grown under aerobic or hypoxic (supplemented with ergosterol, Tween 80, and ethanol) conditions for 8 h. RNA was isolated using a hot-phenol method (4) and reverse transcribed. The resulting cDNA was labeled with Cy3-dUTP or Cy5-dUTP, using a BioPrime kit (Invitrogen), and hybridized to yeast Y6.4 whole ORF microarrays (Toronto Microarray Centre, University Health Network). Fluorescence was quantitated on a GenePix 4000B scanner with GenePix 5.0 software (Axon Instrumentation). Expression values were subjected to total intensity and LOWESS normalization, using MIDAS software (TIGR) (46). Three biological replicates were performed for wild-type (strain FY2609) and *hap1Δ* (strain FY2611) cells, and the normalized data are presented in Table S1 in the supplemental material. The mRNA levels that were significantly changed between aerobic and hypoxic growth of *HAP1* wild-type cells were identified by a SAM (statistical analysis of microarrays) package, using a false discovery rate cutoff of 9.81% (see Table S1 in the supplemental material) (57). In this way, 878 aerobic and 759 hypoxic genes were identified, and subsequent analyses focused on the genes that changed more than twofold (197 and 217 genes, respectively) (see Table S1 in the supplemental material). Hap1-regulated genes were defined as those that changed more than twofold in *hap1Δ* compared to the wild type. Hap1 binding to yeast gene promoters was determined previously by ChIP-chip analysis (22); for the current study, a *P* value of <0.01 was used as a threshold for statistically significant

binding. Forty-two of the 6,307 genes (0.7%) represented on the microarray are involved in ergosterol metabolism; 5 out of 10 (50%) of the *Hap1* genes repressed in hypoxic conditions were in this category, a significant enrichment ($P < 10^{-162}$, as determined by the chi-square test for independence).

Northern hybridization analysis. Northern hybridization analysis was performed as previously described (4). The following probes were amplified by PCR for random ^{32}P labeling (positions are relative to that of *ATG*): *CYC1* (+6 to +279), *ERG2* (+189 to +546), *ERG5* (+47 to +1379), *ERG11* (+15 to +731), *HIS3* (-27 to +376), *HSP82* (-93 to +1269; detects both *HSP82* and *HSC82*), *SNR190* (entire gene), and *SSA1* (+238 to +831; detects all *SSA* genes). Note that, based on a comparison to rRNA levels, the *SNR190* transcript used as a loading control did not change under the different growth conditions in these experiments. Expression was quantitated on a Molecular Dynamics Storm 860 phosphorimager, using ImageQuant TL software (Amersham Biosciences), and the values are presented in the appropriate figure legends. For *ERG5* Northern hybridization analysis, the value for each strain was first normalized to that of the loading control, *SNR190*, and this ratio was then normalized to the ratio for a wild-type strain after a 4-h shift to hypoxic conditions. The numbers presented are the averages of multiple experiments that were performed at least twice and often multiple times.

ChIP analysis. ChIP experiments to examine *Hap1* binding in vivo were performed as previously described (41), using strains that contained the *myc-HAP1* or *myc-TUP1* allele. Formaldehyde cross-linking was carried out for 20 min (*myc-HAP1* strain) or 90 min (*TUP1-myc* strain). For immunoprecipitation, we used an anti-myc antibody (clone A14; Santa Cruz Biotechnology). DNA was quantitated by real-time PCR, using a Stratagene MX3000P (2). Binding to the indicated region was calculated relative to binding to an ORF-free region on chromosome I and was normalized to the level of input chromatin. As a control for shearing efficiency, no binding was detected in the two ORFs adjacent to the *ERG5* regulatory region (positions are relative to that of *ERG5* ATG): *ERG5* (+1272 to +1420) and *SOK2* (-2174 to -2012) (data not shown). To control for background binding, we normalized *myc-Hap1* or *Tup1-myc* binding to the amount of binding detected by ChIP in a strain expressing untagged (wild-type) *HAP1* or *TUP1*. Each experiment was performed at least three times. The primer sequences are listed in Fig. S6 at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>.

Microarray data accession numbers. The microarray data discussed in this publication 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 number GSE8613.

RESULTS

Microarray experiments identify new *Hap1* targets and suggest that *Hap1* is a hypoxic repressor. Heme-dependent activation by *Hap1* during aerobic growth is known to play a widespread role in oxygen-mediated transcription in *S. cerevisiae* (65). However, previous studies have suggested that *Hap1* also has other roles in response to changing oxygen levels (11, 12, 17, 45, 58, 59). In order to determine the global role of *Hap1*, we performed microarray analyses in the sequenced and systematically characterized *S. cerevisiae* S288C genetic background. While previous microarray studies have been done to study *Hap1*-mediated regulation in *S. cerevisiae* (5, 54), none have been done in the S288C background. Such an analysis in the S288C background would likely benefit from the many types of genome-wide studies that have been performed with S288C strains, including genome-wide analysis of *Hap1* binding (22). One drawback of S288C is that it contains a mutant *hap1* allele that causes a partial loss of function (18). Therefore, for our studies, we replaced the S288C *hap1* mutant gene with either a wild-type *HAP1* sequence (hereafter referred to as *HAP1*) or a complete deletion of *HAP1* (*hap1Δ*; see Materials and Methods). Using these strains, we carried out microarray experiments to compare mRNA levels during aerobic and hypoxic growth for both *HAP1* and *hap1Δ* strains.

Consistent with previous studies, our results show that there

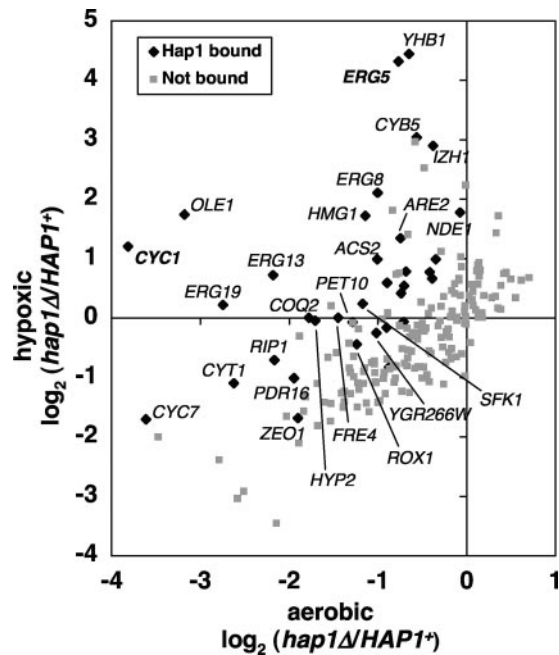


FIG. 1. *Hap1* regulates aerobic genes during both aerobic and hypoxic conditions. Expression microarray experiments revealed 197 genes that were significantly downregulated in wild-type cells upon a shift to hypoxic growth. The *hap1Δ/HAP1* expression ratio of these 197 genes is shown for aerobic and hypoxic cells. The promoters of 34 of these genes (black diamonds) were significantly bound by *Hap1* under aerobic conditions. Points labeled with gene names changed more than twofold in *hap1Δ* compared to *HAP1*. Statistical analyses are described in the Materials and Methods section.

