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Identification of *trans*-acting factors involved in *HEM13* expression

by

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November 1995

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

ISBN 0-612-12502-5

This thesis is dedicated to my parents

Helen P. Orlovsky and Constantin D. Ushinsky

ABSTRACT

HEM13 encodes coproporphyrinogen oxidase, the sixth enzyme of the heme biosynthetic pathway. Expression of *HEM13* is repressed under aerobic conditions. In the absence of heme or oxygen, the expression of *HEM13* increases forty-fold. Mutations defining *trans*-acting factors involved in the regulation of *HEM13* expression were isolated and analyzed. This analysis identified two different alleles of *HAP1*. *HAP1* encodes an activator protein involved in the expression of a number of heme-dependent genes such as *CYC1*, which encodes iso-1-cytochrome *c*, and *ROX1*, which encodes a repressor of *HEM13* expression. The *hap1* mutant allele in one strain confers a phenotype much like that of the *hap1::LEU2* allele in that *HEM13* expression is elevated under repressing conditions and not fully inducible in the absence of heme. In addition, expression of *ROX1* is constitutively low. The other mutant allele of *HAP1* confers a novel phenotype. Expression of *HEM13* is uninducible in a strain containing this other allele of *HAP1* while that of *ROX1* and *CYC1* has become heme-independent. The mutation associated with this mutant allele of *hap1* was localized to a glycine to aspartate change in amino acid 235 of HAP1. DNA binding assays indicated 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. The regulatory sequences involved in *HEM13* expression have been localized to a 365 bp region which contains several distinct positive and negative regulatory elements. Gel shift experiments with overlapping fragments containing sequences from this region of the *HEM13* promoter indicate that multiple factors interact with the elements in this region.

RÉSUMÉ

Le gène *HEM13* code pour l'oxydase de coproporphyrinogène, la sixième enzyme impliquée dans la voie de la biosynthèse de l'hème. L'expression de *HEM13* est réprimée dans les conditions d'aérobiose. En l'absence d'hème ou d'oxygène, l'expression de *HEM13* est augmentée de quarante fois. Des séquences contenant des mutations pour les facteurs agissant en *trans* sur la regulation de l'expression de *HEM13* ont été isolées et analysées. Cette analyse a mené à l'identification de deux différents allèles de *HAP1*. Ce gène code pour une protéine activatrice impliquée dans l'expression de plusieurs gènes comme *CYC1*, qui code pour l'iso-1-cytochrome *c* et *ROX1*, qui code pour un répresseur de *HEM13*. L'allèle mutant *hap1* donne un phénotype semblable à celui du mutant *hap1::LEU2* . L'expression de *HEM13* est augmentée dans les conditions réprimantes et n'augmente pas en absence d'hème. De plus, l'expression de *ROX1* est constitutive et faible dans ce mutant. L'autre allèle mutant de *HAP1* donne un nouveau phénotype. L'expression de *HEM13* n'est pas induite dans une souche qui possède cet autre allèle, alors que l'expression de *ROX1* et *CYC1* devient indépendante de l'hème. Il a été démontré que la mutation associée avec cet allèle de *hap1* est due à un changement d'une glycine en acide aspartique à la position 235 de HAP1. Des expériences de retardement de la migration de l'ADN dans les gels de polyacrylamide ont indiqué que la protéine codée par cet allèle mutant de *HAP1* peut toujours se fixer à l'ADN mais cette fixation n'est pas stimulée par l'hème, contrairement à celle de la protéine HAP1 de type sauvage. Les séquences régulatrices impliquées dans l'expression de *HEM13* ont été localisées dans une région de 365 pb contenant plusieurs éléments de régulation positive et négative . Les expériences de retardement avec des fragments qui chevauchent cette région du promoteur de *HEM13* ont indiqué que plusieurs facteurs sont capables d'interagir avec ces éléments.

ACKNOWLEDGEMENTS

I would to thank first my supervisor Teresa Keng for her monumental support and faith in me, and not to mention her patience. I would also like to thank Rick Stewart for his guidance as my co-supervisor. Thanks to the members of the laboratory past and present, Charlie DiFlumeri, Sandy Weber, Robert Larocque, Catherine Richard and Susan Sillaots, whom I've had the pleasure of knowing. The assistance and friendship of the staff and students of the Microbiology and Immunology department is gratefully acknowledged. Special mention goes to Christaine Maroun for her close friendship and encouragement over the years. Also thanks goes to Angelina Guzzo and Josée Brisebois for their motivation and support for the comprehensive exam.

A special thank you goes to my husband George Szatmari for his encouragement, emotional support and invaluble help in typing and editing the thesis. I would also like to thank George, Christaine, Anna Novoselcev and Sybil Murray-Denis for their assistance in translating the abstract, Sonia Jung for the densitometric analysis and Gary Brazier for the scanned figures. A big thank you goes to my sister Anastasia Ushinsky and my mother Helen for their many hours babysitting. Thank you to my entire family, including Alexandra Ushinsky, James Grant, and Jay Beauchamp for their support. Honorable mention goes to my friends, Lydia Lee, Annie Woo, Louis Collins and Julia Bailey for helping me keep my life in perspective.

I would also like to thank Dr. Reg Storms who inspired and supported me since my undergraduate days. Thanks also to Dr. Elaine Newman who gave me the opportunity to work in her lab.

This work was made possible by the financial support of Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) and the F.C. Harrison Fellowship.

OBJECTIVES OF STUDY

This research project involved the identification of diffusible factors involved in the regulation of expression of the *HEM13* gene in *Saccharomyces cerevisiae*. *HEM13* encodes an enzyme in the heme biosynthetic pathway. This pathway is important in all cells because heme is used as a prosthetic group for many enzymes needed for respiration and oxygen radical removal. In addition, some heme biosynthetic intermediates and their derivatives such as siroheme are important for reactions in other metabolic pathways. In yeast, heme also serves as a regulatory molecule for control of transcription.

HEM13 encodes coproporphyrinogen oxidase, which is the sixth step in the heme biosynthetic pathway. The expression of *HEM13* is negatively regulated by heme and oxygen at the transcriptional level. The *HEM13 cis*-regulatory sequences have been localized to a 365 bp region. Gene products of two loci, *HAP1* and *ROX1*, are believed to form a regulatory pathway that controls *HEM13* expression. *HAP1* encodes a heme-dependent activator and *ROX1* encodes a repressor which is expressed in the presence of heme. β-galactosidase assays of strains containing a *HEM13-lacZ* fusion have shown that *HEM13* transcription is constitutively high in *hap1* and *rox1* mutants.

It has been proposed that in the presence of heme, HAP1 activates the transcription of *ROX1*. ROX1, in turn, represses *HEM13* transcription. In the absence of heme, HAP1 does not function and therefore *ROX1* is not transcribed and expression of *HEM13* is then induced by an activator protein. The factor responsible for *HEM13* transcriptional activation has not yet been identified. This regulatory circuit may be common to all heme- and oxygen-controlled genes such as *ANB1* and *CYC7*, which have also been shown to be

regulated by ROX1. This study aims to further examine the above model, thereby determining the exact role of these two proteins in *HEM13* expression. Other *trans*-acting factors involved in the regulation of *HEM13* expression will also be identified.

The other activators and repressors that regulate *HEM13* expression will be identified by making use of a gene fusion containing the *HEM13* promoter region linked to the *E. coli* reporter gene encoding β-galactosidase. Strains containing such fusions will be mutagenized and activator/repressor mutants will be identified by screening on media containing the chromogenic substrate X-gal, using the intensity of blue color as an indication of promoter strength. Subsequent analysis will include the study of *HEM13* mRNA levels in the mutants in the presence and absence of heme and comparison to levels in wild-type strains. In addition, expression of other heme- and oxygen-regulated genes in these mutants will also be examined.

DNA band retardation assays will be performed using *HEM13* promoter fragments and whole cell extracts from both mutant *rox1* and *hap1* strains and otherwise isogenic wild type strains, in order to see whether interactions of HAP1 and ROX1 as well as other factors with sequences in the *HEM13* promoter can be detected. These assays will be performed using extracts prepared from cells grown in the presence or absence of heme in order to assess the effect of heme on binding. These results will give insight into how heme and oxygen can act to repress expression of *HEM13*.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. A mutagenesis study resulted in the isolation of three mutants with aberrant expression of *HEM13*. Each strain was shown to have a single nuclear mutation which affected *HEM13* expression.

2. Subsequent analysis demonstrated that one mutation was in the *HEM15* gene (*hem15-2*), the gene that encodes ferrochelatase, and the other two mutations represented two different alleles of *HAP1* (*hap1-23* and *hap1-43*) which encodes an activator protein.

