A Novel Allele of *HAP1* Causes Uninducible Expression of *HEM13* in *Saccharomyces cerevisiae*

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ABSTRACT

Transcription of *HEM13* in *Saccharomyces cerevisiae* is repressed by heme and oxygen. We have isolated two mutants in which expression of *HEM13* is aberrant. The mutant alleles in these strains represent two different alleles of *HAP1*. *HAP1* encodes an activator protein whose DNA binding activity is stimulated by heme, and is required for the transcription of *CYC1*, *ROX1* and a number of other heme-dependent genes. One of our mutant alleles confers a phenotype much like that of the *hap1::LEU2* allele. Expression of *HEM13* in a strain with this mutation is elevated under repressing conditions and not fully inducible in the absence of heme. The other mutant allele of *HAP1* we uncovered confers a novel phenotype. A strain containing this allele exhibits heme-independent expression of *CYC1* and *ROX1* and uninducible expression of *HEM13* and *ANB1*. The mutation associated with this novel allele of *HAP1* was localized to a glycine to aspartate change in amino acid 235 of HAP1, between the DNA binding and heme responsive domains. DNA binding assays demonstrated that the protein made from this *HAP1* allele retains the ability to bind DNA, but that unlike wild-type HAP1 protein, this binding is not stimulated by heme.

HEME biosynthesis is an important process in *Saccharomyces cerevisiae* as heme is used as a prosthetic group for many enzymes needed for respiration and oxygen radical removal, as well as for the synthesis of other metabolites such as oleic acid and ergosterol (MATTOON *et al.* 1979). In addition, heme also serves as a regulatory molecule in transcriptional activation of such genes as *CYC1* encoding iso-1-cytochrome *c* (GUARENTE and MASON 1983), *CTT1* encoding catalase T (HÖRTNER *et al.* 1982) and in the transcriptional repression of such genes as *ANB1* encoding the translational factor eIF-5A (LOWRY and LIEBER 1986), *COX5b* encoding an isoform of subunit V of cytochrome *c* oxidase (HODGE *et al.* 1989; TRUEBLOOD *et al.* 1988) and *HEM13* encoding coproporphyrinogen oxidase, the sixth enzyme in the heme biosynthetic pathway (ZAGOREC and LABBE-BOIS 1986).

Mechanisms by which heme acts to regulate gene expression were revealed by the isolation of *trans*-acting regulatory mutants which are defective in regulation of heme-regulated genes. The *hap1* mutation was isolated as one which is unable to activate *CYC1* expression from the upstream activation site 1 (UAS1) (GUARENTE *et al.* 1984). It has subsequently been shown that *HAP1* encodes an activator protein the DNA-binding activity of which is stimulated by heme. (GUARENTE *et al.* 1984; PFEIFER *et al.* 1987a). *HAP1* is also found to be required for the expression of *COR2*, *CYB2*, *CYC7*, *CYT1*, *HMG1*, *ERG11* and *CTT1* genes (DORSMAN and GRIVELL 1990; LODI and GUIARD 1991; PFEIFER *et al.* 1987b;

SCHNEIDER and GUARENTE 1991; THORSNESS *et al.* 1989; TURI and LOPER 1992; WINKLER *et al.* 1988). *HAP1* encodes a protein of 1483 amino acids. The functional domains of the *HAP1* gene product have been mapped and include a zinc finger DNA binding domain from amino acid 1–148, and a putative heme-binding domain from amino acid 247–444. The carboxy-terminal 176 amino acids of HAP1 are found to be required for activation of transcription (PFEIFER *et al.* 1989). Several mutant alleles of *HAP1* with different phenotypes have been isolated. Most of the mutant alleles are unable to synthesize a functional HAP1 protein and have the same phenotype as the *hap1::LEU2* disrupted allele (GUARENTE *et al.* 1984). One mutant allele, *HAP1-18*, produces a protein that is able to differentiate between the HAP1 binding sites in *CYC1* UAS1 and *CYC7* (CLAVILIER *et al.* 1969). The HAP1-18 protein is unable to bind to the site in *CYC1* UAS1 but retains the ability to bind to its site in *CYC7* (PFEIFER *et al.* 1987b). Additional HAP1-positive control mutants which are unable to activate transcription of *CYC7* or both *CYC1* and *CYC7* have also been isolated. These mutant alleles have amino acid substitutions in residues that surround the zinc finger. However, DNA binding activities of the mutant proteins are normal, indicating that the mutants are defective in activation of transcription (TURCOTTE and GUARENTE 1992).

The *rox1* mutation was isolated as one which allowed constitutively high levels of expression of the anaerobic gene *ANB1* and *ROX1* is defined to encode a repressor (LOWRY and ZITOMER 1984). DNA sequence analysis revealed that the ROX1 protein comprises 386 amino

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TABLE 1
Strains used in this study

Strain	Genotype	Source
TKY18	<i>MATa ade1 ura3-52 leu2-3 leu2-112 Δhem1</i>	T. KENG
TKY22	<i>MATa ade1 ura3-52 leu2-3 leu2-112 Δhem1 Δtrp1::hisG</i>	T. KENG
TKY24	<i>MATa ade1 ura3-52 leu2-3 leu2-112 hap1::LEU2 Δhem1 Δtrp1::hisG</i>	T. KENG
TKY24α	<i>MATα ade1 ura3-52 leu2-3 leu2-112 hap1::LEU2 Δhem1 Δtrp1::hisG</i>	This study
SCI4	<i>MATa ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1</i>	This study
SCI4αΔ1	<i>MATα ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 Δtrp1::hisG</i>	This study
SCI4.4	<i>MATa ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 hap1-23</i>	This study
SCI4.3	<i>MATa ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 hap1-43</i>	This study
SCI4.2	<i>MATa ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 hem15-2</i>	This study
SCI4.4 6d	<i>MATα ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 hap1-23 Δtrp1::hisG</i>	This study
SCI4.3 2b	<i>MATα ade1 ura3-52 leu2-3 leu2-112 UAS_{GAL} HEM13::LEU2 Δhem1 hap1-43 Δtrp1::hisG</i>	This study
TKY24HL	As for TKY24, <i>HEM13-lacZ::TRP1</i>	This study
SCI4αΔ1HL	As for SCI4αΔ1, <i>HEM13-lacZ::TRP1</i>	This study
SCI4.4Δ1HL	As for SCI4.4Δ1, <i>HEM13-lacZ::TRP1</i>	This study
SCI4.3 2bHL	As for SCI4.3 2b, <i>HEM13-lacZ::TRP1</i>	This study

acids and has an amino-terminal portion that resembles high mobility group DNA-binding proteins (BALASUBRAMANIAN *et al.* 1993). The transcription of *ROX1* has been shown to be heme-dependent. Thus, the ROX1 repressor is synthesized only in the presence of heme and can function to repress *ANB1* expression only in the presence of heme (LOWRY and ZITOMER 1988). The ROX1 repressor has also been found to be required for the repression of *COX5b*, another anaerobic gene (HODGE *et al.* 1989).

HEM13 is the only gene encoding a heme biosynthetic enzyme that is heme- and oxygen-regulated. Expression of *HEM13* is repressed by heme and oxygen (ZAGOREC and LABBE-BOIS 1986; ZAGOREC *et al.* 1988). However, the effects of anaerobiosis and heme deficiency are not additive. As heme is a molecule that is made only under aerobic conditions, it can serve as an intracellular indicator of oxygen tension. For *HEM13*, the effect of oxygen on gene expression is mediated by the intracellular levels of heme, and many genes which are regulated by oxygen are also similarly regulated by heme.