are a large number of aerobic-specific and hypoxic-specific mRNAs in *HAP1* strains (5, 32–34, 53, 54). Specifically, our experiments identified 197 aerobic genes and 217 hypoxic genes (see Table S1 in the supplemental material; see Materials and Methods for statistical analysis). Approximately 29% of the hypoxic genes, including *TIR1* and *HEM13*, and ~14% of the aerobic genes, including *CYC1* and *ROX1*, have been previously described (13, 20, 39, 64). Furthermore, our microarray results for *hap1Δ* mutants show that approximately 58% of hypoxic mRNAs and approximately 45% of aerobic mRNAs are dependent upon *Hap1* for their regulation (see Table S1 in the supplemental material). Thus, our results are consistent with those of previous studies that showed that *S. cerevisiae* regulates transcription of hundreds of genes in response to different oxygen levels and that *Hap1* plays a prominent role in this regulation.

To gain insight into the number of genes that are directly controlled by *Hap1*, we compared our microarray data to previously published results that analyzed genome-wide binding by *Hap1*. Although these binding studies (22) were performed under aerobic conditions, this comparison suggests that all of the *Hap1*-dependent genes expressed in hypoxic conditions are indirectly regulated by *Hap1*, consistent with the previously proposed model that this mode of *Hap1* control occurs via regulation of the repressor *Rox1* (28). In contrast, among the 89 *Hap1*-dependent aerobic genes that we identified, 25 (28%) are putative direct targets of *Hap1* (Fig. 1; see the gene list in Fig. S2 at <http://genetics.med.harvard.edu/~winston>

/Winston%20Lab%20Links.html) (22). These genes fell into two overlapping sets with respect to their regulation by Hap1: aerobically activated and hypoxically repressed. First, in agreement with its known role as an aerobic activator, Hap1 was required for the aerobic expression of 19 (76%) of these 25 putative direct targets. Six of these 19 genes, including *ROX1*, *CYC1*, and *CYC7*, were previously known to be regulated by Hap1 (42), while most of the genes represent previously unknown Hap1 targets. The latter group contains genes involved in respiration (for example, *COQ2* and *RIP1*) and lipid metabolism (*ERG13*, *ERG19*, *SFK1*, and *PDR16*), among other functions. We did not identify all known Hap1 target genes by this analysis, perhaps due to differences in strain backgrounds or hypoxic conditions. For example, *QCR2* was previously identified as an aerobic gene activated by Hap1 (14). Our analysis suggests that *QCR2* is indeed activated by Hap1 but that it is not aerobic nor significantly bound by Hap1 (although the level of binding is quite close to the threshold) (see Table S1 in the supplemental material).

In addition to its role as an aerobic activator, Hap1 was required for the repression of 10 genes during hypoxic growth, a condition in which Hap1 has been generally viewed as inactive (42, 65). Half of the Hap1-repressed genes that were detected by our microarrays (*HMG1*, *CYB5*, *ERG5*, *ERG8*, and *ARE2*) are involved in the metabolism of ergosterol, many more than would be expected by chance ($P < 10^{-162}$; see Materials and Methods). These data suggest that Hap1 plays a significant but to date largely unexamined role as a hypoxic repressor. This role is the focus of the remaining experiments in this study.

Hap1 is a hypoxic repressor of *ERG* genes. We wanted to test by independent methods whether Hap1 functions as a direct transcriptional repressor under hypoxic conditions. Since many of the genes that were identified as repressed by Hap1 during hypoxic growth are involved in ergosterol metabolism, we focused on three ergosterol biosynthetic genes (*ERG2*, *ERG5*, and *ERG11*). One of these genes, *ERG5*, was in the group of 10 aerobic genes identified by our microarray analysis as direct targets of Hap1 repression during hypoxic growth. *ERG2* and *ERG11* were initially identified by our microarrays as genes subject to Hap1 repression during hypoxic growth (see Table S1 in the supplemental material), and our subsequent analysis, described below, has shown that they are indeed aerobic genes. Furthermore, genome-wide analysis suggests that Hap1 binds to their promoters (22). In addition, *ERG11* was previously suspected to be Hap1 repressed (12, 17, 59). All three genes contain consensus Hap1 binding sites that are conserved among the sequenced sensu stricto yeast strains (*ERG5* is described below; for *ERG2* and *ERG11*, see Fig. S3 at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>).

To measure mRNA levels for *ERG2*, *ERG5*, and *ERG11* during aerobic and hypoxic growth, we performed Northern analysis. Our results (Fig. 2) show that *ERG2*, *ERG5*, and *ERG11* are all expressed during aerobic growth and repressed during hypoxic growth in *HAP1* cells. Importantly, the hypoxic repression of these genes is Hap1 dependent, as they are no longer repressed, but rather are induced, in a *hap1Δ* mutant. In addition, Hap1 appears to have a modest role in activation of *ERG5* during aerobic growth, as *hap1Δ* caused a reproducible