3. The expression of other heme-regulated genes (*CYC1, CYC7, ROX1* and *ANB1*) was also affected in strains that contain the mutations. It was further demonstrated that the same mutation in each strain affected expression of *HEM13* and *CYC1*.

4. *hap1-43* represented a novel allele of *HAP1*. The DNA sequences encoding the DNA binding domain, the dimerization domain, the adjacent heme responsive domain and the activation domain of both wild-type *HAP1* and *hap1-43* were determined and compared with each other. This comparison found a one base pair change at position +704 of the coding reigon of *hap1-43*.

5. It was demonstrated that this one base pair change was responsible for the phenotype conferred by *hap1-43*.

6. *In vitro* DNA binding experiments demonstrated that the HAP1-43 protein exhibited heme-independent binding to the UAS1 of *CYC1*.

7. *In vitro* DNA binding experiments with various fragments of the *HEM13* promoter and whole cell extracts of wild-type cells (TKY22) showed strong protein-DNA interactions in the region from position -326 to -121 upstream of the transcription initiation site. Five distinct complexes (A - E) can be resolved.

8. *In vitro* DNA binding experiments with a DNA fragment containing sequences from position -326 to -121 of the *HEM13* promoter and whole cell extracts from wild-type (TKY22), *hap1::LEU2* (TKY24), *rox1::LEU2* (CDY1), and *hap1-43* (SCI4.3) strains confirmed that HAP1 does not bind to the *HEM13* promoter. ROX1 binding cannot be detected under the experimental conditions used, thus suggesting that the complexes formed involves the binding of other regulatory proteins to the *HEM13* promoter region.

9. The protein-DNA interactions detected as complexes A and B were siginificantly decreased in the presence of heme. Complex C and D interactions were also decreased in the presence of heme. Complex E levels remained the same in wild-type cells grown in either the presence or absence of heme, but were significantly increased in *rox1::LEU2* extracts and in *hap1-43* extracts.

10. *In vitro* DNA binding experiments with a series of smaller overlapping fragments containing the region from position -326 to -121 upstream of the *HEM13* transcription initiation site were carried out. From this analysis, complexes A and B can be deduced to involve sequences from position -326 to -293 of the *HEM13* promoter. Complex D and another complex found only in

the absence of heme were formed when sequences from position -284 to -250 were present. In addition, the sequences required for formation of complex C were localized to a region spanning position -230 to -214. Complex E formation required the presence of sequences from position -295 to -280. Complex F, which comigrates with complex E, was formed with sequences from position -175 to -160.

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INTRODUCTION

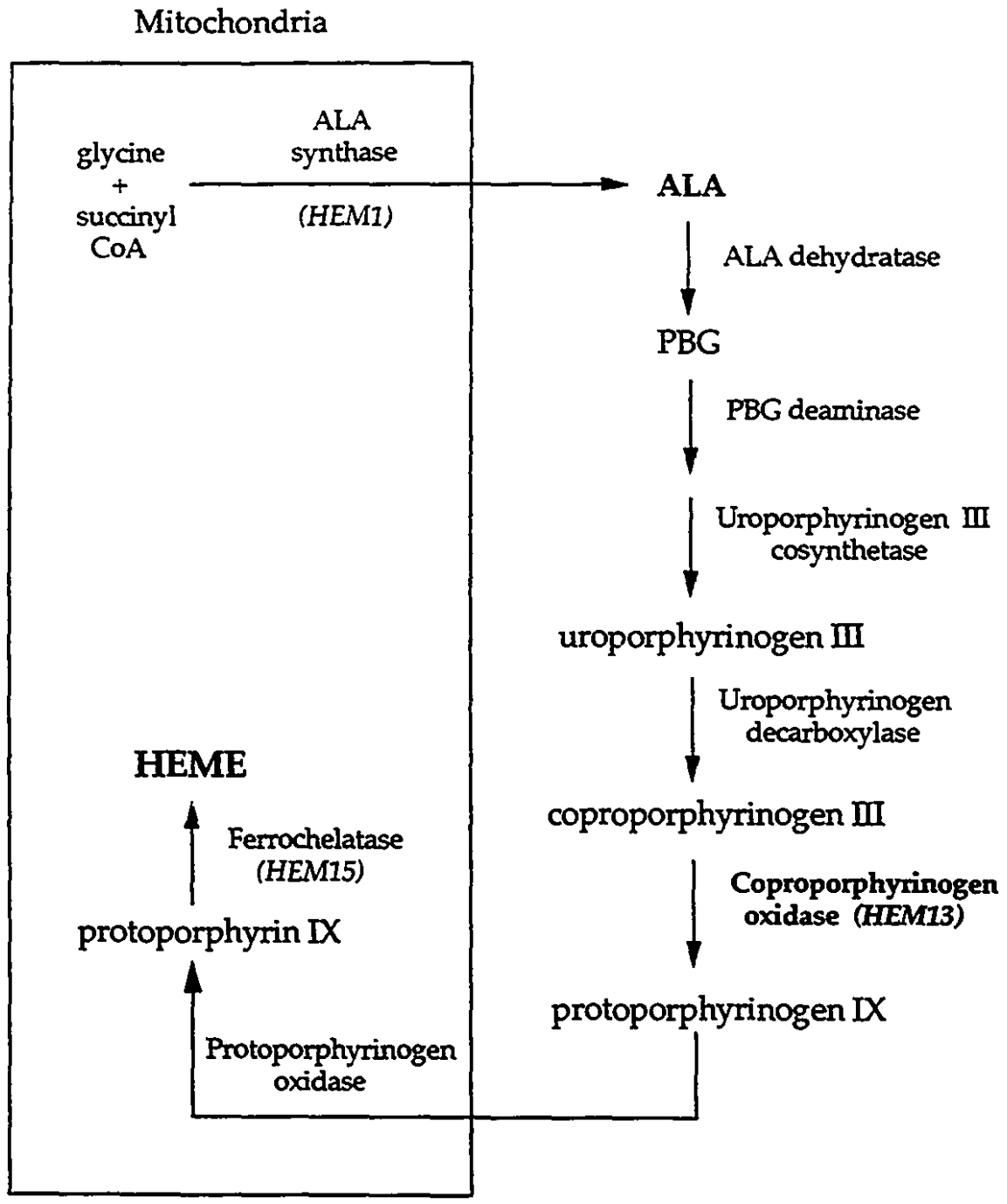
Heme biosynthetic pathway

Heme is an important molecule in all organisms, and hemoproteins carry out many important functions in cells. For example, in yeast, the cytochromes *a*+*a*3, *b*, *c*1 and *c* are associated with the respiratory chain bound to the inner membrane of mitochondria. In addition, cytochrome *c* peroxidase found in the intermembrane space of mitochondria, as well as catalase A and T present in the cytosol, are involved in detoxifying metabolic byproducts. Cytochrome b_5 is involved in oxidative desaturation of fatty acids and cytochrome P_{450} catalyzes the first step in the ergosterol biosynthetic pathway by participating in the oxidative removal of the 14α-methyl group of lanosterol. In addition, the siroheme-containing sulfite reductase catalyzes an early step in the methionine biosynthetic pathway (Labbe-Bois and Labbe, 1990). In mammalian physiology, many of the functions of heme as the prosthetic group of hemoproteins mediating oxygen transport and storage, and of cytochromes involved in generating cellular energy via respiration, are preserved. Heme is also involved in the formation of certain steroid hormones, and participates in some reduction reactions. In addition, heme also plays a role in the detoxification of drugs and in the regulation of protein synthesis. It has also been implicated in modifying cellular development (Martasek *et al.*, 1994).

In *Escherichia coli* and *Salmonella typhimurium*, heme functions both in respiration and in oxygen defense (Xu *et al.*, 1992). The heme biosynthetic pathway (Figure 1) is comprised of eight steps, seven of which are common to all organisms. In some bacteria, yeast, avian and mammalian cells, δ-aminolevulinate (ALA) is formed via the Shemin pathway, where the condensation of succinyl-CoA and glycine is catalyzed by the enzyme ALA-

Figure 1. The heme biosynthetic pathway in yeast. The pathway is comprised of eight steps. The first step and the last two steps occur in the mitochondria and the remaining steps occur in the cytosol.

The Heme biosynthetic pathway in Yeast



synthase. However, in chloroplasts and green algae, in cyanobacteria, eubacteria (including *E. coli*) and archaebacteria, ALA is formed via the C_5 pathway from the 5-carbon skeleton of glutamate (Jahn *et al.*, 1992), which will not be discussed here.