We have begun an analysis of the *cis*- and *trans*-regulatory elements involved in the regulation of *HEM13* expression in an effort to understand how the yeast cell regulates the expression of enzymes in the heme biosynthetic pathway. We have previously demonstrated that *HEM13* expression is under the control of the ROX1 repressor and that transcription of *ROX1* is activated by HAP1 (KENG 1992).

We are also interested in identifying other factors involved in regulation of *HEM13* expression. Using the chromogenic substrate X-Gal, a mutagenized strain containing a *HEM13-lacZ* fusion was screened for mutants defective in the normal regulation of *HEM13* expression. Two mutant strains in which regulation of expression of *HEM13* was affected were isolated. These strains were found to have altered expression of other heme- and oxygen-regulated genes such as *CYC1*, *ANB1* and *ROX1*, and are found to contain two different alleles of *HAP1*. Genetic and molecular characterization indicate that one

of these *hap1* mutant alleles encodes a protein that still retains the ability to bind DNA, even though unlike the wild-type HAP1 protein, this binding is not stimulated by the addition of heme to the binding reaction.

MATERIALS AND METHODS

Strains and growth conditions: *S. cerevisiae* strains used in this study are listed in Table 1. Strains were propagated in either YEP or synthetic medium as described (SHERMAN *et al.* 1986). YEP medium consists of 1% yeast extract and 2% Bacto-peptone. Dextrose or galactose was added to a final concentration of 2%. Synthetic medium contains 0.67% yeast nitrogen base without amino acids and is supplemented with 2% dextrose or galactose and 1 mg/ml casamino acids. Other nutritional supplements were added to a final concentration of 40 µg/ml. Agar was added at 2% for plates. Yeast X-Gal plates were prepared as described and were used to estimate the level of expression of β-galactosidase from *lacZ* fusion plasmids (GUARENTE 1983). Diploids were induced to sporulate on sporulation plates prepared as described (SHERMAN *et al.* 1986). 5-Fluoro-orotic acid (FOA) plates were prepared as described (BOEKE *et al.* 1984).

All the yeast strains used contain *hem1* deletions. *HEM1* encodes the first enzyme in the heme biosynthetic pathway, δ-aminolevulinic synthase. The product of δ-aminolevulinic synthase is δ-aminolevulinic acid (ALA). *Δhem1* strains can grow only when the medium is supplemented with 50 µg/ml δ-aminolevulinic acid (ALA), under which conditions ALA is taken up and converted into heme via the heme biosynthetic pathway (GOLLUB *et al.* 1977). Alternatively, *Δhem1* cells can also be grown if the medium is supplemented with 0.005% Tween 80 (oleic acid), 20 µg/ml ergosterol and 40 µg/ml methionine (TEM). These supplements represent products of other biosynthetic pathways that require heme or its biosynthetic intermediates. Deuteroporphyrin IX, when added, was at a concentration of 5 µg/ml.

Plasmids, transformation and DNA techniques: Plasmids containing various fusions used in these experiments are listed in Table 2. YCp13Z contains 1.45 kb of upstream non-coding sequences and 34 codons of *HEM13* fused to *lacZ* on a centromeric plasmid and was used as an indicator for expression of *HEM13* in these experiments (RICHARD 1990). pTP101 was a gift of M. HALDI

TABLE 2

Plasmids used in this study

Plasmid	Fusion or gene	Source
YCp13Z	<i>HEM13-lacZ</i>	C. RICHARD
YCpSD5	<i>UAS_{GAL}-CYC1-lacZ</i>	T. KENG
pBL101	<i>CYC1-lacZ</i>	L. GUARENTE
pTP101	<i>CYC7-lacZ</i>	M. HALDI
pTKACZ	<i>ANB1-CYC1-lacZ</i>	T. KENG
Ylp13Z-T	<i>HEM13-lacZ</i>	T. KENG
YEpHEM15	<i>HEM15</i>	T. KENG
YCpHAP1	<i>HAP1</i>	K. PFEIFER

Plasmids YCp13Z, YCpSD5, pBL101, pTKACZ and YCpHAP1 are centromeric plasmids with the *ARS4-CEN1* region. pTP101 and YEpHEM15 have the 2- μ m origin of replication, while Ylp13Z-T is an integrative plasmid. Ylp13Z-T contains the *TRP1* marker. All other plasmids contain the *URA3* marker.

and contains a *CYC7-lacZ* fusion. pBL101 contains a *CYC1-lacZ* fusion while pTKACZ contains a fusion of the regulatory sequences of *ANB1* to a *CYC1-lacZ* fusion. YCpSD5 contains *UAS_{GAL}* fused to a *CYC1-lacZ* fusion on an *ARS-CEN* plasmid. YEpHEM15 and YCpHAP1 contain the entire upstream regulatory and coding sequences of the *HEM15* and *HAP1* genes, respectively. Yeast strains were transformed either with the lithium acetate transformation protocol (ITO *et al.* 1983) or by disruption with glass beads (COSTANZO and FOX 1988). Restriction digests, DNA end modification, agarose gel electrophoresis and Southern analysis were performed by standard techniques (SAMBROOK *et al.* 1989).

Mutagenesis and screen for mutants: Mutagenesis was carried out in strain SCI4, a derivative of the $\Delta hem1$ strain TKY18. In order to allow isolation of activator mutants that may abolish expression from the *HEM13* promoter and result in heme auxotrophy, a copy of *HEM13* under the control of *UAS_{GAL}* was introduced into TKY18. A *Sma*I to *Xho*I fragment from the yeast *GAL1-GAL10* promoter (*UAS_{GAL}*) was fused to the *Dra*I to *Sph*I fragment which contains the TATA and transcriptional initiation sites in addition to the entire coding region of *HEM13* (GUARENTE *et al.* 1982; ZAGOREC *et al.* 1988). In medium with galactose, expression of *HEM13* from this fusion is able to allow complementation of the *hem13-1* mutant allele. The *UAS_{GAL}-HEM13* fusion was integrated at the chromosomal *LEU2* locus in TKY18, as was confirmed by DNA hybridization analysis (Southern) (data not shown). The resulting strain, SCI4, was transformed with YCp13Z and mutagenized using ethyl methanesulfonate (Kodak-Eastman), according to the procedure described by SHERMAN *et al.* (1986). SCI4 was first grown at 30° on YEPD plates supplemented with ALA for two to three days. The colonies were collected and re-suspended in 10 mM potassium phosphate, pH 7.0. The suspension was pipetted into sterile screw cap tubes into which 0, 20, 40 or 60 μ l ethyl methanesulfonate were added. The tubes were incubated at 30° for 60 min and washed once with 5% sodium thiosulfate and twice with 10 mM potassium phosphate, pH 7.0. The mutagenized

cells were diluted to yield approximately 200 colonies per plate and spread onto synthetic galactose plates supplemented with ALA. After incubation at 30° for 4 days, the colonies were replica plated onto yeast X-Gal plates supplemented with either ALA or Tween 80, ergosterol and methionine. Colonies which exhibited a greater or lower intensity in blue color compared to wild-type strain SCI4 were patched onto synthetic galactose plates containing ALA. The patches were then allowed to grow at 30° for 2–3 days and were replica plated again onto yeast X-Gal plates with either ALA or Tween 80, ergosterol and methionine. If the changes in blue color were again observed, the isolates were assayed for β -galactosidase activity using the substrate *o*-nitrophenyl- β -D-galactopyranoside (GUARENTE 1983).

Expression of other heme-regulated genes: To assess the expression of *UAS_{GAL}-CYC1-lacZ*, *CYC1-lacZ*, *ANB1-CYC1-lacZ* and *CYC7-lacZ* in the mutant strains, the strains were grown under non-selective conditions and plated on to YEPD plates. Colonies which had lost the YCp13Z plasmid were screened on minimal plates without uracil. Such colonies were then transformed with plasmids YCpSD5, pBL101, pTKACZ and pTP101 which respectively carry the indicated fusions.