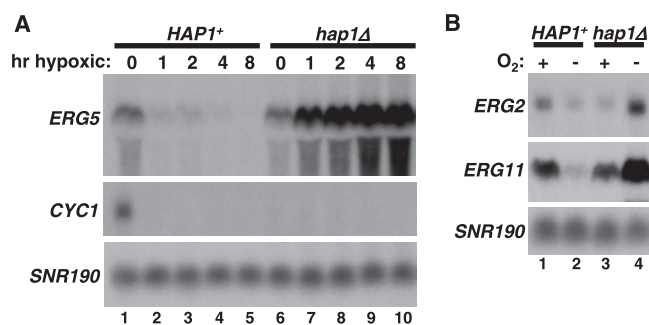


FIG. 2. Hap1 is both an aerobic activator of *CYC1* and a hypoxic repressor of *ERG* genes. (A) *ERG5* and *CYC1* mRNA levels were measured by Northern analysis in a *HAP1* strain (FY2609) and a *hap1Δ* strain (FY2611) grown in hypoxic conditions for the indicated times. Shown is a typical Northern blot with the averages of the results of multiple Northern analyses (as described in Materials and Methods) as follows: lane 1, 5.4; lane 2, 1.7; lane 3, 2.0; lane 4, 1.0; lane 5, 0.9; lane 6, 4.5; lane 7, 8.7; lane 8, 11.8; lane 9, 15.9; lane 10, 19.8. hr, hour. (B) Northern analyses were performed to measure *ERG2* and *ERG11* mRNA levels in *HAP1* (FY2609) and *hap1Δ* (FY2611) strains grown aerobically (+) and hypoxically (–) for 8 h. The averages of the quantitations from Northern analyses for *ERG2* are as follows: lane 1, 1.8; lane 2, 1.0; lane 3, 1.3; lane 4, 4.8. The averages of the quantitations from Northern analyses for *ERG11* are lane 1, 2.9; lane 2, 1.0; lane 3, 2.7; lane 4, 7.3.

decrease in *ERG5* aerobic mRNA levels. However, *hap1Δ* did not cause a detectable defect in *ERG2* or *ERG11* activation during aerobic growth. An activating role for Hap1 in the aerobic expression of *ERG* genes has been previously shown (9, 29, 52, 56). To contrast the role of Hap1 as a hypoxic repressor with its role as an aerobic activator (44), we also measured *CYC1* mRNA levels in *HAP1* and *hap1Δ* strains. In *HAP1* strains, *CYC1* transcription mimics that of the three *ERG* genes, on during aerobic growth and off during hypoxic growth (Fig. 2A). In striking contrast to the regulation of *ERG2*, *ERG5*, and *ERG11*, however, a *hap1Δ* mutation abolished aerobic expression of *CYC1*, while not affecting its lack of expression during hypoxic growth. These results demonstrate that Hap1 plays a significant role in transcriptional repression of a specific set of genes during hypoxic growth.

Previous studies that examined Hap1 DNA binding throughout the genome (22) and specifically at the *ERG3* gene (9) used aerobic growth conditions. To determine whether Hap1 acts directly to repress transcription of *ERG2*, *ERG5*, and *ERG11* during hypoxic growth, we performed Hap1 ChIP experiments to compare Hap1 binding in both growth conditions. In this experiment, we included *CYC1* for comparison. Our results (Fig. 3) show that Hap1 is specifically associated with the promoters of *ERG2*, *ERG5*, and *ERG11* under both aerobic and hypoxic conditions and suggest that there is a greater level of binding under hypoxic conditions when Hap1 represses transcription of these genes. The level of binding to *ERG2* was significantly less than to *ERG5* and *ERG11*, correlating with the more modest regulation of this gene by Hap1. As expected, Hap1 bound specifically to the *CYC1* promoter under aerobic conditions, when Hap1 is known to activate *CYC1* (Fig. 3). Under hypoxic conditions, Hap1 was also bound to *CYC1*, despite the fact that Hap1 does not function at *CYC1* under these conditions. Taken together, our microarray, Northern

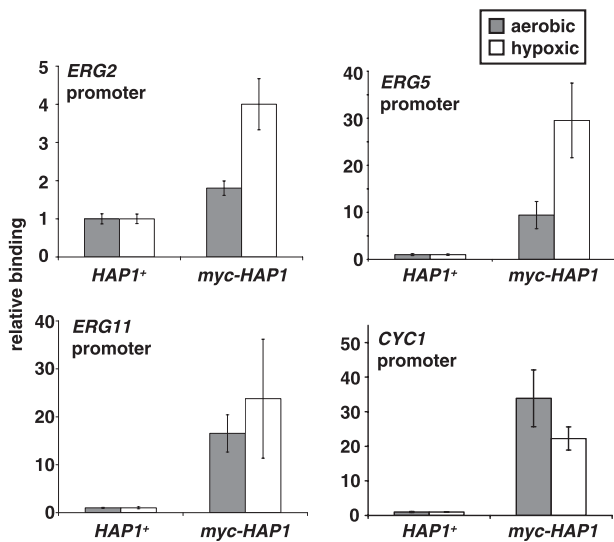


FIG. 3. Hap1 binds to the promoters of *ERG* and *CYC1* genes under aerobic and hypoxic conditions. Hap1 binding was measured by ChIP (see Materials and Methods), using an α -myc antibody, and by quantitating the following gene sequences by real-time PCR: *ERG2* (−491 to −371), *ERG5* (−323 to −189), *ERG11* (−607 to −490), and *CYC1* (−397 to −268). Cells expressing Hap1 fused to a myc epitope tag (*myc-HAP1*, strain FY2612 or FY2613) or untagged *HAP1* (*HAP1*⁺, strain FY2609 or FY2610) were grown under aerobic and hypoxic conditions for 4 h. Shown are the means and standard errors of at least three independent experiments.

analysis, and ChIP results are most consistent with the conclusion that Hap1 has two distinct and direct regulatory functions in vivo: transcriptional activation during aerobic growth and transcriptional repression during hypoxic growth.

Tup1 is required for Hap1-mediated repression. Transcriptional repressors often function through the recruitment of corepressor complexes. One candidate corepressor complex to function with Hap1 in repression is Tup1/Ssn6 (40), previously shown to play roles in repression during hypoxic growth (for examples, see references 1, 8, and 11). We tested whether Tup1/Ssn6 is required for Hap1-mediated repression of *ERG5*.

Our Northern blots show that Tup1, like Hap1, is required for *ERG5* repression under hypoxic conditions but has little role in *ERG5* aerobic activation (Fig. 4A). In contrast, neither the aerobic activation nor the hypoxic repression of *CYC1* is dependent upon Tup1, consistent with a role for Tup1 specifically in Hap1-dependent repression. To test whether the role of Tup1 is direct, we performed ChIP experiments (Fig. 4B). These experiments revealed that Tup1 is physically associated with the *ERG5* promoter preferentially under hypoxic conditions when the gene is repressed. Furthermore, the association of Tup1 is Hap1 dependent. These results strongly suggest that Hap1 functions as a direct transcriptional repressor under hypoxic conditions, at least in part by the recruitment of the corepressor Tup1/Ssn6.