ALA synthase

The first step in the heme biosynthetic pathway is the formation of δ-aminolevulinic acid (ALA). The synthesis of ALA is carried out either by ALA synthase in the mitochondrial matrix in yeast and in mammalian cells or by glutamyl tRNA dehydrogenase in *E. coli* (reviewed in Pinkham and Keng, 1994, Straka *et al.*,1990). Glutamyl tRNA dehydrogenase is encoded by the *E. coli* *hemA* gene. The heme pathway is feedback regulated at the level of *hemA* expression in order to supply heme as it is required. The factors involved in this regulation include ArcA, which functions as an activator of *hemA* transcription, Fnr, which represses *hemA* in the absence of oxygen, and Integration Host Factor (IHF). In addition, in a *hemA* mutant the expression of a *hemA-lacZ* gene fusion increases in both the presence or absence of oxygen; however addition of ALA to the fusion strain restores expression to wild-type levels, suggesting the involvement of transcriptional regulation in the heme pathway (Darie and Gunsalus, 1994).

In mammalian cells, there are two ALA synthase genes (ALAS1 and ALAS2), which are found on different chromosomes and are regulated in different manners (Ferriera and Gong, 1995). ALAS1 is a housekeeping ALA synthase, which is ubiquitously expressed, but chiefly made in the liver to provide heme for various cytochrome P_{450} proteins (May and Bawden, 1989), The enzyme is feedback inhibited by heme. This inhibition occurs on several levels, including direct inhibition of ALAS1 activity, repression of transcription and translation, and inhibition of translocation into the mitochondria. ALAS2 is

expressed in erythroid cells, and is mainly involved in the production of heme for hemoglobin, which accounts for approximately 85% of heme synthesis in the adult (May and Bawden, 1989). In differentiating erythrocytes, globin and heme biosynthetic enzymes are expressed co-ordinately. This regulation of ALAS2 expression is on several levels. In addition to regulation on the transcriptional level, there is also regulation of translation of the mRNA via interactions with an Iron Responsive Element (IRE) (this element is not present in ALAS1), and an IRE binding protein as well as posttranscriptional regulation (Ferriera and Gong, 1995). Interestingly, ferrochelatase (the terminal enzyme of the heme biosynthetic pathway) has been shown to bind to ALAS2 IRE (Ferreira, 1995). In erythroid cells, ALAS2 does not appear to be feedback inhibited by heme.

In summary, ALAS1 is negatively regulated by heme and ALAS2 is positively regulated by heme. ALAS1 is expressed in cells (mainly liver) which have lower requirement for heme while ALAS2 is expressed in erythroid cells, which have a greater demand for heme.

In yeast, ALA synthase is encoded by the *HEM1* gene. Yeast ALA synthase, unlike higher eukaryotic ALA synthases (i. e. ALAS1) is not feedback-inhibited by heme (Urban-Grimal and Labbe-Bois, 1981). In a detailed promoter analysis, expression of *HEM1* was found to be essentially constitutive as a result of interactions between two different regulatory systems, and not positively induced by heme as was found for ALAS2 from higher eukaryotes (Keng and Guarente, 1987). In addition, overexpression of *HEM1* in yeast led to increases in ALA synthase activity and intracellular levels of ALA, but not to changes in the levels of heme, porphyrins or cytochromes, suggesting that synthesis of ALA is not the rate limiting step in heme biosynthesis(Arrese *et al.*, 1983).

ALA dehydratase

The second step in the heme biosynthetic pathway is catalyzed by ALA dehydratase in the cytosol. Two molecules of ALA are condensed with the subsequent loss of two water molecules to form monopyrrole porphobilinogen (PBG) (Straka *et al.*, 1990). This step is the first common step in the biosynthesis of all tetrapyrroles. The ALA dehydratase protein is highly conserved from *E. coli* to humans (Jaffe, 1995). This enzyme is very sensitive to lead, and the enzymatic activity decreases as lead levels increase (Abraham, 1991). Lead poisoning has been shown to cause multiple abnormalities in porphyrin metabolism, since it affects the heme pathway at multiple steps (Straka *et al.*, 1990). It has also been found that there are subtle differences in the 5' untranslated regions (UTR) in erythroid and housekeeping ALA dehydratase mRNAs, the production of which are controlled by different promoters (Ponka and Schulman, 1993). In yeast, ALA dehydratase is encoded by the *HEM2* gene. This gene is postulated to be the rate limiting step of the heme biosynthetic pathway. *HEM2* expression has been examined and found not to be regulated by carbon source, nor by intracellular heme levels (Pinkham and Keng, 1994).

PBG deaminase

The third step of the pathway is catalyzed by a cytosolic enzyme, porphobilinogen deaminase, which condenses four molecules of PBG in a head-to-tail manner to form hydroxymethylbilane. In higher eukaryotes, there are two types of PBG deaminase expressed; one in all tissues, and one exclusively in erythroid tissues. These two proteins are, in fact, two isoenzymes encoded by two mRNA species transcribed from a single structural gene with two different promoter sites (Straka *et al.*, 1990). The two mRNA species differ only in their 5' termini. A housekeeping promoter drives expression of PBG deaminase mRNA in all tissues while an erythroid-specific promoter is responsible for expression in

erythroid tissues. The two promoters are regulated independently as the deletion of the housekeeping promoter does not lead to the activation of the erythroid-specific promoter which is situated 3 kb downstream. The erythroid-specific ALAD promoter bears homologies to the ALAS promoter and the β-globin gene promoters (May and Bawden, 1989; Ponka and Schulman, 1993) and is bound by a common erythroid-specific factor GATA-1 and the erythroid-2 nuclear factor (NF-E2) (Sassa, 1990; Ponka and Schulman, 1993).

In yeast, PBG deaminase is encoded by the *HEM3* gene. It was found that transcription of *HEM3* is not affected by carbon source, or by intracellular heme levels, or by levels of heme biosynthetic intermediates (Keng *et al.*, 1992). This type of regulation resembles that of *HEM1* in nature (Pinkham and Keng, 1994).

Uroporphyrinogen III synthase

The fourth step in the heme biosynthetic pathway is the cyclization of hydroxymethylbilane to form uroporphyrinogen III by the cytosolic enzyme uroporphyrinogen III synthase. This reaction is not well characterized, but in yeast, does not appear to be the rate limiting step in the heme biosynthetic pathway (Pinkham and Keng, 1994). It is interesting to note that in the comparison of uroporphyrinogen III synthase from different sources, the amino acid sequences show a very low level of identity with a total of five invariant residues. This is unusual, since the other enzymes in the heme pathway display higher levels of amino acid identity with one another (Shoolingin-Jordan, 1995). The yeast gene for uroporphyrinogen III synthase (*HEM4*) has recently been sequenced and shows 18-23% identity with uroporphyrinogen III synthase from other sources (Amillet and Labbe-Bois, 1995).

Uroporphyrinogen decarboxylase

The fifth step of the pathway is catalyzed by another cytosolic enzyme, uroporphyrinogen decarboxylase, which decarboxylates each of the four

carboxymethyl substituents leaving a methyl group in its place, generating coproporphyrinogen III. In higher eukaryotes, there is tissue-specific control of expression of uroporphyrinogen decarboxylase, which is expressed as a single mRNA (Sassa, 1990). In yeast, there is no accumulation of uroporphyrinogen or any other intermediate in wild-type cells grown in a variety of growth conditions, suggesting this is not the rate-limiting step in the pathway (Labbe-Bois and Labbe, 1990). The gene encoding uroporphyrinogen decarboxylase has been cloned and sequenced and its expression examined. It was found that the gene is induced by nonfermentable carbon sources but is not regulated by heme levels (DiFlumeri *et al.*, 1992).

Coproporphyrinogen oxidase

The sixth step in the heme biosynthetic pathway is catalyzed by coproporphyrinogen oxidase. This enzyme carries out the oxidative decarboxylation of two of the four carboxyethyl side chains to vinyl groups, yielding protoporphyrinogen IX. This enzyme is cytosolic in yeast (Camadro *et al.*, 1986) but is localized on the outer surface of the inner mitochondrial membrane in higher eukaryotes (Taketani *et al.*, 1994). In *E. coli*, there are two major steps in the heme biosynthetic pathway which are regulated by oxygen; the first is the first step of the pathway, while the second is in the sixth step of the pathway, coproporphyrinogen oxidase (Troup *et al.*, 1994). Interestingly, there are two coproporphyrinogen oxidase genes in *E. coli*: *hemF* which encodes an enzyme that functions in aerobic conditions and *hemN* which encodes a coproporphyrinogen oxidase that functions in anaerobic conditions. The aerobic coproporphyrinogen oxidase requires oxygen, has a predicted amino acid sequence that is 44% identical with that of the yeast enzyme, and can complement a yeast coproporphyrinogen oxidase mutant. The anaerobic enzyme does not display this level of identity (Troup *et al.*, 1994).