Genetic analysis: The mating type of SCI4 was switched by transformation with the plasmid pGALHO (kindly provided by J. PINKHAM) and growth on galactose. The mating type of segregants were determined by mating with *MATa met13* and *MATa met13* tester strains. A *trp1* deletion was then introduced into SCI4 α using a *Eco*RI-*Bgl*II fragment from pNKY1009 (a gift of N. KLECKNER) which contains a *trp1* deletion disrupted by the *hisG::URA3::hisG* cassette (ALANI *et al.* 1987). Transformants were first isolated as uracil prototrophs and tested for tryptophan auxotrophy. All tryptophan auxotrophs were streaked on to fresh FOA plates, the FOA resistant colonies were tested for both uracil and tryptophan auxotrophies (BOEKE *et al.* 1984). This resulted in SCI4 $\alpha\Delta 1$, a *MATa* $\Delta trp1::hisG$ derivative of SCI4, which was crossed with each of the isolated mutants harboring YCp13Z. The diploids were isolated, induced to sporulate and the tetrads were dissected using standard yeast techniques (SHERMAN *et al.* 1986). The phenotype of each germinated spore was assessed by examining expression of *HEM13-lacZ* from YCp13Z in β -galactosidase assays. To assess expression of *CYC1-lacZ*, segregants were cured of YCp13Z by growth under non-selective conditions and transformed with plasmid pBL101 which contains the *CYC1-lacZ* fusion. The transformants were assayed for β -galactosidase activity. Complementation analysis was performed by crossing the segregants which have mutant phenotypes as well as the appropriate mating types and nutritional auxotrophies. The diploids were isolated and then assayed for β -galactosidase activity expressed from YCp13Z.

β -Galactosidase assays: Strains were grown in synthetic glucose media to OD₆₀₀ of approximately 1. The

cells were permeabilized with chloroform and sodium dodecyl sulfate and β -galactosidase activity was assayed as described (GUARENTE 1983). Each determination was performed in duplicate. The results presented are the averages of the duplicate assays of at least two independent determinations or transformants. Sample errors were less than 20%.

RNA hybridization (Northern blot) analysis: Total RNA was isolated from strains grown in synthetic medium supplemented with 50 μ g/ml ALA or with Tween, ergosterol and methionine (GUARENTE *et al.* 1984). The RNA samples were separated on 1% agarose gels run in MOPS-formaldehyde buffer as described elsewhere (SAMBROOK *et al.* 1989) and transferred onto FLASH membranes (Stratagene). Antisense RNA Probes were synthesized using SP6 RNA polymerase (Pharmacia) or T7 RNA polymerase (GIBCO/BRL) and [α - 32 P]CTP (ICN Biomedicals). The *HEM13* probe was transcribed from plasmid pAMH13 which contains a 1.9-kb *DraI-SphI* fragment of *HEM13* cloned into the *HincII* and *SphI* sites of pAM18. The *ROX1* probe used was transcribed from the plasmid pAMROX1-XH which contains a 2.0-kb *XbaI-HindIII* fragment from YCpROX1 (kindly provided by R. ZITOMER) subcloned into the vector pAM18. Actin message was probed with antisense RNA synthesized from pSPactin which contains a 3.3-kb *EcoRI-BamHI* fragment cloned into pSP65. Hybridizations were carried out under conditions previously described (SHACKLEFORD and VARMUS 1987).

Amplification and sequencing of the *hap1-43* allele: Chromosomal DNA was isolated from the wild-type strain SCI4 and the mutant strain SCI4.3 and used as templates in amplification reactions (SAIKI *et al.* 1988). Each functional domain of HAP1 was amplified using Taq DNA polymerase (BIO/CAN) as per manufacturer's instructions. For the heme-responsive domain from position +713 to +1358 of the *HAP1* coding region, the primers used for amplification were 5'-ACTGGTTGTC-TATCATGAAA-3' and 5'-GCGGGATGCGAACCAGC-ATG-3'. The activation domain from positions +3899 to +4934 of the *HAP1* coding region was amplified using primers 5'-CCATGAACCAGCTATACGGG-3' and 5'-ACCAGGTGTTTGTATTACGGG-3'. Lastly, to amplify the DNA binding domain within positions -74 to +739, primers 5'-TCTCCAGTCGCAGGCAAG-3' and 5'-GGTCACCCTTTCATGATAG-3' were used. Two independent amplification reactions each of wild-type and mutant templates were carried out for each domain. The amplified DNA fragments were cloned into the TA cloning phagemid vector, pTZ/PC (a gift of D. Y. THOMAS). Single-stranded DNA was isolated and sequenced using Sequenase (U.S. Biochemical Corp.) or T7 DNA polymerase (Pharmacia) according to instructions suggested by the suppliers. A minimum of two independent clones from each independent amplification reaction were sequenced.

Reconstruction of the *hap1-43* allele: The entire *HAP1* coding and regulatory sequences are present on the two micron containing plasmid pHAP1-S (a gift of B. TURCOTTE). This plasmid was digested with *ScaI*, treated with shrimp alkaline phosphatase (U.S. Biochemical Corp.) and ligated to the 1.7-kb *ScaI* fragment from pRS316 (SIKORSKI and HIETER 1989). This replaces the two micron origin of replication with ARS-CEN sequences to yield pHAP1ARS-CEN. This plasmid was then digested with *HindIII* and *BstEII* and treated with shrimp alkaline phosphatase. pTZ/PC DB3-12, containing an amplified fragment from positions -74 to +739 of the *hap1-43* allele, was also digested with *HindIII* and *BstEII* and the 390-bp fragment containing the *hap1-43* mutation was isolated and ligated to the prepared pHAP1ARS-CEN backbone. The resulting plasmid, pHAP1-43, contains the entire *HAP1* coding region with the G to A substitution at position +704.

To assess the effect of pHAP1-43 on expression of *HEM13-lacZ*, Ylp13Z-T, an integrative plasmid with a *HEM13-lacZ* fusion and a *TRP1* marker was linearized with *XbaI* and introduced into the *hap1* disrupted strain TKY24 to give strain TKY24HL. The reconstructed plasmid pHAP1-43 or the wild-type pHAP1ARS-CEN was individually introduced into TKY24HL and the effect of each plasmid on expression of the *HEM13-lacZ* fusion was determined.

Cell extract preparation: Extracts were prepared as described (PFEIFER *et al.* 1987a) with a few modifications. Cells grown to an A_{600} value of 1.5 were harvested by centrifugation and then frozen for a minimum of 1 hr at -70°. They were then washed with 0.1 volume extraction buffer [200 mM Tris-HCl, pH 8.0, 400 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 7 mM 2-mercaptoethanol]. The cell pellet was resuspended in extraction buffer and was disrupted by agitation at 4° with a vortex mixer in the presence of 0.67 volume of acid washed glass beads (0.45-mm diameter). Extracts were incubated for 30 min on ice and then centrifuged for 45 min at 12,000 rpm. The supernatant was collected and the proteins were precipitated by the addition of 100% (NH₄)₂SO₄ in 20 mM HEPES pH 8.0, 5 mM EDTA to a final (NH₄)₂SO₄ concentration of 50%. The pellet was resuspended in 20 mM HEPES pH 8.0, 5 mM EDTA, 1 mM PMSF, 7 mM 2-mercaptoethanol and 20% glycerol. Protein concentrations were determined using the Bio-Rad protein assay.