Multiple Hap1 binding sites contribute to *ERG5* regulation. Studies of DNA binding by Hap1 to known target genes have identified a consensus site for Hap1 binding that contains two tandem CGG triplets separated by 6 bp, although functional variations of this consensus have been identified (43, 65). We identified two putative Hap1 sites within the 750 bp between the *ERG5* start codon and the stop codon of the adjacent gene (*SOK2*). Both sites are conserved among the *Saccharomyces* sensu stricto yeast species (7), although the proximal site 1 exhibits greater conservation than site 2 (Fig. 5A). To test whether the sites are important for *ERG5* regulation, the 12-bp sites were mutated and then analyzed for effects on *ERG5* mRNA levels during both aerobic and hypoxic growth. Northern blotting revealed that while each site is required for full activation of *ERG5* during aerobic growth, only the site closer to *ERG5*, site 1, is required for repression of *ERG5* during hypoxic growth (Fig. 5B). In this mutant (*erg5-1*), some derepression of *ERG5* is observed during hypoxic growth, although the level is significantly less than that seen in a *hap1Δ* mutant (Fig. 5B, compare lane 6 to lane 10). The double site mutant (*erg5-1,2*) (Fig. 5B, lanes 7 and 8) exhibited an *ERG5* expression pattern similar to that of the site 1 mutant, further suggesting that only site 1 plays a role in *ERG5* repression. These results demonstrate that these two Hap1 binding sites participate in *ERG5* regulation. However, they are not sufficient for

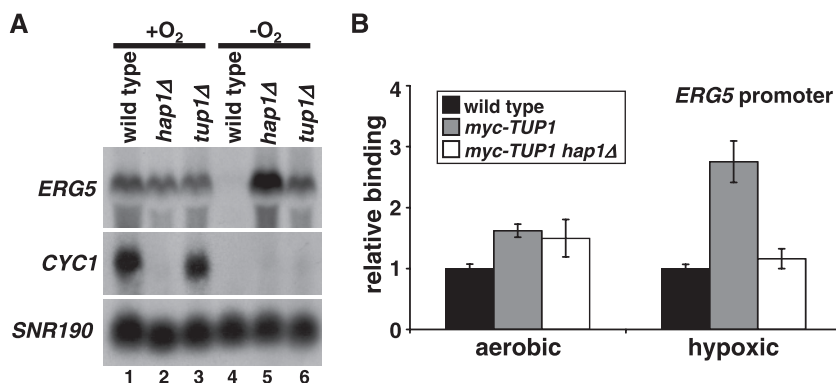


FIG. 4. Hap1 represses *ERG5* through recruitment of the Tup1 corepressor. (A) *ERG5* and *CYC1* expression were monitored by Northern blotting of wild-type (FY2609), *hap1Δ* (FY2611), and *tup1Δ* (FY2673) cells grown hypoxically (−O₂) or aerobically (+O₂) for 4 h. The averages of the quantitations from Northern analyses are as follows: lane 1, 9.4; lane 2, 3.8; lane 3, 6.5; lane 4, 1.0; lane 5, 13.2; lane 6, 7.3. (B) Tup1 binding to the *ERG5* promoter (−323 to −189) was monitored by ChIP experiments in aerobic or hypoxic conditions, using an untagged (wild-type, FY2609), *TUP1-myc* (FY2674), or *TUP1-myc hap1Δ* (FY2675) strain. Shown are the means and standard errors for three independent experiments.