The *hemF* gene of *E. coli* is controlled by two promoter elements, one for the expression of the operon, one specific for *hemF* (Troup *et al.*, 1994). *hemN* is weakly expressed in aerobic conditions and is induced three-fold under anaerobic conditions. Much like *E. coli*, *S. typhimurium* also has two genes encoding coproporphyrinogen oxidase. The *E. coli* and *S. typhimurium hemF* genes are very homologous (up to 92% homology), as are the *hemN* genes, and the amino acid sequence of the enzyme encoded by the *S. typhimurium hemF* gene is also 44% identical to the yeast enzyme. The existance of two bacterial enzymes suggests that there is strict regulation of their corresponding genes according to growth conditions, which are likely to be determined by oxygen availability. Since molecular oxygen participates directly in the reaction of the aerobic enzyme as an electron acceptor, coproporphyrinogen oxidase may be the logical step in the heme biosynthetic pathway at which bacteria sense the availability of oxygen for respiration and adjust synthesis of heme accordingly (Troup *et al.*, 1995; Xu *et al.*, 1992; Xu and Elliot, 1993).

In humans, there is a single coproporphyrinogen oxidase enzyme, which is 52% identical to the yeast enzyme on the amino acid level. The yeast enzyme is 27 amino acids shorter and does not contain a leader peptide for mitochondrial import (Martasek *et al.*, 1994; Taketani *et al.*, 1994). A detailed examination of the coproporphyrinogen oxidase gene promoter in humans by primer extension and RNAse protection assays indicates the presence of multiple transcription initiation sites. This suggests that a single promoter is active but differentially regulated in erythropoetic and nonerythropoetic cells (Delfau-Larue *et al.*, 1994). The promoter itself has no TATA or CAAT boxes, but does contain six Sp1 sites, four GATA and CACCC boxes. The GATA binding sites found in combination with CACCC boxes or Sp1 sites usually indicate the presence of promoter regions of genes specifically expressed or up-regulated in erythroid cells

(Grandchamp *et al.*, 1995). Interestingly, the expression of mouse coproporphyrinogen oxidase, an enzyme which is 86% homologous to the human enzyme, is transcriptionally induced during erythroid differentiation in mouse cells in culture (Kohno *et al.*, 1993). Even in photosynthetic organisms such as the green alga *Chlamydomonas reinhardtii* or in higher plants, expression of coproporphyrinogen oxidase is induced when there is increased demand for heme (Madsen *et al.*, 1993; Hill and Merchant, 1995). Thus, a common theme amongst the various organisms is the up regulation of coproporphyrinogen oxidase activity in response to increased demand for heme biosynthesis.

In yeast, coproporphyrinogen oxidse is encoded by the *HEM13* gene. The enzyme encoded by this gene has an absolute requirement for molecular oxygen for its activity, as is also found for the human enzyme (Tait, 1978). Early studies showed that the activity of coproporphyrinogen oxidase is increased in heme biosynthetic mutants and in wild-type cells grown in anaerobic conditions (Urban-Grimal and Labbe-Bois, 1981; Rytka *et al.*, 1984). In extracts prepared from anaerobically grown cells, the level of coproporphyrinogen oxidase activity is increased up to 40-fold when compared to levels found in extracts prepared from aerobically-grown cells (Zagorec and Labbe-Bois, 1986). A similar increase in coproporphyrinogen oxidase activity was found in heme biosynthetic mutants defective in either the first or last step of the pathway when compared to their wild-type counterparts, suggesting that both heme and oxygen have negative effects on the expression or the activity of coproporphyrinogen oxidase. The effects of heme and oxygen on the level of coproporphyrinogen oxidase activity are not additive as there was no demonstrable change in the levels of enzyme activity in a heme-deficient strain grown aerobically and anaerobically (Zagorec and Labbe-Bois, 1986). The level of coproporphyrinogen oxidase activity in the heme-deficient strain was constitutively high and was not affected by oxygen

tension, indicating that oxygen exerts its effects on enzyme activity via heme. To determine the mechanism by which heme and oxygen can affect coproporphyrinogen oxidase activity, steady-state levels of coproporphyrinogen oxidase protein and mRNA were measured. Both protein and mRNA levels decreased in wild-type cells grown in aerobic conditions or in *hem1* mutant cells grown in the presence of ALA or heme, thus indicating that heme and oxygen affected enzymatic activity by modulating the expression of enzyme in the cells at the transcriptional level. Moreover, the effect of oxygen on enzyme and *HEM13* mRNA levels is mediated by heme as its absence coupled with anaerobiosis did not lead to further increases in either mRNA or protein levels (Zagorec and Labbe-Bois, 1986).

HEM13 has been cloned and sequenced and it was directly demonstrated in Northern blot analysis that *HEM13* is negatively regulated by heme and oxygen at the transcriptional level. When the *HEM13* promoter was fused to the *lacZ* gene, expression of β-galactosidase activity from this fusion was similarly regulated. A preliminary deletion analysis showed that sequences upstream of -340 are necessary for high levels of expression in anaerobic conditions (Zagorec *et al.*, 1988). Other aspects of *HEM13* expression will be discussed later in this thesis.

Protoporphyrinogen oxidase

The seventh step of the pathway is catalyzed by protoporphyrinogen oxidase, localized to the inner mitochondrial membrane. Protoporphyrinogen IX is oxidized to yield protoporphyrin IX. Protoporphyrinogen oxidase has recently been cloned in humans but little information exists on its expression (Nishimura *et al.*, 1995). Mouse protoporphyrinogen oxidase cDNA has been isolated and sequenced and is found to be 86% identical to the human cDNA. In experiments with murine erythroleukemic cells, it was found that induction of differentiation

resulted in increased mRNA and enzyme activity levels for the last three steps (coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase) of the heme biosynthetic pathway (Taketani *et al.*, 1995). In yeast, the enzymatic activity is increased two-fold in cells grown in a nonfermentable carbon source (Poulson and Polgase, 1974). Similar to coproporphyrinogen oxidase, protoporphyrinogen oxidase levels are increased in a heme biosynthetic mutant (Camadro *et al.*, 1982). In addition, a protoporphyrinogen oxidase mutant lacked cytochromes and accumulated protoporphyrinogen. The pool of free iron in this strain was significantly lower than in a wild-type strain (Urban-Grimal and Labbe-Bois, 1981; Camadro *et al.*, 1982). The significance of this is still unclear.

Ferrochelatase

The final step of the heme biosynthetic pathway is catalyzed by ferrochelatase, which attaches the ferrous iron to protoporphyrin IX at the matrix face of the inner mitochondrial membrane. In humans, there appears to be two mRNA species encoding ferrochelatase, one of which is associated with erythroid differentiation. The two mRNAs have been shown to differ at their 3' ends but are transcribed from one ferrochelatase gene which has two putative polyA signals. Examination of the promoter region demonstrated the existence of sites for the NF-E2 factor (Ponka and Schulman, 1993). It is interesting to note that when a subunit of NF-E2 (MafK) is conditionally overexpressed in erythroleukemic cells, it caused accumulation of hemoglobin indicating overexpression of both heme biosynthetic enzymes and globin genes (Igarashi *et al.* 1995). In yeast, *HEM15* encodes ferrochelatase, and its activity and transcription were found to be increased when the cells were grown in the presence of a nonfermentable carbon source (Labbe-Bois, 1990). *HEM15* is not affected by intracellular heme levels although it was found to be induced when the intracellular concentration of iron was low (Pinkham and Keng, 1994).

Heme regulatory functions in higher eukaryotes

The heme biosynthetic pathways are not regulated in the same manner in *E. coli*, yeast and humans, but appear to be more adapted to the specific needs of each organism. However, the common theme that emerges is that heme is involved in some form of regulation of its own synthesis. The function of heme as a regulatory molecule is not confined to controlling the activity of the heme biosynthetic pathway. Other examples of heme regulatory functions have been identified in higher eukaryotes. The best studied example is in regulation of globin translation during the erythrocyte differentiation. At the reticulocyte stage of differentiation, the cell has lost 85% of the total proteins as well as its nucleus, so that no new RNA can be produced and any regulation of expression observed can only be on the level of translation. At this stage, over 90% of proteins synthesized consists of the α and β globin chains. Synthesis of the two chains must be coordinately regulated together with the availability of heme. Initial observations indicate that translation of globin chains has an absolute requirement for iron, for if iron is limiting, protein synthesis is inhibited and is restored only when iron is reintroduced (Traugh, 1989). The translation inhibitor was subsequently found to be a heme-regulated inhibitor (HRI), a protein kinase whose activity is regulated by heme. The kinase phosphorylates the α-subunit of the initiation factor eIF2α under conditions of heme deficiency. This phosphorylation results in the inhibition of protein synthesis (Wek, 1994). Increasing the heme availability results in the inhibition of HRI, the dephosphorylation of eIF2α and the resumption of protein synthesis (Traugh, 1989).