Probe for DNA binding assay: The 86-bp probe containing UAS1 of *CYC1* was isolated from pLG669ZΔ229-178 by digestion with *XhoI* and *SmaI* and purification from a 5% polyacrylamide gel. The fragment was labeled using Klenow fragment and [α - 32 P]TTP (ICN Biomedicals). The Klenow enzyme was inactivated by incubation at 75° for 10 min and the labeled DNA was purified using

a Nick-Spin column (Pharmacia) per the manufacturer's instructions.

Gel electrophoresis DNA binding assay: Protein-DNA complexes were resolved on high ionic strength polyacrylamide gels as described (PFEIFER *et al.* 1987a) with some modifications. Binding reactions were carried out in 20 μ l volumes containing 4 mM Tris-HCl pH 8.0, 60 mM KCl, 2 mM MgCl₂, 4% glycerol, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 0.5 ng radioactive DNA probe, 1 μ g sonicated salmon sperm DNA and 10 μ g protein. Hemin was added to a final concentration of 40 μ M when used. Reaction mixes were incubated 20 min at room temperature and loaded immediately onto 4% polyacrylamide gel (acrylamide to bisacrylamide weight ratio 40:1) in 1 $\times$ TBE buffer. Each gel was pre-run for 30 min at 25 mA in 1 $\times$ TBE buffer. Gels were electrophoresed at 25 mA at room temperature. They were then transferred to Whatman 3MM paper, dried, and autoradiographed.

RESULTS

Mutagenesis results: We screened 31,500 independent colonies as described in MATERIALS AND METHODS. Ninety-four of these still showed a mutant phenotype when retested on X-Gal plates and were assayed for *HEM13-lacZ* expression in liquid assays. Of these, 10 were found to have aberrant expression of β -galactosidase activity from the fusion plasmid YCp13Z. To determine whether the differences in expression of *HEM13-lacZ* were due to plasmid-borne mutations or *trans*-acting mutations, the ten mutants were grown under nonselective conditions. Cured strains were retransformed with the original YCp13Z plasmid or YCpSD5, a low copy number plasmid with the same ARS-CEN sequences as YCp13Z but which contains a *UAS_{GAL}-CYC1-lacZ* fusion. These transformants were assayed and three were found to retain aberrant expression of *HEM13-lacZ*, indicating that the original mutations in these strains were not plasmid-associated. Furthermore, expression of *UAS_{GAL}-CYC1-lacZ* in these strains was not affected (results not shown), suggesting that the mutations did not affect replication or copy number of ARS-CEN plasmids.

The levels of expression of *HEM13-lacZ* in these mutant strains are shown in Table 3, together with those observed for the parent strain. One of the three mutants, SCI4.4, showed an increase in *HEM13-lacZ* expression to 30 units in the presence of heme and a decreased level of expression of 75 units in the absence of heme when compared to the wild-type strain, making *HEM13-lacZ* expression in this strain much like that in the strain TKY24 which contains the *hap1::LEU2* allele (Table 3). In another mutant, SCI4.3, there was a low level of *HEM13-lacZ* expression of 5 units in the presence of heme. This level of expression remained low at twelve units in the absence of heme. Expression of *HEM13-*

TABLE 3

Expression of *HEM13-lacZ* in the wild-type and mutant strains

Strain ^a	Relevant genotype	Units of β -galactosidase activity ^b		
		ALA	TEM	+DP
SCI4	<i>HAP1 HEM15</i>	6	228	6
TKY24	<i>hap1::LEU2 HEM15</i>	33	48	33
SCI4.4	<i>hap1-23 HEM15</i>	30	75	25
SCI4.3	<i>hap1-43 HEM15</i>	5	12	7
SCI4.2	<i>HAP1 hem15-2</i>	34	342	255
SCI4.2/ YEpHEM15	<i>HAP1 hem15-2/HEM15</i>	2	239	ND

^a All strains harbor YCp13Z which contains 34 codons of *HEM13* fused to *lacZ* and has *URA3* as a selectable marker.

^b Assays were done on transformed strains grown in synthetic glucose medium supplemented with δ -aminolevulinic acid (ALA) or Tween 80, ergosterol and methionine (TEM). Assays were also done on transformants grown in media containing Tween 80, ergosterol and methionine and supplemented with deuteroporphyrin IX (+DP). Values are presented as Miller units and represent the averages of duplicate assays of at least two independent determinations, ND = not determined.

lacZ in the remaining mutant strain SCI4.2 was elevated to 34 units under repressing conditions but normally derepressed in the absence of heme.

Because *HEM13* expression is derepressed in the absence of heme, an increase in *HEM13-lacZ* expression under repressing conditions could be due to an additional defect in the heme biosynthetic pathway besides *hem1* such that intracellular levels of heme remain low even in the presence of ALA. Such mutants would be expected to have normal levels of *HEM13-lacZ* expression under inducing conditions and therefore, have a phenotype much like strain SCI4.2. To eliminate such mutants from our screen, expression of *HEM13-lacZ* in strains SCI4.4, SCI4.3 and SCI4.2 was determined in the presence of the heme analog deuteroporphyrin IX. Addition of deuteroporphyrin IX to the Δ *hem1* mutant, SCI4, grown in the absence of heme resulted in repression of *HEM13* expression. The results in Table 3 demonstrate that the addition of deuteroporphyrin IX to the mutant SCI4.4 did not completely repress expression of *HEM13-lacZ*; expression was decreased from 75 to 25 units. These observations parallel those made in strain TKY24, a *hap1::LEU2* disrupted strain; addition of deuteroporphyrin IX also did not repress expression in this strain. Interestingly, mutant SCI4.2 appeared to be insensitive to added deuteroporphyrin IX; the level of expression of *HEM13-lacZ* remained high at 255 units and is comparable to that observed in SCI4.2 grown in the absence of ALA. This suggests that deuteroporphyrin IX may not be transported into SCI4.2 such that it cannot act to repress expression. Alternatively, because deuteroporphyrin IX must be complexed with ferrous ion by the ferrochelatase enzyme in order for it to be able to repress transcription of *HEM13*, the mutation in SCI4.2

FIGURE 1.—*HEM13* transcription in the mutant strains SCI4.4 and SCI4.3. Total RNA was isolated from the wild-type strain SCI4, the *hap1-23* mutant strain SCI4.4 and the *hap1-43* mutant strain SCI4.3 grown in the presence of ALA (+ALA) or Tween 80, ergosterol and methionine (–ALA). 30 µg of total RNA were run on a 1% agarose gel in MOPS-formaldehyde buffer as described in MATERIALS AND METHODS. RNA samples were transferred to FLASH membranes and probed with radiolabeled *HEM13* RNA probes. The filter was then stripped and reprobed with the *ACT1* probe.

may be a leaky mutation in *HEM15*, the structural gene for ferrochelatase (GUARENTE *et al.* 1984). To examine this possibility, SCI4.2 was transformed with YEpHEM15, a 2-µm plasmid which contains the *HEM15* coding region. This plasmid was able to restore expression of *HEM13-lacZ* back to normal (Table 3). This indicates that the mutation which affected expression in SCI4.2 is probably a leaky mutation in *HEM15* or one which affected expression of *HEM15*. Based on these observations, the mutant strain SCI4.2 was not further characterized.

Northern analysis of the mutants: To verify that the isolated mutations affected transcription of *HEM13*, we examined *HEM13* RNA levels in a RNA hybridization (Northern blot) experiment. Total RNA was isolated from SCI4.3, SCI4.4 as well as the parent strain SCI4 grown in the presence of the heme precursor ALA or in its absence. Analyses of these RNA samples show that *HEM13* transcript levels parallel those of β -galactosidase activity expressed from the *HEM13-lacZ* fusion in these strains (Figure 1). Densitometric analysis revealed that in the wild-type strain *HEM13* transcript levels are induced some 24-fold by the absence of ALA. In strain SCI4.3, the level of *HEM13* mRNA is identical to the wild-type strain under repressing conditions and increased only 1.6-fold in the absence of ALA. In strain SCI4.4, quantitation of *HEM13* message levels indicate that it was approximately 10-fold higher than in the wild-type strain under repressing conditions and not induced by the absence of ALA.