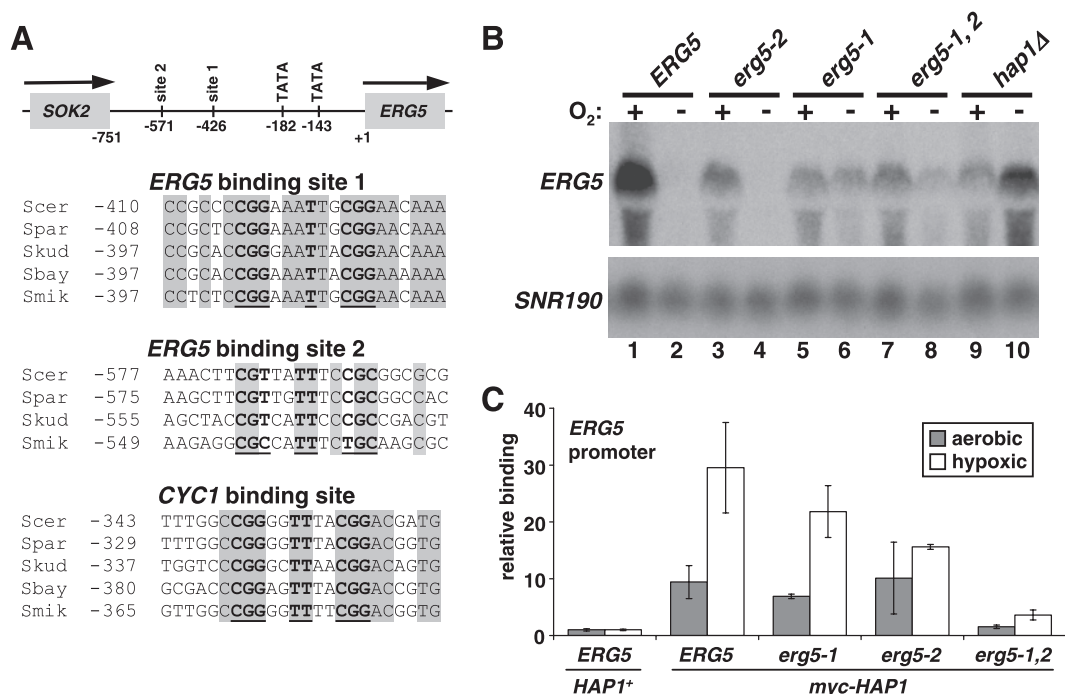


FIG. 5. Two Hap1 binding sites contribute to *ERG5* regulation. (A) At the top is a diagram of the structure of the *ERG5* promoter, with numbers denoting locations relative to the *ERG5* ATG. The two consensus Hap1 binding sites within the *ERG5* promoter of the related yeast species: *S. cerevisiae* (Scer), *S. paradoxus* (Spar), *S. kudravzevii* (Skud), *S. bayanus* (Sbay), and *S. mikitae* (Smik). For site 2, there is no consensus site in *S. bayanus*. Also shown is the alignment for UAS1 from the *CYC1* promoter. The numbers denote the first nucleotide of each of the sequences relative to ATG. Nucleotides shown in bold are consensus Hap1 positions, while the nucleotides in shaded boxes denote conserved positions. Site 1 is the reverse complement. (B) *ERG5* mRNA levels were measured by Northern blotting in strains containing a wild-type *ERG5* promoter (*ERG5*, FY2612), *ERG5* Hap1 site 2 mutant (*erg5-2*, FY2624), *ERG5* Hap1 site 1 mutant (*erg5-1*, FY2626), *ERG5* with both sites mutant (*erg5-1,2*, FY2628), and *hap1Δ* (FY2611). Strains that are not *hap1Δ* contain the *myc-HAP1* allele. Cells were grown hypoxically (-) or aerobically (+) for 4 h. Shown is one of the Northern blots from multiple experiments. The averages of the quantitations from multiple Northern analyses are as follows: lane 1, 9.4; lane 2, 1.0; lane 3, 6.1; lane 4, 1.6; lane 5, 6.4; lane 6, 2.9; lane 7, 3.6; lane 8, 2.8; lane 9, 3.8; lane 10, 13.2. (C) Hap1 binding to the *ERG5* promoter (-323 to -189) was monitored by ChIP in the following strains: untagged *HAP1* (*ERG5*, *HAP1*⁺, FY2610), *myc-HAP1* (FY2612 or FY2613), *myc-HAP1 erg5-1* (FY2625), *myc-HAP1 erg5-2* (FY2623), and *myc-HAP1 erg5-1,2* (FY2627). Shown are the means and standard errors for three independent experiments. Note that the data for untagged *HAP1* and *myc-HAP1* are also shown in Fig. 3.

conferring the full level of Hap1-dependent repression of *ERG5* that occurs during hypoxic growth.

To test whether the binding of Hap1 to the *ERG5* promoter depends on site 1 and site 2, we performed ChIP experiments using wild-type and site mutant strains. Our results (Fig. 5C) show that each site contributes to Hap1 binding at *ERG5*. In the individual site 1 or site 2 mutants, there were modest decreases in Hap1 binding, while in the double mutant, Hap1 binding was greatly diminished, although it was still greater than background levels. From these results, we concluded that Hap1 binds to each site in vivo.

Our analysis of the two Hap1 consensus binding sites suggested that additional *ERG5* promoter elements are likely required for *ERG5* repression during hypoxic growth. To identify such promoter elements, we systematically mutated 100-bp sequences in the large intergenic region 5' of *ERG5* (Fig. 6A). Northern blots of strains containing these mutations showed that two regions, 4 and 5, are required for full repression. The sequences changed by mutation 5 contain Hap1 binding site 1, and mutation 5 had a modest effect on *ERG5* repression, similar to that of mutation 1 (compare *erg5-1* in Fig. 5B with *erg5-5* in Fig. 6B). In contrast, mutation 4 caused a dramatic

effect on *ERG5* repression, similar to that caused by a *hap1Δ* mutation. Interestingly, although this 100 bp does not contain a consensus Hap1 binding site, it does contain five conserved Hap1 half-sites, more than would be expected by chance ($P < 10^{-26}$) (Fig. 6C). Such half-sites have been shown to be sufficient for Hap1 binding in certain sequence contexts (25, 43). Indeed, mutating region 4 caused a reduction in Hap1 binding at *ERG5* (Fig. 6D) that was similar in degree to the mutation of either of the full Hap1 sites (Fig. 5C).

To define more precisely the sequences within region 4 that are required for repression, we constructed and analyzed mutations that change shorter sequences. First, three mutations that alter 50 bp each were constructed within the region 4 sequence (mutations 7, 8, and 9) (Fig. 7A and 6C). Our results (Fig. 7B) show that each one of these mutations confers a significant level of derepression, with the strongest derepression observed in the region 8 mutant, which alters three of the five half-sites. Then we constructed a series of five mutations across region 4 that altered only 20 bp each. All of these changes caused derepression, with the strongest effects observed when mutating the middle of region 4 (Fig. 7C), consistent with the pattern of derepression seen with the 50-bp