In higher eukaryotes, heme is also involved in regulation of protein transport. There is evidence that high levels of heme in liver cells inhibit the intracellular transport of ALAS into mitochondria, resulting in the accumulation

of the enzyme in the cytosol (Padmanaban *et al.*, 1989). Interestingly, the heme regulatory motif (HRM) that mediates heme inhibition of ALAS transport into mitochondria is similar to the HRM sequences on HRI (Wek, 1994). However, it is not yet known whether heme binds directly to these sequences.

Another example of heme-mediated regulation in higher eukaryotes is the regulation of expression of the erythropoetin gene. This gene encodes a hormone which stimulates red blood cell production under low oxygen (hypoxic) conditions. It is primarily produced in kidney and liver tissues and travels to the bone marrow, where it stimulates the proliferation and differentiation of erythroid cells by stimulating the activity of the AP1 factor (Patel and Sytkowski, 1995) and stimulating transcription of the erythroid specific GATA-1 factor (Busfield *et al.*, 1995). Expression of the erythropoetin gene is mediated by a heme protein which is postulated to function as an oxygen sensor (Goldberg *et al.*, 1988). This response can be inhibited by blocking heme synthesis. In addition, the *cis*-acting sequences responsible for this response have been localized. Deletion of these sequences results in the loss of response to hypoxic conditions. These sequences have been shown to bind to specific DNA-binding proteins (Blanchard *et al.*, 1993).

Heme regulatory functions in yeast

In yeast, heme is also involved in different aspects of regulation. Heme seems to play a similar role in regulation of translation. It has been demonstrated that translation of catalase T mRNA is regulated by heme levels. An *in vitro* translation system that is made from heme-deficient cells is unable to support translation of catalase T mRNA. However, when heme was added directly to the *in vitro* translation system made from these deficient cells, translation of the mRNA was observed, thus demonstrating heme's direct role in translational control (Hamilton *et al.*, 1982).

Heme also plays a role in protein transport in yeast. Experiments have demonstrated that covalent heme attachment to apo-cytochrome-c_1, a subunit of ubiquinol-cytochrome *c* reductase, is required for its proper mitochondrial localization (Dumont *et al.*, 1987; Zollner *et al.*, 1992).

The most well characterized regulatory functions of heme in yeast are its roles in both the repression and activation of gene expression. The effects of oxygen on gene expression in yeast are mediated by changes in heme levels. Because of the two oxygen-dependent steps in the heme biosynthetic pathway, heme is made only when oxygen in present and can serve as a good indicator of oxygen tension. Expression of many genes induced or repressed by oxygen is regulated by heme in a similar manner. Many experiments have demonstrated that aerobic growth of a heme mutant strain fails to induce expression of oxygen-dependent genes while addition of heme to an anaerobic culture will bring about repression of an oxygen-repressed gene. Taken together, these observations indicate that heme mediates the effects of oxygen on gene expression. To better understand the role of heme in regulation of gene expression in yeast, a brief overview of the effects of oxygen on yeast physiology will be presented.

Oxygen has profound effects on the energy metabolism of most organisms. Oxygen tension determines whether ATP will be produced primarily via respiration-linked oxidative phosphorylation or glycolysis-linked substrate level phosphorylation. Oxidative phosphorylation is more efficient than glycolysis in capturing the redox energy of reduced substrates. The ratio of ATP produced by these two processes affects the amount of biomass produced, among other things. Thus, oxygen tension may be an important environmental and developmental signal for the regulation of cell growth and differentiation (Poyton and Burke, 1992).

Growth in a nonfermentable carbon source such as lactate can take place only in the presence of oxygen. Under these conditions, yeast cells metabolize the carbon souce via the Krebs cycle or tricarboxylic acid (TCA) cycle with ATP generated via respiration (Figure 2). The hexose phosphates that are necessary for biosynthetic reactions are generated via gluconeogenesis. Most of the gluconeogenic steps are catalyzed by glycolytic enzymes with the exception of two irreversible steps which are catalyzed by the unique gluconeogenic enzymes fructose bisphosphatase (FBP1 in yeast) and phosphoenolpyruvate carboxylase (PCK1 in yeast). However, when grown on a fermentable carbon source such as glucose, yeast cells metabolize the carbon source via the glycolytic pathway (Figure 3). Although the pyruvate that is formed theoretically can be further metabolized via the TCA cycle, it is decarboxylated and reduced to ethanol instead. When glucose is depleted, the ethanol that is produced during glycolysis will be metabolized. This mode of growth potentially gives yeast cells a selective advantage in that they can quickly convert glucose to ethanol which can be toxic to a competing organism, or cannot be utilized by the competing organism for growth (Ronne, 1995). Since yeast cells preferentially undergo fermentative growth on glucose, catabolite repression is involved in regulation of genes encoding enzymes involved in respiratory functions (Rosenblum-Vos *et al.*, 1991) and the TCA cycle enzymes (Repetto and Tzagoloff, 1989; Lombardo *et al.*, 1992).

Figure 2. Overview of metabolic pathways in yeast during growth on a nonfermentable carbon source, ethanol. Abbreviations: Glu-6-P, glucose-6-phosphate; Fru-6-P, fructose-phosphate; Fru 1,6-P, fructose 1,6-bisphosphate; PEP, phosphenolpyruvate; NADint, NADH dehydrogenase (interior); NADext, NADH dehydrogenase (exterior); QH2, ubiquinol; bc1, ubiquinol-cytochrome *c* oxidoreductase; c, cytochrome *c*; cco, cytochrome *c* oxidase. Not drawn are other pathways involved in metabolizing other nonfermentable carbon sources. Also not drawn is the NADH produced from glyoxylate cycle or from gluconeogenesis.