Genetic analysis: The two mutants, SCI4.3, and SCI4.4 were individually crossed with the wild-type strain

TABLE 4

Diploid analysis of mutant strains

Parental strains ^a	β -Galactosidase activity ^b	
	ALA	TEM
SCI4 × SCI4 α Δ1	2	177
SCI4 × TKY24 α (<i>hap1::LEU2</i>)	2	143
SCI4.4 × SCI4 α Δ1	2	156
SCI4.3 × SCI4 α Δ1	1	64

^a Each mutant strain harboring YCp13Z, an *ARS4-CEN1* plasmid that carries a *HEM13-lacZ* fusion with a *URA3* marker, was mated with the wild type strain SCI4 α Δ1. Diploids were selected on plates without tryptophan and uracil.

^b Assays were performed on strains grown in synthetic glucose medium supplemented with 50 µg/ml δ -aminolevulinic acid (ALA) which is equivalent to heme-sufficient conditions or with Tween 80, ergosterol and methionine (TEM), equivalent to heme-deficient conditions, as described in MATERIALS AND METHODS. Units are reported as Miller units and represent the averages of duplicate assays of at least two independent determinations.

SCI4 α Δ1 to determine if the respective mutations were dominant or recessive. Table 4 summarizes the results of the β -galactosidase assays on the resulting diploids. The mutation in strain SCI4.4 is clearly recessive; the level of *HEM13-lacZ* expression of two units under repressing and 156 units under derepressing conditions in the heterozygous diploid is identical to that found with the wild-type homozygous diploid (SCI4 × SCI4 α Δ1). However, the heterozygous diploid formed when SCI4.3, the mutant strain which exhibited an uninducible level of expression of *HEM13-lacZ*, was crossed with the wild-type strain gave a decreased level of expression under inducing conditions (TEM), giving 64 units of activity instead of 177 units seen in a wild-type diploid.

To determine if each of the mutant phenotypes segregates as a single nuclear mutation, the described diploids were induced to sporulate and the resultant tetrads dissected. Expression of *HEM13-lacZ* was then determined in the germinated spores. In each case, a segregation pattern of 2 mutant:2 wild type in a minimum of five tetrads indicated that the phenotypes of both mutant strains are due to single nuclear mutations.

Complementation analysis: To determine the number of complementation groups represented by the two mutations, each of the mutant strains or a segregant from the cross with wild type was crossed with one another and the expression of *HEM13-lacZ* from YCp13Z was measured in the diploids. In addition, because the mutant strain SCI4.4 has a phenotype very similar to that of a *hap1::LEU2* mutant, we also wanted to determine if it indeed contained a mutant allele of *HAP1* by examining expression of *HEM13-lacZ* in the diploid formed from a cross of SCI4.4 with the *hap1::LEU2* strain TKY24 α . The results shown in Table 5 indicate that the recessive mutation in SCI4.4 is unable to complement a *hap1::LEU2* mutation. Expression of *HEM13-lacZ* in this diploid (TKY24 α × SCI4.4) was 10 units in the presence of heme and 28 units under in-

TABLE 5

Complementation analysis of mutant strains

Parental strains ^a	Units of β -galactosidase activity ^b	
	+ALA	+TEM
SCI4 $\times$ SCI4 Δ 1	2	177
TKY24 $\times$ TKY24 α	12	34
SCI4.4 6d $\times$ SCI4.4	11	33
SCI4.3 2b $\times$ SCI4.3	2	7
TKY24 α $\times$ SCI4.4	10	28
TKY24 α $\times$ SCI4.3	2	7
SCI4.4 6d $\times$ SCI4.3	2	7

^a See Table 1 for complete genotypes.

^b Strains SCI4.3 and SCI4.4 harbored YCp13Z, an ARS-CEN plasmid containing a *HEM13-lacZ* fusion. These were mated with the indicated strains which all contain a $\Delta trp1::hisG$ marker. Diploids were selected on synthetic glucose plates lacking tryptophan and uracil. The diploids were grown in synthetic glucose medium supplemented with 50 μ g/ml δ -aminolevulinic acid (ALA) or with Tween 80, ergosterol and methionine (TEM). Units reported are Miller units and represent the averages of duplicate assays performed in at least two separate determinations.

ducing conditions, and was identical to expression in a *hap1::LEU2* homozygous diploid (TKY24 $\times$ TKY24 α). This indicated that SCI4.4 was a *hap1* mutant. The *hap1* allele in this strain was named *hap1-23*.

Interestingly, the allele in SCI4.3 is completely dominant over the *hap1-23* and *hap1::LEU2* alleles; expression of *HEM13-lacZ* in the SCI4.4 6d $\times$ SCI4.3 and TKY24 α $\times$ SCI4.3 diploids is extremely low and similar to that observed in SCI4.3 2b $\times$ SCI4.3 diploids. This suggests that the mutation present in SCI4.3 may be a mutation of *HAP1*.

To determine whether SCI4.3 contains a mutation in *HAP1*, diploids from the TKY24 α $\times$ SCI4.3 cross were induced to sporulate and the resulting tetrads were analyzed for the pattern of expression of *HEM13-lacZ*. In each of the six tetrads examined, two spores showed elevated expression of *HEM13-lacZ* of 20–30 units in the presence and absence of heme, a phenotype characteristic of the *hap1::LEU2* mutation, and two spores showed uninducible levels of expression of *HEM13-lacZ*, the phenotype associated with the mutation in SCI4.3 (data not shown). This analysis indicates that the mutation in SCI4.3 represents a novel allele of *HAP1* which affects expression of *HEM13* in a manner very different from the *hap1* disruption. This allele is designated *hap1-43*.

Plasmid complementation of *hap1* mutants: To further confirm that strains SCI4.4 and SCI4.3 each has a mutant allele of *HAP1*, derivatives of these strains containing an integrated copy of *HEM13-lacZ* at the *TRP1* locus were each transformed with YCpHAP1 (a gift of K. PFEIFER). The results are summarized in Table 6. The presence of the vector YCp50 in either strain has little effect on *HEM13-lacZ* expression. Normal regulation of *HEM13-lacZ* expression in strain SCI4.4 Δ 1HL was completely restored when transformed with YCpHAP1; ex-

TABLE 6

Complementation with a plasmid copy of *HAP1*

Strains ^a	Plasmid	β -Galactosidase activity ^b	
		ALA	TEM
SCI4 α Δ 1HL	None	2	239
SCI4.4 Δ 1HL	YCp50	18	35
SCI4.4 Δ 1HL	YCpHAP1	3	280
SCI4.3 2bHL	YCp50	<1	5
SCI4.3 2bHL	YCpHAP1	<1	65

^a Each strain has an integrated copy of *HEM13-lacZ* at the *TRP1* locus.

^b Assays were performed on strains grown in synthetic glucose medium supplemented with 50 μ g/ml δ -aminolevulinic acid (ALA) or with Tween 80, ergosterol and methionine (TEM) as described in MATERIALS AND METHODS. Units are reported as Miller units and are averages of duplicate assays performed on at least two independent transformants.

pression is now 3 units in the presence of heme and induced to 280 units in the absence of heme. However, in strain SCI4.3 2bHL harboring YCpHAP1, *HEM13-lacZ* expression was only partially restored under inducing conditions to 65 units. These observations are similar to those observed in the diploid analysis when each mutant strain was crossed with a wild-type strain (Table 4) and further supports the idea that each strain contains a mutant allele of *HAP1*.