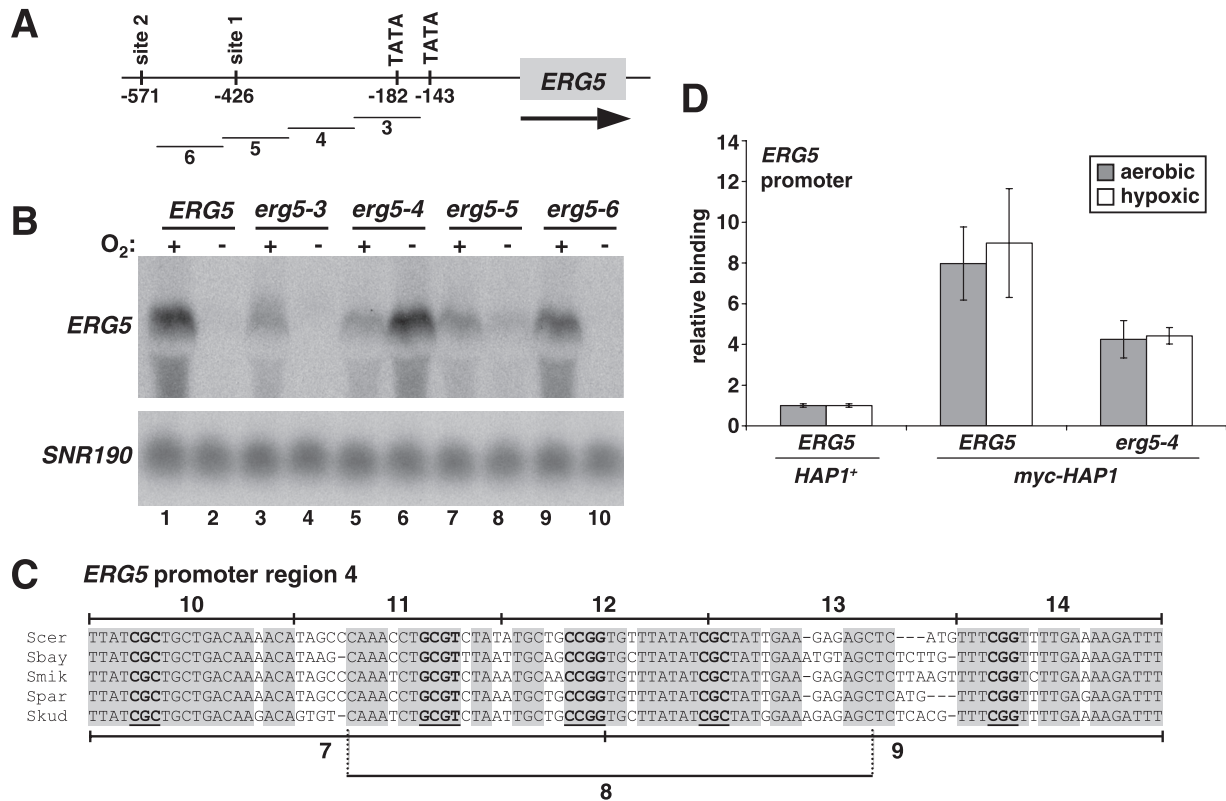


FIG. 6. Additional *ERG5* promoter elements are important for repression. (A) Structure of the *ERG5* promoter denoting regions 3 to 6 that were mutated. (B) *ERG5* mRNA levels were measured by Northern blotting in strains containing the following *ERG5* promoter mutations: *ERG5* (FY2609), *erg5-3* (FY2618), *erg5-4* (FY2619), *erg5-5* (FY2620), or *erg5-6* (FY2621). Cells were grown aerobically (+) or hypoxically (-) for 4 h. Shown is one of the Northern blots from multiple experiments. The averages of the quantifications are as follows: lane 1, 9.4; lane 2, 1.0; lane 3, 6.2; lane 4, 0.8; lane 5, 4.5; lane 6, 9.2; lane 7, 4.4; lane 8, 2.5; lane 9, 9.7; lane 10, 0.8. (C) Conservation of region 4 in the sequenced sensu stricto yeast species *S. cerevisiae* (Scer), *S. paradoxus* (Spar), *S. kudravzevii* (Skud), *S. bayanus* (Sbay), and *S. mikitae* (Smik). Putative Hap1 half-sites in the *ERG5* promoter are denoted in bold and are underlined, while the nucleotides in the shaded boxes denote conserved nucleotides. Regions 7 to 14 are the same regions noted and analyzed for Fig. 7. Five conserved half-sites were found in region 4, more than the 0.2 CGG or CGC sites expected by chance ($P < 10^{-26}$, as determined by the chi-square test for independence). The background rate was determined by counting the number of conserved sites (on either strand) within 5,000 bp of the promoter sequence (specifically within -100 to -600 bp of ATG) of 10 randomly chosen genes. (D) Hap1 binding to the *ERG5* promoter (-482 to -367) was monitored by ChIP experiments with the following strains: untagged *HAP1* (*HAP1*⁺ *ERG5*, strain FY2610), *myc-HAP1* (strain FY2612), and *myc-HAP1 erg5-4* (strain FY2644). Shown are the means and standard errors of three independent experiments. The lower level of Hap1 binding to the *ERG5* promoter compared to the results shown in Fig. 3 and 5C may be due to strain or primer differences; the primers used for the experiments in Fig. 3 and 5C could not be used here because one of the primers hybridized to the mutated region 4.

changes. These results demonstrate that besides Hap1 binding site 1, another region of the *ERG5* promoter that contains multiple Hap1 half-sites is required for Hap1-mediated repression. The promoters of *ERG2*, *ERG11*, and *CYC1* also contain multiple conserved half-sites (see Fig. S4 at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>). The fact that such sites are present at both Hap1-repressed and Hap1-activated genes suggests that they are not the determining factor in the role of Hap1 at a promoter.

Hsp70 is partially required for *ERG5* repression, while Hsp90 is dispensable. Previous studies have shown that Hap1 is physically associated with the chaperones Hsp70 and Hsp90 and that these associations are important in regulating Hap1-mediated activation of *CYC1* (24, 35, 36). To test whether these proteins also regulate Hap1 repression of *ERG5*, we analyzed *ERG5* mRNA levels in strains that can be depleted for these essential chaperones (see Materials and Methods). Briefly, to test the role of Hsp70, we used a

mutant strain in which the one remaining Hsp70 gene, *SSA1*, was under the control of the *GAL1* promoter. Our results show that when cells are depleted for Hsp70, there is a modest derepression of *ERG5* during hypoxic growth (see Fig. S5A at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>; compare lane 4 to lane 8). Contrary to previous results (24), however, depletion of Hsp70 in our strains had no effect on the repression of *CYC1* (see Fig. S5A at <http://genetics.med.harvard.edu/~winston/Winston%20Lab%20Links.html>; compare lane 2 to lane 6). We next tested the role of Hsp90 by deleting the *HSP82* gene and placing the *HSC82* gene under *GAL1* control. In contrast to earlier observations (36), Hsp90 did not appear to play a role in *CYC1* activation in our strains (Fig. S5B). Upon Hsp90 depletion, the levels of *CYC1* mRNA showed the same mild reduction in both the control and