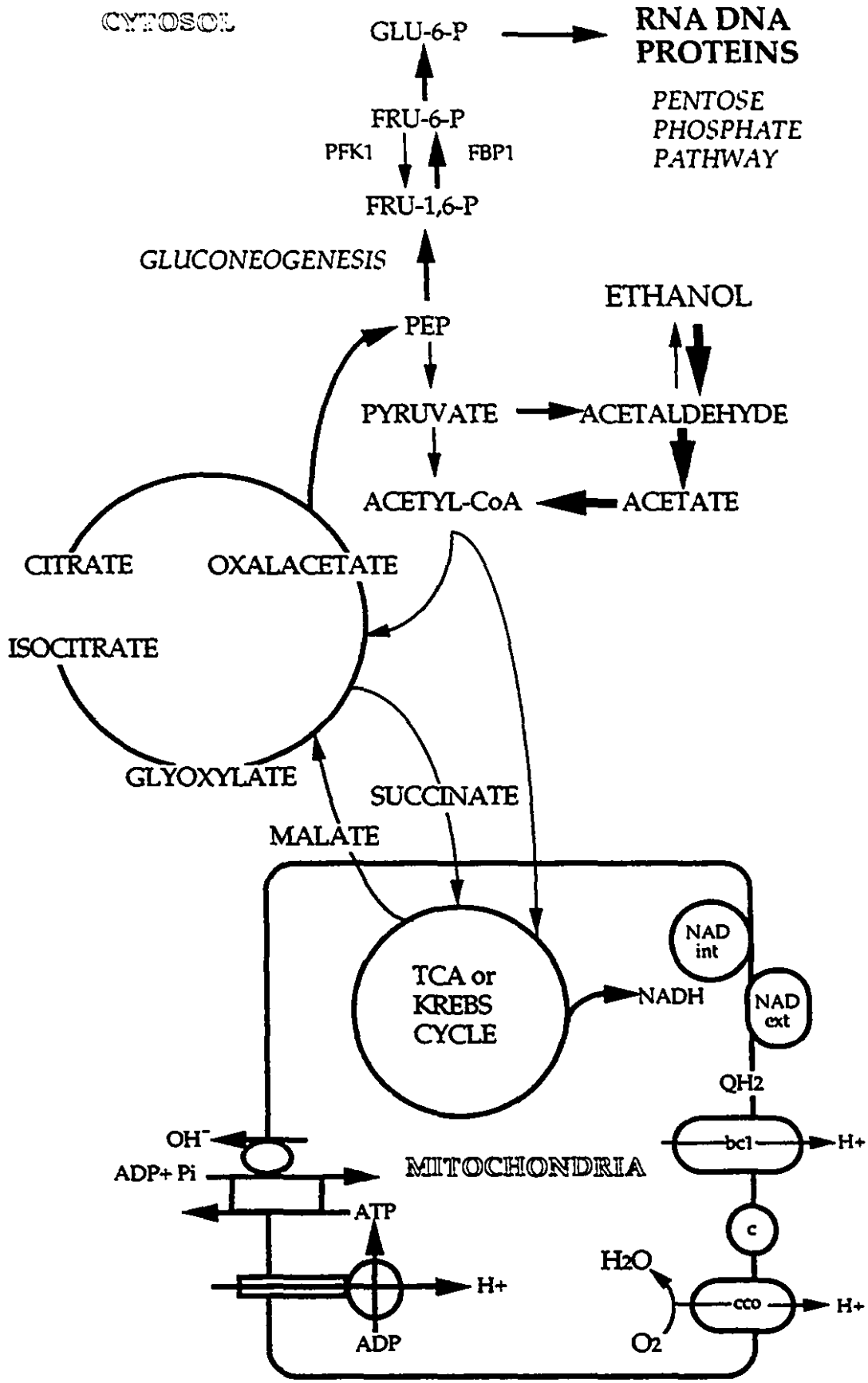
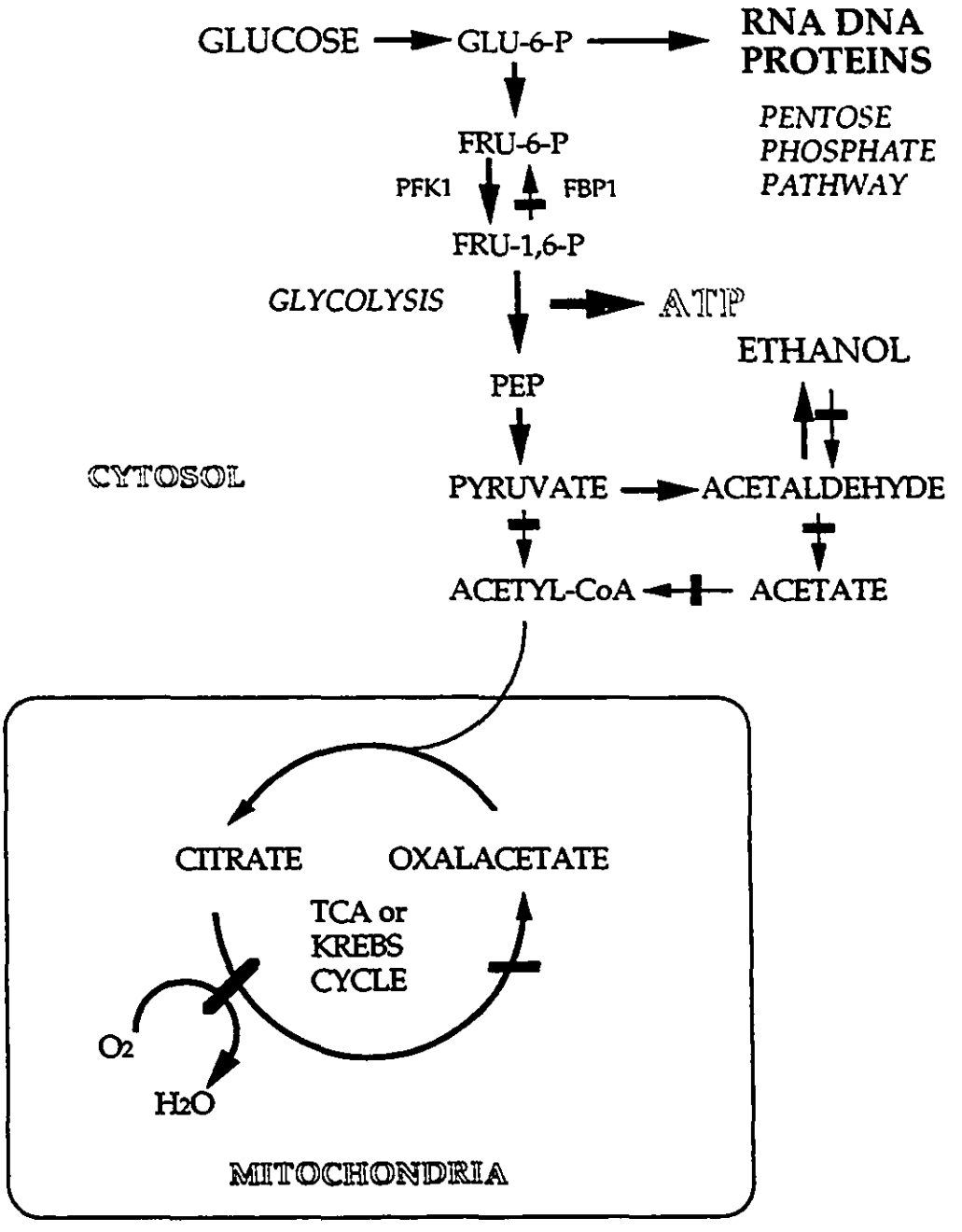

CYTOSOL
GLU-6-P
RNA DNA
PROTEINS
PENTOSE
PHOSPHATE
PATHWAY
FRU-6-P
PFK1
FBP1
FRU-1,6-P
GLUCONEOGENESIS
ETHANOL
PEP
PYRUVATE
ACETALDEHYDE
ACETYL-CoA
ACETATE
CITRATE
OXALACETATE
ISOCITRATE
GLYOXYLATE
SUCCINATE
MALATE
TCA or
KREBS
CYCLE
NADH
NAD
int
NAD
ext
QH2
bc1
H+
OH⁻
ADP+ Pi
MITOCHONDRIA
ATP
c
H2O
cco
H+
ADP
O2

Figure 3. Overview of metabolic pathways involved in growth of yeast on glucose. See Figure 2 for abbreviations. Specific pathways for the utilization of other carbon sources, glyoxylate cycle and glycolysis are not shown.
▬ represents glucose repressed enzymes.


GLUCOSE
GLU-6-P
RNA DNA
PROTEINS
PENTOSE
PHOSPHATE
PATHWAY
FRU-6-P
PFK1
FBP1
FRU-1,6-P
GLYCOLYSIS
ATP
ETHANOL
PEP
CYTOSOL
PYRUVATE
ACETALDEHYDE
ACETYL-CoA
ACETATE
CITRATE
OXALACETATE
TCA or
KREBS
CYCLE
O2
H2O
MITOCHONDRIA

Heme and oxygen regulated genes

TCA cycle and glyoxylate enzymes

In *Saccharomyces cerevisiae*, expression of a number of genes is regulated by oxygen and by heme. These genes can be divided into different classes. The first class includes enzymes in the TCA cycle and the glyoxylate cycle. The TCA cycle, in addition to providing carbon skeletons used in many biosynthetic reactions also generates NADH which drives ATP synthesis. All of the TCA cycle genes are transcribed in the nucleus, translated in the cytoplasm and like higher eukaryotes, the enzymes are transported into the mitochondria. Citrate synthase catalyzes the condensation of acetyl coenzyme A and oxaloacetate to give citrate, and is the rate-limiting step of the TCA cycle. In yeast, there are two genes that encode citrate synthase, *CIT1* and *CIT2*. CIT1 is the major form of citrate synthase in cells growing on a nonfermentable carbon source and it is the mitochondrially located enzyme. CIT2 is located in the cytosol and is the citrate synthase required for the glyoxylate cycle which takes place in the cytosol. Not surprisingly, only expression of *CIT1* is derepressed in media with a nonfermentable carbon source (Kim *et al.*, 1986).

Another important reaction in the TCA cycle, the oxidative decarboxylation of α-ketoglutarate to succinyl-CoA and CO_2, is catalyzed by the α-ketoglutarate dehydrogenase complex (KGDC). There are three enzyme components to this complex: α-ketoglutarate dehydrogenase, encoded by *KGD1*, dihydrolipoyl transsuccinlyase, encoded by *KGD2* and dehydrolipoyl dehydrogenase, encoded by *LPD1*. All three genes are transcriptionally regulated in the same manner by a global regulatory pathway responsible for the regulation of a number of genes involved in mitochondrial oxidative metabolism (Bowman *et al.*, 1992; Repetto and Tzagoloff, 1989; 1990). Coordinate regulation of citrate synthase, pyruvate dehydrogenase, α-ketoglutarate

dehydrogenase complex and aconitase was observed in *CIT1* and *CIT2* double mutants (Kispal *et al.*, 1988).