Effect of the mutations on expression of other heme- and oxygen-regulated genes: We were interested to see how the expression of other heme- and oxygen-regulated, and *HAP1*-regulated genes was affected by the *hap1-23* and *hap1-43* mutant alleles. These genes and promoters include *CYC1*, encoding iso-1-cytochrome *c*, a gene which is positively regulated by heme via the *HAP1* activator protein; *CYC7*, encoding iso-2-cytochrome *c*, a gene which is not regulated by heme but is under the control of *HAP1* (GUARENTE *et al.* 1984), and *ANB1*, encoding an isoform of eIF-5A, which is negatively regulated by heme (LOWRY and LIEBER 1986). The results are summarized in Table 7. The *hap1-23* mutant allele which results in an elevated expression of *HEM13-lacZ* under repressing conditions, renders expression from the *CYC1* and *CYC7* promoters uninducible. However, expression from an *ANB1-CYC1-lacZ* fusion is elevated under repressing (+ALA) conditions and decreased to seven units under inducing (+TEM) conditions when compared to the *HAP1* strain SCI4. These results are similar to that observed with strain TKY24 containing a disrupted allele of *HAP1*. The *hap1-43* mutant allele in SCI4.3 which leads to an uninducible level of expression of *HEM13-lacZ* also has a very strong effect on expression of *ANB1-CYC1-lacZ*; expression is dramatically lowered to less than one unit under heme-deficient conditions. However, expression driven by the *CYC1* promoter in this mutant strain is greatly increased to 32 units under the same heme-deficient conditions, indicating that *CYC1-lacZ* expres-

TABLE 7
Expression of *CYC1-lacZ*, *ANB1-CYC1-lacZ* and *CYC7-lacZ* in the mutant strains

Strain (<i>HAP1</i> allele)	Units of β -galactosidase activity ^a					
	<i>CYC1-lacZ</i>		<i>ANB1-CYC1-lacZ</i>		<i>CYC7-lacZ</i>	
	ALA	TEM	ALA	TEM	ALA	TEM
SCI4 (<i>HAP1</i>)	80	<1	2	238	12	12
TKY24 (<i>hap1::LEU2</i>)	3	3	10	3	2	<1
SCI4.4 (<i>hap1-23</i>)	6	2	11	7	4	2
SCI4.3 (<i>hap1-43</i>)	102	32	<1	<1	11	4

^a *CYC1-lacZ*, *ANB1-CYC1-lacZ* and *CYC7-lacZ* were introduced into the respective strains on plasmids pBL101, PTKACZ and pTP101. Assays were performed on transformants grown in the presence of ALA, representing heme-sufficient conditions, or in the presence of Tween 80, ergosterol and methionine (TEM), representing heme-deficient conditions. Results are averages of duplicate assays performed on at least two colonies from each transformation.

sion in this strain has been rendered heme-independent. *CYC7* expression does not appear to be altered significantly in this strain in the presence of heme, remaining at eleven units but is decreased to four units in the absence of heme.

These assays were performed using the original isolates of the mutant strains. To demonstrate that a single mutation in each of the mutant strains affected expression of both *HEM13* and *CYC1*, a complete tetrad from a cross of each strain with wild-type was transformed with the *CYC1-lacZ* plasmid pBL101, and separately with YCp13Z which contains the *HEM13-lacZ* fusion. In each case, the mutation that affected *HEM13* expression cosegregated with that affecting *CYC1* expression (data not shown). Therefore, we conclude it is the same mutation in each strain that is affecting expression of both *HEM13* and *CYC1*.

Because the mutations affected expression of more than one heme-regulated gene, they may cause respiratory deficiency. We examined the ability of the mutants to grow in the presence of non-fermentable carbon source. Each mutant strain was found to be able to grow using lactate as a carbon source.

***hap1-43* affects transcription of *ROX1*:** The observation that *CYC1* expression has become heme-independent in strain SCI4.3 suggested a mechanism by which the mutant *hap1-43* allele could cause uninducible expression of *HEM13*. It has previously been shown that the *HAP1* gene product is required for the activation of *ROX1* transcription in the presence of heme. *ROX1* functions as a repressor of *HEM13* transcription and expression of *ROX1* in a heme- and *HAP1*-independent manner resulted in repression of *HEM13* expression that is also heme- and *HAP1*-independent (KENG 1992). We thus wished to examine if *ROX1* transcription in strain SCI4.3, which contains the *hap1-43* allele, has also been rendered heme-independent. Total RNA was isolated from the wild-type strain and the two mutant strains grown in the presence or absence of ALA. Northern blot analysis and densitometry of these RNA samples show that *ROX1* transcript levels cannot be detected in the *HAP1* strain in the absence of ALA. Ad-

dition of ALA to the medium results in induction of high levels of *ROX1* message. In comparison, in strain SCI4.4, the level of *ROX1* RNA is low but detectable in the absence of ALA in the medium. This level is not significantly altered with ALA in the medium. Conversely, in strain SCI4.3, expression of *ROX1* has become constitutively high and heme-independent (Figure 2).

Localization of the mutation in the *hap1-43* allele: The heme-independent expression of *CYC1* and *ROX1* in strain SCI4.3 indicated that *hap1-43* represented an allele of *HAP1* which has a novel phenotype. To localize the mutation in the *hap1-43* allele, we made use of DNA amplification techniques to examine defined regions of the *HAP1* coding region in SCI4.3. It has previously been demonstrated that the *HAP1* gene product contains at least three functional domains: the DNA binding domain from amino acid 1 to 148, a proposed heme-binding domain from amino acid 247–444, and the transcription activation domain from amino acid 1308–1483. An internal region from amino acid 445–1308 may be involved in regulating the activity of the *HAP1* protein. We targeted our initial amplifications to the DNA binding, heme-binding and activation domains. Each domain was amplified and cloned from genomic DNA isolated from SCI4.3, alongside with amplifications of the corresponding domains from DNA isolated from the wild-type strain SCI4. Sequence analysis of the clones and comparisons of the mutant and wild-type sequences indicate that amongst the three domains, there is only one change, a G to A substitution, at position +704 of the *HAP1* coding region (Figure 3). This results in the replacement of the glycine codon with an aspartate codon at amino acid 235 of *HAP1*, between the DNA binding and the proposed heme-binding domains.

To demonstrate that this glycine to aspartate substitution results in the phenotype associated with the *hap1-43* allele, we reconstructed this mutation into a *HAP1* clone in an ARS-CEN vector. The 390-bp *HindIII*-*BstEII* fragment derived from an amplification reaction of genomic DNA from SCI4.3, containing the sole G to A substitution at position +704 in the *HAP1* coding region was used to replace the corresponding fragment in

FIGURE 2.—*ROX1* transcription in the mutant strains SCI4.4 and SCI4.3. Total RNA was prepared from the SCI4 (wild-type) and the two mutant strains SCI4.4 (*hap1-23*) and SCI4.3 (*hap1-43*). The cultures were supplemented with ALA (+ALA) or with Tween 80, ergosterol and methionine (–ALA). Samples of 30 μ g of total RNA were run on a 1% agarose gel in MOPS-formaldehyde buffer as described. RNA samples were transferred on to FLASH membranes and probed with radioactively labeled *ROX1* RNA probe. The filter was stripped and reprobed with *ACT1* probe.