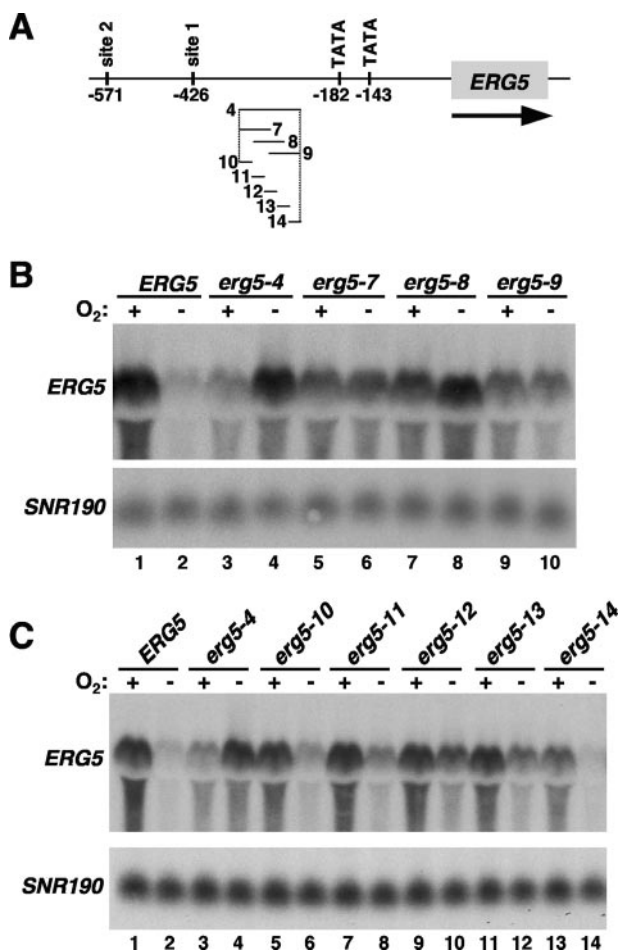


FIG. 7. Multiple sequences within *ERG5* region 4 are required for repression. (A) Structure of the *ERG5* promoter denoting regions 4 and 7 to 14 that were mutated. (B) Effect of 50-bp mutations in region 4 on *ERG5* expression. Northern blotting was performed for strains containing the following *ERG5* promoter alleles: *ERG5* (FY2612), *erg5-4* (FY2644), *erg5-7* (FY2645), *erg5-8* (FY2646), and *erg5-9* (FY2647). The averages of the quantitations from multiple Northern blotting experiments are as follows: lane 1, 9.4; lane 2, 1.0; lane 3, 3.7; lane 4, 8.1; lane 5, 6.0; lane 6, 4.1; lane 7, 6.2; lane 8, 4.3; lane 9, 3.9; lane 10, 2.6. (C) Effect of 20-bp mutations on *ERG5* expression. Northern blotting was performed for strains containing the following *ERG5* promoter alleles: *ERG5* (FY2612), *erg5-4* (FY2644), *erg5-10* (FY2639), *erg5-11* (FY2640), *erg5-12* (FY2641), *erg5-13* (FY2642), and *erg5-14* (FY2643). Cells were grown aerobically (+) or hypoxically (-) for 4 h. The averages of the quantitations from Northern analyses are as follows: lane 1, 9.4; lane 2, 1.0; lane 3, 3.7; lane 4, 8.1; lane 5, 4.9; lane 6, 1.7; lane 7, 5.6; lane 8, 2.1; lane 9, 5.1; lane 10, 3.3; lane 11, 6.1; lane 12, 2.9; lane 13, 9.2; lane 14, 1.7.

depletion strains, possibly a result of the shift in carbon source. Similarly, there was no effect on *ERG5* mRNA levels under any growth condition. Taken together, our results suggest that Hsp70 contributes modestly to the ability of Hap1 to serve as a repressor during hypoxic growth. However, the small effect of Hsp70 depletion on *ERG5* derepression in comparison to the large effect of a *hap1Δ* mutation suggests that additional factors play significant roles in determining the activity of Hap1.

***ERG* genes and *CYC1* are differentially regulated at intermediate heme levels.** Our studies have shown that *ERG5* and

other genes are repressed during hypoxic growth by Hap1. As activation of *CYC1* and other aerobic genes by Hap1 is heme dependent (19, 65), we tested whether repression of *ERG5* by Hap1 is also regulated by heme. To do this, we added heme to cells grown hypoxically, a condition in which heme is normally absent, and measured *ERG5* mRNA levels. Our results show that the addition of heme causes derepression of *ERG5* transcription (Fig. 8A, compare lanes 2 and 3). In contrast, the addition of heme had no effect on *ERG5* expression in *hap1Δ* cells (Fig. 8A, compare lanes 5 and 6). Thus, Hap1 is a repressor of *ERG5* only in the absence of heme.

The two roles of Hap1, aerobic activation of *CYC1* and hypoxic repression of *ERG* genes, appear to accomplish the same expression pattern of aerobic-specific transcription. To test whether there might be differences of biological consequence between these two modes of regulation by Hap1, we measured *ERG2*, *ERG5*, *ERG11*, and *CYC1* mRNA levels at intermediate heme levels. To do this, we used a *hem1Δ* mutant which cannot synthesize δ -ala, a precursor in the synthesis of heme. In this mutant, heme levels can be adjusted by adding different amounts of δ -ala to the growth medium. Our results (Fig. 8B and C) show that, as expected, when δ -ala levels (and heme levels) were increased, *ERG* and *CYC1* mRNA levels increased. However, there was a clear difference between the two sets of genes, as the three *ERG* genes were expressed at low levels of δ -ala while *CYC1* was not detectably expressed until significantly higher levels of δ -ala were added. As expected, in the absence of Hap1, *ERG5* was constitutively repressed while *CYC1* was not detectably transcribed, demonstrating that Hap1 mediated these changes in expression in response to the changes in heme levels (Fig. 8B). These results show that the different modes of Hap1 regulation result in different levels of gene expression at intermediate oxygen levels.

DISCUSSION

In order to understand oxygen-mediated transcription in *S. cerevisiae*, we set out to identify the direct targets of the transcriptional regulator Hap1. Our microarray experiments, combined with previous in vivo DNA binding studies (22), allowed a comprehensive identification of putative Hap1 direct targets, many of which were previously unidentified. Surprisingly, we found that a significant number of these genes are repressed by Hap1 during hypoxic growth, including many involved in ergosterol metabolism. We focused on three genes in the ergosterol biosynthetic pathway that Hap1 represses (*ERG2*, *ERG5*, and *ERG11*), as well as one gene that Hap1 activates, *CYC1*, and showed by ChIP experiments that Hap1 binds to their regulatory regions during both hypoxic and aerobic growth. Additional experiments have suggested a model in which Hap1 represses transcription during hypoxic growth by recruiting the corepressor Tup1/Ssn6. Furthermore, our experiments, combined with previous studies, have established that the dual activity of Hap1 is controlled by heme levels, with activation dependent on the presence of heme and repression dependent upon its absence. Finally, our results suggest that the two activities of Hap1 allow it to differentially modulate gene expression at intermediate oxygen levels, a condition often encountered by cells. Taken together, our findings alter the pre-