Enzymes in respiration and energy generation

The second class of genes under oxygen and heme control are the genes involved in respiration and the generation of energy. The mitochondrial electron transport chain is composed of four enzyme complexes: NADH dehydrogenase, succinate dehydrogenase, coenzyme Q (ubiquinol) cytochrome *c* oxidoreductase, cytochrome *c* oxidase as well as cytochrome *c* and coenzyme Q_1. Together these components comprise minimally 30 different proteins, including five different cytochromes and a number of electron carrying prosthetic groups. In yeast, all but four are encoded by nuclear genes (Trawick *et al.*, 1989).

Yeast mitochondria contain at least two different NADH dehydrogenases. Each enzyme is bound to the inner mitochondrial membrane; one faces the matrix side and is responsible for the oxidation of NADH produced by the TCA cycle and the other faces the intermembrane space and is involved in the oxidation of NADH produced in cytosolic metabolic reactions. The internal NADH dehydrogenase is functionally similar to Complex I found in higher eukaryotes and the external NADH dehydrogenase is equivalent to the malate/aspartate shuttle present in higher eukaryotes (Marres *et al.* ,1991; de Vries *et al.*, 1992). NADH dehydrogenase was isolated from yeast and found to be a single subunit with a molecular mass of 53 kDa containing a noncovalently-linked FAD prosthetic group. The level of this enzyme is decreased when the cells are grown in glucose (de Vries and Grivell, 1988). The gene for this NADH dehydrogenase has been isolated and it encodes a protein of 513 amino acids which is 22.2% identical to the *E. coli* NADH dehydrogenase. In addition, it was found that this gene encodes the internal NADH dehydrogenase (Marres *et al.*, 1991; de Vries *et al.*, 1992).

Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone. It is composed of four proteins, Ip - iron protein subunit and the flavoprotein subunits SDH 1, 3, and 4. It was demonstrated that levels of all four proteins are coordinately induced in the presence of a nonfermentable carbon source (Lombardo *et al.*, 1992; Daignon-Fornier *et al.*, 1994). Repression by glucose also involves the control of the turnover rate of the mRNAs for the Ip subunit and the flavoprotein subunits. The half-life of the RNAs was greater than 60 minutes in cells growing in a nonfermentable carbon source and less than five minutes in glucose grown cells. This is dependent on sequences in the 5' untranslated region and the 5' exonuclease encoded by the *XRN1* gene (Cereghino *et al.*, 1995). Cytochrome *b* is associated with this complex and is made up of two integral membrane proteins which anchor the complex to the mitochondrial membrane. Cytochrome *b*, encoded by *CYB3*, was also shown to be regulated by carbon source (Abraham *et al.*, 1994).

Coenzyme Q (ubiquinol) cytochrome *c* oxidoreductase (cytochrome *bc*1 complex) has nine subunits of which only one is encoded by mitochondrial DNA. The complex transfers electrons from ubiquinol to cytochrome *c*. This reaction is coupled with proton translocation, and generates ATP. Several subunits of this complex have been examined. *COR2* (*QCR2*) encodes a 40 kDa nonheme subunit of this complex and *CYT1* encodes the cytochrome *c*1 component. Both have been found to be induced in the presence of a nonfermentable carbon sources and heme (Dorsman and Grivell, 1990; Oechsner *et al.*, 1992). Other components have been identified: the Rieske iron-sulfur protein, a 17 kDa protein (Kim and Zitomer, 1990), in addition to two other subunits of the *bc*1 complex (*QCR6* and *QCR10*). *QCR6* regulates the activity of the *bc*1 complex by processing the cytochrome *c*1 component (Yang and

Trumpower, 1994). The presence of *QCR10* during assembly of the bc_1 complex is required for stable association with the iron-sulfur protein (Brant *et al.*, 1994). However, the regulation of the synthesis of these proteins has not yet been investigated .

CYB2 encodes cytochrome b_2 (L[+] lactate cytochrome *c* oxido reductase), which catalyzes the transfer of electrons from L(+) lactate to cytochrome *c*. *CYB2* is induced in the presence of lactate and heme and is repressed in the presence of glucose or the absence of heme (Lodi and Guiard, 1991).

Cytochrome *c* transfers electrons from cytochrome *c* reductase complex to cytochrome *c* oxidase complex (Poyton and Burke, 1992). Cytochrome *c* in yeast exists as two isoenzymes which differ by only 16% at the amino acid level. These isoenzymes are encoded by two genes, *CYC1* and *CYC7*. Both enzymes can function interchangeably in the electron transport chain. The genes are regulated in similar fashion: they are both induced on the transcriptional level in the presence of heme and are further induced by nonfermentable carbon sources. However, the overall level of expression of *CYC1* is far greater than that of *CYC7*, although the CYC7 apoprotein is stable when cells are in heme deficient conditions, and CYC1 apoprotein is not (Guarente and Mason, 1983; Prezant *et al.*, 1987).

The cytochrome *c* oxidase complex is the terminal member of the mitochondrial respiratory chain in eukaryotes. This enzyme catalyzes the reduction of oxygen to water and is the only irreversible step in the mitochondrial electron transport chain. It is thus an important control point in the regulation of electron flow through the chain as well as in the overall regulation of cellular energy metabolism (Cumsky *et al.*, 1987). Cytochrome *c* oxidase is located in the inner mitochondrial membrane and is composed of nine polypeptide subunits. In yeast, six of these subunits are encoded by the nucleus

and three are encoded by mitochondrial DNA. The mitochondrially-encoded subunits (Cox1, 2, 3) contain the catalytic redox center of the enzyme and the nuclear-encoded subunits modulate intracellular levels of cytochrome *c* oxidase. Two non-identical isoforms of a nuclear encoded subunit, Cox5a and Cox5b, were found. These isoforms are encoded by separate genes, but show 66% identity at the protein level and are functionally interchangeable (Cumsky *et al.*, 1985; 1987). They alter the turnover number of the holocytochrome *c* oxidase by altering the rate of at least one of the intramolecular electron transport reactions of the holoenzyme (Poyton and Burke, 1992). The regulation of several of the nuclear-encoded subunits has been examined. Most of the genes encoding the subunits (Cox 4, 5a, 6, 7, 8, 9) are transcriptionally activated by the presence of heme and oxygen. It is interesting to note that the gene encoding the Cox5b subunit is repressed by the presence of oxygen and heme. This observation may reflect another level of regulation of the activity of cytochrome *c* oxidase in response to varying environmental conditions. Depending on the oxygen tension, Cox5a and Cox5b isoforms are present in different ratios and can alter the catalytic function of the holoenzyme, as the Cox5b isoform increases the turnover rate of the holoenzyme (Hodge *et al.*, 1989; Poyton and Burke, 1992). It has been previously noted that cytochrome *c* oxidase activity (like that of other respiratory chain components) is decreased when cells are grown on glucose and other fermentable carbon sources. Therefore, it is not surprising that expression of genes encoding some of the subunits (Cox4, 5a, 5b, 6) are also regulated by carbon source (Trawick *et al.*, 1989; Hodge *et al.*, 1990; Silve *et al.*, 1992).

It is also interesting to note that a similar type of organization of the genes encoding subunit 5 of cytochrome *c* oxidase has been found in other fungi. There are two *COX5* genes in six other *Saccharomyces* species including *S. kluyveri* which is distinctly related to *S. cerevisiae* (Cumsky *et al.*, 1987). From that analysis

of Cox5a/5b isolated from yeast and from the partial amino-terminal amino acid sequencing studies of tissue-specific isologs in porcine and bovine tissue, it is clear that the genes encoding these two proteins, like the two isologs of cytochrome c (*CYC1* and *CYC7*) are derived from multigene families (Trueblood and Poyton, 1987).

The last component involved in the mitochondrial electron transport chain is the ADP/ATP carrier which is a nuclear-encoded integral protein of the inner mitochondrial membrane through which ADP and ATP are exchanged between the mitochondria and the cytosol. There are three different genes, *AAC1*, *AAC2*, and *AAC3* which encode ADP/ATP carriers in yeast. Expression of these genes is under the control of environmental factors. *AAC1* and *AAC2* are preferentially expressed under derepressing conditions (nonfermentable carbon source) in the presence of oxygen and heme, whereas *AAC3* is preferentially expressed under heme and oxygen-limiting conditions (Sabova *et al.*, 1993; Betina *et al.*, 1995). *AAC2* is the predominant form of the ADP/ATP carrier in cells, since both *AAC1* and *AAC3* are weakly expressed (Betina *et al.*, 1995).