FIGURE 3.—The sequence at position +704 of the *HAP1* coding region is altered in the *hap1-43* allele. The top panel depicts the DNA sequence corresponding to positions +688 to +720 of the *HAP1* coding region and the corresponding amino acid sequence of that region. The arrow indicates the change caused by the *hap1-43* mutation. The bottom panel diagrams the functional domains of HAP1 (PFEIFER *et al.* 1989; KIM *et al.* 1990) and the location of the *hap1-43* mutation with respect to these domains.

the *HAP1* coding region in plasmid pHAP1ARS-CEN. This plasmid, pHAP1-43, was introduced into the *hap1::LEU2* disrupted strain TKY24HL and its effect on expression of the integrated *HEM13-lacZ* fusion was assessed. pHAP1ARS-CEN, containing the wild-type allele of *HAP1* and pRS316, containing only vector sequences, served as controls.

The results (Table 8) indicate that expression of *HEM13-lacZ* in strain TKY24HL is not affected by in-

TABLE 8

Gly to Asp substitution at residue 235 of *HAP1* is responsible for the phenotype associated with the *hap1-43* allele

Plasmid	<i>HAP1</i> allele	Units of β -galactosidase activity ^a	
		ALA	TEM
pRS316	<i>hap1::LEU2</i>	17	37
pHAP1-S	<i>hap1::LEU2/HAP1</i>	2	210
pHAP1ARS-CEN	<i>hap1::LEU2/HAP1</i>	2	184
pHAP1-43	<i>hap1::LEU2/hap1-43</i>	4	18

^a Each plasmid was introduced into strain TKY24HL which contains the *hap1::LEU2* allele and a copy of *HEM13-lacZ* fusion integrated at the *TRP1* locus. Assays were performed on cells grown in the presence of ALA or in its absence (TEM). The results are the averages of duplicate assays performed on at least two independent transformants.

roduction of the vector pRS316. However, regulation is returned to normal with the introduction of the wild-type allele of *HAP1* on pHAP1-S or pHAP1ARS-CEN. Introduction of the mutation on the plasmid pHAP1-43 into TKY24HL dramatically reduced expression of *HEM13-lacZ* in the absence of heme, such that it is now only 18 units. The levels of expression indicate that the glycine to aspartate change at position 235 in the *HAP1* coding region is responsible for the phenotype associated with the *hap1-43* mutant allele.

Gel electrophoresis DNA binding assays: Studies on *CYC1* expression indicated that the heme-dependent expression of *CYC1* and other HAP1-regulated genes is due to the heme-induced DNA binding activity of the HAP1 activator (PFEIFER *et al.* 1989). The heme-independent expression of *CYC1* in the SCI4.3 mutant strain suggests that the DNA binding activity of the HAP1-43 protein may be heme-independent. To investigate this possibility, we examined the DNA binding activity of the various mutant HAP1 proteins. Figure 4A shows binding assays performed using extracts prepared from cells grown under heme-sufficient (+ALA) conditions. Two complexes were detected using the 86-bp probe from UAS1 of *CYC1*. The complex with the faster mobility is RC2, an otherwise uncharacterized binding activity that has been previously observed (PFEIFER *et al.* 1987a). Addition of heme to the binding reactions does not change the level of RC2 complex formed. The complex with slower mobility contains HAP1 protein. Addition of heme to the binding reactions stimulated HAP1 binding to the fragment twofold as determined by densitometry (compare lanes 1 and 5). Strain SCI4.4 containing the *hap1-23* mutant allele, like strain TKY24 which contains the *hap1::LEU2* disruption, is unable to make a HAP1 protein that could be detected in the binding assays (lanes 2, 4, 6 and 8). Interestingly, the HAP1-43 protein made in strain SCI4.3 still retains the ability to bind DNA. However, unlike the wild-type HAP1 protein, the binding activity of this altered protein is not

FIGURE 4.—Gel electrophoresis DNA binding assay. Whole cell extracts were prepared from strains containing *HAP1* (TKY22), *hap1::LEU2* (TKY24), *hap1-23* (SCI4.4), and *hap1-43* (SCI4.3) alleles grown in the absence or presence of heme. The extracts were incubated with an 86 base pair probe containing UAS1 of *CYC1* at room temperature for 20 min. The protein-DNA complexes were then resolved on a 4% high ionic strength polyacrylamide gel. In panel A, all extracts were prepared from cells grown in the presence of ALA. Lanes 1–4 also contain 40 μ M hemin added directly to the binding reaction. In lanes 5–8, no hemin was added to the binding reactions. Lane 9 represents DNA probe alone. In panel B, the extracts were prepared from cells grown in the absence of ALA. Reactions in lanes 1 and 3 contain 40 μ M hemin in the binding reactions while the reactions in lanes 2 and 4 do not contain any additional hemin.

stimulated by the presence of heme in the binding reaction, as confirmed by densitometric analysis (lanes 3 and 7).

Figure 4B shows the binding assays performed using extracts prepared from cells grown in the absence of ALA in the medium. Interestingly, HAP1 protein made in the wild-type *HAP1* strain, TKY22, grown under heme-deficient conditions and in the absence of heme in the binding reactions is still able to bind to DNA (see lane 2). Addition of heme to the binding reaction enhances the DNA binding activity approximately twofold (compare lanes 1 and 2). The HAP1-43 protein made under heme-deficient conditions is also able to bind DNA. However, densitometry of the autoradiograms revealed that this binding activity is not stimulated by the addition of heme in the binding reaction (lanes 3 and 4).

DISCUSSION

HEM13 is a member of a group of genes which are negatively regulated by oxygen and heme. Other members of this group include *ANB1*, *COX5b* and *HMG2* (LOWRY and LIEBER 1986; TRUEBLOOD *et al.* 1988; HODGE *et al.* 1989; THORNESS *et al.* 1989). Regulation of *HEM13* expression involves the HAP1 and ROX1 proteins (Figure 5). In the presence of heme, HAP1 activates transcription of *ROX1*, and the ROX1 gene product represses *HEM13* expression. In the absence of heme, *ROX1* is not transcribed and *HEM13* expression is induced. Using expression of a *HEM13-lacZ* fusion on a low copy number plasmid as an indicator of *HEM13* expression, we have observed that heme represses ex-

pression approximately 40-fold in the strain SCI4. In an effort to understand how heme functions to repress expression, we have isolated two mutants which are defective in this regulation of *HEM13* expression. Our analysis indicates that the mutations in strains SCI4.4 and SCI4.3 represent two very different alleles of *HAP1*.

The *hap1-23* allele in SCI4.4 confers a phenotype very much like that of the *hap1::LEU2* allele in that expression of *HEM13* and *ANB1* in mutant strains is increased under repressing conditions and decreased under derepressing conditions, suggesting a role for HAP1 in the activation of *HEM13* expression under inducing conditions. In addition, expression of *CYC1* and *CYC7* is significantly decreased both in the presence and absence of heme. *ROX1* transcription is also affected in strain SCI4.4. A low level of *ROX1* message can be detected in this *hap1-23* mutant strain in the absence of heme, in contrast to the undetectable levels seen in a *HAP1* strain. However, this low level of expression cannot be induced by heme. These observations are similar to what we have seen previously in a *hap1::LEU2* mutant strain (KENG 1992) and suggest a mechanism by which HAP1 can play a role in regulating expression of *HEM13* under derepressing conditions. In addition to being an activator of *ROX1* transcription in the presence of heme, HAP1 may function as a repressor of *ROX1* transcription in the absence of heme. In the *hap1-23* mutant strain, *ROX1* expression is increased in heme-deficient conditions because of the absence of repression by HAP1. This expression leads to a decreased level of expression of *HEM13* under inducing conditions.