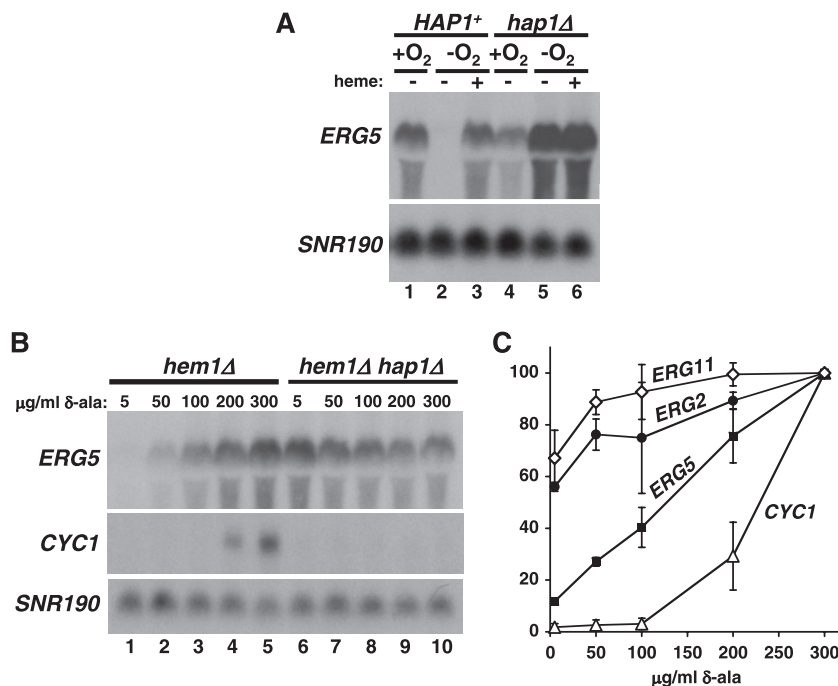


FIG. 8. *ERG5* and *CYC1* are differentially regulated at intermediate heme levels. (A) *ERG5* expression was monitored by Northern blotting in *HAP1*⁺ (FY2609) and *hap1Δ* (FY2611) cells grown hypoxically ($-O_2$) or aerobically ($+O_2$) for 8 h in the presence (+) or absence (-) of 50 μ g/ml heme. The averages of quantitations are as follows: lane 1, 7.5; lane 2, 1.0; lane 3, 15.5; lane 4, 6.1; lane 5, 25.2; lane 6, 20.6. (B) Northern blotting was performed to measure *ERG5* and *CYC1* mRNA levels in *hem1Δ* (FY2637) and *hem1Δ hap1Δ* (FY2638) mutants. Cells were grown in media containing the indicated concentrations of δ -ala for 4 h. (C) *ERG2*, *ERG5*, *ERG11*, and *CYC1* mRNA levels in *hem1Δ* cells were quantitated as described in the Materials and Methods section. Shown are the means and standard errors for three independent experiments.

vailing view of Hap1 as an activator of aerobic genes, providing strong evidence that it plays an equally significant role as a repressor under hypoxic conditions.

One key question raised by these results is how heme controls the switch of Hap1 between activator and repressor. Mammalian nuclear hormone receptors are one previously studied example of factors that can act both positively and negatively. These factors repress transcription in the absence of ligand through the recruitment of corepressor complexes. In the presence of ligand, the corepressor complexes are displaced in favor of coactivators (3, 63). In the case of Hap1, heme may act in a similar manner, dictating the differential association of factors with Hap1. Indeed, we have shown here that Tup1, a subunit of the Tup1/Ssn6 corepressor complex, is required for *ERG5* repression and that Hap1 recruits Tup1 to the *ERG5* promoter specifically during hypoxic growth. Hap1 presumably functions as an aerobic activator through the recruitment of a coactivator yet to be identified. It will be important to determine how heme regulates Hap1 binding to these and other factors.

Previous studies have yielded ambiguous results regarding the possibility that Hap1 might directly repress transcription during hypoxic growth. A number of studies had suggested that Hap1 can act as a repressor under such conditions (11, 12, 17, 45, 58, 59), and recent evidence has shown that Hap1 directly represses transcription of its own gene in a heme-independent fashion during aerobic growth (25). Prior to our studies, however, there was no conclusive evidence that Hap1 plays a direct role as a repressor during hypoxic growth. With respect to the

ability of Hap1 to bind DNA during hypoxic growth, one study found that Hap1 isolated from heme-deficient cells could bind DNA *in vitro* (17) and a recent study also suggested that Hap1 can bind DNA in heme-deficient cells (25). Other studies, however, suggested that Hap1 has reduced affinity for DNA in the absence of heme (24, 35, 58, 65). Our ChIP and mutational results strongly suggest that Hap1 can bind DNA *in vivo* in the absence of heme to directly repress the transcription of particular genes. The fact that Hap1 binds *in vivo* under both aerobic and hypoxic conditions suggests that the conversion of Hap1 between activator and repressor may occur at a step subsequent to DNA binding. There are several examples of transcription factors that bind to their sites under conditions where they do not normally act, including the well-characterized activator Gal4 (15, 47).

A related issue is the nature of the promoter elements at Hap1 target genes that determine whether Hap1 acts as an activator or repressor. Our results have shown that the region of the *ERG5* promoter that is most strongly required for Hap1-dependent repression contains five conserved Hap1 half-sites. Such half-sites have been shown to allow Hap1 binding (43) and may be responsible for Hap1 binding to its own promoter, which lacks full Hap1 sites (25). An examination of three genes that are repressed by Hap1 and one gene that is activated by Hap1 (Fig. S4) showed that all four contain several half-sites in a variety of positions, making it unlikely that half-sites are sufficient to confer Hap1-mediated repression. In addition, previous studies suggested that the specific sequence of Hap1 binding sites can determine the level of activation by Hap1

without affecting its DNA binding by an allosteric effect (21, 30). Similarly, the sequence of binding sites might determine repression by Hap1. Additional experiments are required to resolve the role of promoter elements in determining whether Hap1 acts as an activator or as a repressor.

In both humans and yeast, normal sterol levels are critical for proper membrane function. Sterol biosynthesis is tightly controlled, and it is well known that in humans aberrant cholesterol levels can lead to many serious diseases (16, 51). In *S. cerevisiae*, two transcription factors, Upc2 and Ecm22, have previously been demonstrated to be required for the activation of transcription of at least some *ERG* genes in response to low sterol levels (10, 60). Our work has extended our understanding of the regulation of ergosterol levels in *S. cerevisiae* by elucidating the central role played by Hap1 in modulating the level of *ERG* gene expression in response to oxygen levels. The finding that Hap1 does not fully repress *ERG* transcription at intermediate heme levels suggests that cells are able to synthesize ergosterol as long as there is some oxygen available. This ability may be critical for growth, given that oxygen excludes the import of exogenous ergosterol (37, 55). In contrast, the Hap1-activated gene required for aerobic respiration, *CYC1*, may not be expressed at intermediate heme levels, because cells can generate sufficient ATP in low-oxygen conditions through fermentation. Thus, the fact that the repression and activation functions of Hap1 respond differentially to intermediate heme levels allows *S. cerevisiae* to differentially regulate two key metabolic pathways in response to changing oxygen levels.

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