FIGURE 5.—Regulation of *HEM13* expression in strains SCI4, SCI4.4 and SCI4.3. In the wild-type strain, SCI4, *HEM13* transcription is repressed by heme via the action of HAP1 and ROX1. HAP1 activates *ROX1* transcription in the presence of heme and may repress *ROX1* transcription in the absence of heme. In strain SCI4.4, no functional HAP1 protein is made, resulting in a low constitutive level of *ROX1* transcription. This results in an elevated level of *HEM13* transcription in the presence of heme and not fully inducible level of transcription in the absence of heme. In strain SCI4.3, the HAP1-43 protein is able to activate transcription of *ROX1* both in the presence or absence of heme. *HEM13* transcription is constitutively repressed.

However, *ROX1* expression cannot be induced by heme because of the absence of HAP1 activation, resulting in the absence of full repression of *HEM13* expression (Figure 5). The possible role of HAP1 as a repressor under heme-deficient conditions is currently under investigation.

The *hap1-43* allele represents an allele of *HAP1* that has a novel and dramatic phenotype. Expression of *HEM13-lacZ* and *ANB1-CYC1-lacZ* is rendered uninducible in the absence of heme. Expression of *CYC1* and *ROX1* is also drastically affected such that there is a significant level of expression of both genes in the absence of heme. It has previously been demonstrated that expression of *ROX1* would lead to repression of *HEM13* expression (KENG 1992). Therefore, the expression of *ROX1* in the absence of heme observed in strain SCI4.3 would lead to repression of *HEM13* transcription even under heme-deficient conditions (Figure 5). The effect of the *hap1-43* allele on expression of *CYC7* cannot be easily interpreted because the low level expression of *CYC7* is the result of interactions between multiple regulatory elements which include HAP1 and ROX1 proteins. The decrease in expression of *CYC7-lacZ* observed in strain SCI4.3 grown in the absence of ALA may represent the effect of heme-independent *ROX1* expression on transcription.

The *HAP1* gene product is a protein of 1483 amino acids which has heme-dependent DNA binding activity (PFEIFER *et al.* 1987a, 1989). Studies on the HAP1 protein indicate that the amino terminal 148 amino acids are associated with DNA binding activity and include the domain that is required for dimerization of HAP1 (ZHANG *et al.* 1993). The region between amino acids 247 and 444 contains seven repeats of the KCPVDH motif and is postulated to be responsible for the heme response. Deletion of this region results in a HAP1 protein that is able to exhibit a high degree of DNA binding

activity *in vitro* and to activate expression *in vivo* even in the absence of heme (PFEIFER *et al.* 1989; FYTLOVICH *et al.* 1993). Between these two domains is a poly-glutamine-rich region between amino acids 177 and 189. The carboxy-terminal 176 amino acids of HAP1 is required for transcription activation while the internal region from residue 445–1308 is involved in regulating the activity of the protein (KIM *et al.* 1990). We have localized the mutation associated with the *hap1-43* allele to a G to A substitution at position +704 of the *HAP1* coding region. This results in a replacement of glycine with aspartate at residue 235 of HAP1, between the defined DNA binding and heme responsive domains (Figure 3).

An interesting comparison can be made between the phenotypes conferred by the *hap1-43* allele and the *HAP1ΔK* allele that is deleted for the heme-responsive domain between amino acids 247 and 444. Although both alleles allow for heme-independent expression of *CYC1* and other HAP1-regulated genes, the mutations associated with these two alleles fall within different regions of the HAP1 coding region. In addition, unlike *HAP1ΔK* which allows for at least a fourfold increase in *CYC1* expression over that found in a wild-type *HAP1* strain grown in the presence of heme (PFEIFER *et al.* 1989; FYTLOVICH *et al.* 1993), the *hap1-43* allele is not associated with significantly elevated levels of *CYC1-lacZ* expression under such conditions.

Recently, observations made in two different laboratories have led to the proposal of alternate mechanisms by which heme can stimulate expression of HAP1-activated genes. Analysis of the interactions between HAP1 and UAS1 of *CYC1* demonstrated that heme is required for the dimerization of HAP1 and dimer formation is essential for DNA binding activity (ZHANG *et al.* 1993). Promotion of dimerization with antibodies di-

rected against the amino terminus of HAP1 bypasses the heme requirement for binding. Heme is postulated to bind to the KCPVDH motifs that are found within the heme responsive domain between residues 247 and 444. In the absence of heme, this domain is proposed to block dimerization. Parallel studies on the interaction of HAP1 with the regulatory sequences UAS1-B2 of *CYB2* were conducted by FYTLOVICH *et al.* (1993). Using a strain that overproduced HAP1, they were able to detect specific interactions of HAP1 with its binding site *in vitro*, even in the absence of heme in the binding reactions. However, such complexes gave rise to a diffuse band shift that is of low mobility, and can be detected only if the binding reactions were conducted at 5°. Addition of heme to the reaction changes this pattern to a well defined band shift that is of higher mobility. Overproduction of a modified allele of HAP1 which contains a deletion of the heme-responsive domain from amino acid 247–444 results in formation of the well defined band shift with the higher mobility both in the presence or absence of heme in the binding reactions. These observations led to the proposal that the HAP1 protein is able to bind to DNA in the absence of heme. However, this HAP1 protein is unable to activate transcription because it is found in a complex with as yet unidentified X factor(s) to give the diffuse pattern in gel electrophoresis DNA binding assays. Addition of heme to the binding reaction allows factor X to dissociate from the complex and the bound HAP1 to activate transcription of *CYB2*. Both these studies examined only the effects of heme in *in vitro* binding reactions as all extracts were prepared from wild-type strains that were heme-sufficient. Under such conditions, there are high levels of expression of HAP1-dependent genes such as *CYC1*.

In this study, we were unable to detect a HAP1-DNA complex using extracts prepared from a strain that contains the *hap1-23* allele. Similarly, we were also unable to detect a complex using extracts prepared from a *hap1::LEU2* disrupted strain. This is consistent with the lack of induction of *ROX1* and *CYC1* transcription by heme that is observed in these strains. The *hap1-43* allele gave rise to a protein that binds DNA. The mobility of the complex is identical to that formed with extracts prepared from the *HAP1* strain. However, binding of the HAP1-43 protein to DNA is not stimulated by addition of heme to the binding reaction. Surprisingly, we were able to detect a significant level of wild-type HAP1-DNA complex formation using extracts prepared from cells grown in the absence of heme and without addition of heme to the binding reactions. Under these conditions, expression of *CYC1* and other HAP1-activated genes is very low. These observations suggest that heme plays a role not only in enhancing the DNA binding activity of HAP1 protein but also in stimulating the ability of bound HAP1 to activate transcription.

If the HAP1-43 protein were able to bind DNA and activate transcription in a heme-independent manner,

the *hap1-43* allele would be dominant over the *hap1-23* or *hap1::LEU2* alleles. One might also expect that the *hap1-43* allele would be dominant over the wild-type *HAP1* allele. In our analysis, the *hap1-43* mutant allele is dominant over the *hap1-23* and *hap1::LEU2* alleles in that expression of *HEM13* in a *hap1-43/hap1-23* or a *hap1-43/hap1::LEU2* diploid is uninducible. It is interesting to note that in a *hap1-43/HAP1* diploid or in a *hap1-43* strain transformed with YCpHAP1, expression of *HEM13-lacZ* can be derepressed only to approximately 65 units and not to the level of approximately 180 units observed in a *HAP1* homozygous diploid or to 280 units as seen in a *hap1-23* strain transformed with the plasmid containing the wild-type allele of *HAP1* (Tables 4 and 6). These observations suggest that the *hap1-43* allele is not completely recessive to *HAP1*. This lack of complete dominance of the *hap1-43* allele may be due to the formation of mixed dimers of HAP1, containing both wild-type HAP1 and HAP1-43 proteins, which are heme-dependent for its activity.

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