Sterol biosynthesis

Another class of heme and oxygen-regulated genes is represented by several genes involved in sterol biosynthesis. The sterol biosynthetic pathway produces two products which are important for respiration: ubiquinone, a component of the electron transport chain, and the side chain of heme, a cofactor of cytochrome *c* oxidase. The demand for these two components is higher in high oxygen and heme conditions. In addition, the sterol pathway provides structural components for cell wall biosynthesis as well as components necessary for protein synthesis, protein glycolysation and cell cycle control (Casey *et al.*, 1992; Lorenz and Parks, 1991). HMG-CoA reductase is an early enzyme of the sterol biosynthetic pathway and exists as two isoforms encoded by two separate

genes, *HMG1* and *HMG2*. It is interesting to note that in many organisms, sterol biosynthesis is partially controlled by the regulatory activity of this enzyme, as it is the rate-limiting step. In yeast, *HMG1* and *HMG2* are inversely regulated by heme and oxygen; *HMG1* is induced in the presence of heme and *HMG2* is repressed by heme. The result of this is that overall, there is a constant level of HMG-CoA reductase in the cell. However, the two isoenzymes are located in different places in the cell, which may suggest the two genes are responding to the different demands of this pathway (Thorsness *et al.*, 1989; Casey *et al.*, 1992). Lanosterol 14α-demethylase, encoded by *ERG11* in yeast, is a member of the cytochrome P_{450} gene family. This gene family encodes heme-containing monooxygenases catalyzing the oxidation of biosynthetic intermediates, drugs, pesticides, and in higher eukaryotes, promutagens and carcinogens. In yeast, *ERG11* catalyzes an essential reaction in the biosynthesis of ergosterol, which is the predominant sterol in yeast. The expression of this gene is induced by glucose, heme and low levels of oxygen.

NADPH-cytochrome P_{450} reductase (encoded by the *CPR1* gene) is another component of the P_{450} system and is coordinately expressed with *ERG11* and *HMG2*. This coordinate expression may reflect a mechanism that allows cells to withstand periods of anaerobiosis (Turi and Loper, 1992).

Enzymes involved in detoxification of oxygen metabolites

A fourth class of heme and oxygen-regulated genes are those involved in detoxification of oxygen metabolites. Mitochondrial respiration involves the transfer of four electrons to oxygen. The transfer of one or two electrons to oxygen can also occur during respiration, leading to the formation of superoxide radicals and hydrogen peroxide. Yeast cells are able to remove superoxide radicals with superoxide dismutase and hydrogen peroxide with catalase. There are two catalases in yeast. Cytoplasmic catalase T encoded by the *CTT1* gene

and peroxisomal catalase A is encoded by the *CTA1* gene. Both genes are induced in the presence of oxygen and heme and are glucose repressed (Hortner *et al.*, 1982). *CTT1* expression is also induced by suboptimal nutrient supply, signalled by the Ras2 protein and cAMP-dependent protein kinase, (Bissinger *et al.*, 1989) and by heat shock (Wieser *et al.*, 1991), suggesting that catalase T may serve a protective role in the cell.

Superoxide dismutases are metalloenzymes which convert the superoxide radicals to hydrogen peroxide and oxygen. Two superoxide dismutases are found in yeast cells. *SOD1* encodes the Cu-Zn superoxide dismutase present in the cytosol. Expression of this gene requires copper and expression is further induced when cells are grown on a nonfermentable carbon source and repressed in limiting heme or oxygen conditions. *SOD2* encodes the Mn superoxide dismutase present in the mitochondria, and is regulated by oxygen and by a nonfermentable carbon source in a manner similar to that of *SOD1*. However, the *SOD* genes are not induced by nutrient limitation or by heat shock (Galiazzo and Labbe-Bois, 1993).

Translation initiation factor eIF5A

Another class of heme and oxygen-regulated genes is represented by genes encoding the two isoforms of the translation initiation factor eIF5A. This translation factor is a highly conserved protein (yeast eIF5A is 63.4% identical to human eIF5A [Metha *et al.*, 1990]) that promotes the formation of the first peptide bond during protein biosynthesis. This protein has a unique and essential covalent modification of one of its lysine residues, which is modified by spermidine to form hypusine. It is interesting to note that the yeast eIF5A can substitute for the human eIF5A found in HeLa cells (Schnier *et al.*, 1991), or the human cDNA eIF5A, when expressed in yeast, can substitute for the yeast protein *in vivo* (Schwelberger *et al.*, 1993). In yeast the two isoforms are 90%

identical, and are functionally interchangeable. However, they are inversely regulated by heme and oxygen. *ANB1* (also known as *tr-2*, *TIF51B*, *HYP2*) is preferentially expressed in low oxygen and heme conditions, whereas *TIF51A* (also known as *tr-1*, *HYP2*) is preferentially expressed in high oxygen and heme conditions (Mehta *et al.*, 1990; Schneir *et al.*, 1991; Schwelberger *et al.*, 1993; Wohl *et al.*, 1993). It is worth noting that *ANB1* is immediately upstream of the *CYC1* gene on chromosome 10 in yeast and *TIF51A* is in the same region of chromosome 5 as is *CYC7* indicating that they are duplicated gene clusters (Kang *et al.*, 1992; Melnick and Sherman, 1993).

Yeast hemoglobin

A sixth class of heme and oxygen-regulated genes is the *S. cerevisiae* hemoglobin (YHG) which is a flavoprotein of unknown function. This protein has sequence similarities to the hemoglobins of *Candida* and several bacterial species. There is high expression of *YHG* in log phase and in high heme and oxygen conditions, although there is detectable expression of *YHG* in oxygen-deficient conditions (Crawford *et al.*, 1995).

Heme biosynthetic pathway enzymes

The last class of heme and oxygen-regulated genes includes genes encoding enzymes in the heme biosynthetic pathway. The *HEM1* and *HEM3* genes appear to be expressed constitutively. However, an analysis of the *HEM1* promoter showed that there are sequences present which mediate induction by heme (Keng and Guarente, 1987; Keng *et al.*, 1992). Of the other genes in this pathway, only the gene encoding coproporphyrinogen oxidase is regulated by heme and oxygen (Zagorec *et al.*, 1988).

Upon examination of all the different classes of genes regulated by heme and oxygen, there appears to be a functional link between the genes encoding proteins involved in respiration and the electron transport chain, and those

encoding proteins involved in the detoxification of oxygen metabolites and sterol biosynthesis, in addition to others. It would make sense that these genes would be coordinately regulated by common regulatory factors. Characteristics of the regulatory factors involved are revealed when the genes are further classified into three different groups: 1) genes regulated by heme and carbon source, 2) genes induced by heme, and 3) genes repressed by heme.

Genes regulated by heme and carbon source

The aerobic respiratory system of yeast is subject to extensive catabolite control. In other words, yeast cells grown in glucose have decreased formation of mitochondrial cytochromes as well as other respiratory enzymes. Decreases in cytochrome production are also accompanied by decreased heme biosynthesis. Genetic studies of yeast mutants with altered catabolite repression have revealed the existence of a regulatory network having branches controlling different sets of enzymes. One branch controls the enzymes for gluconeogenesis while another controls enzymes required for the utilization of other carbon sources other than glucose. Yet another branch controls mitochondrial respiratory enzymes (Borralho *et al.*, 1989).

HAP2 / 3 / 4 / 5 complex

The regulatory complex that is responsible for the regulation of mitochondrial respiratory enzymes is the HAP2/3/4/5 complex. This complex was initially identified as a mutation (*hap2-1)* that did not allow derepression of *CYC1* expression in a nonfermentable carbon source (Guarente *et al.*, 1984). The *cis*-acting element responsible for this derepression was localized to a region with sequence 5' TGATTGGT 3', called UAS2 in *CYC1* (Oleson *et al.*, 1987). Mutations in either *hap2* or *hap3* abolished expression from *CYC1* UAS2 and demonstrated a global defect in the synthesis of cytochromes and other proteins involved in respiratory functions, evidenced by the inability of the mutant strains to grow on

a nonfermentable carbon source (Pinkham and Guarente, 1985). The levels of all cytochromes in the mutant strains were decreased to 40-50% to those found in a wild-type strain. Interestingly the level of heme biosynthesis also was decreased by 40-50% (Mattoon *et al.*, 1990).

It was also noted that in cells lacking normal levels of intracellular heme, the basal level of expression from UAS2 is decreased significantly (Guarente *et al.*, 1984). In later studies, expression driven by the HAP2 complex was found to be ten-fold lower in heme deficient cells as compared to levels in cells synthesizing heme. This decrease in expression in heme-deficient strains was observed whether expression was driven by a HAP2/3/4/5 complex from *CYC1* UAS2 or by a LexA-HAP2 complex from the *lexA* operator. Although the exact mechanism of heme involvement in the HAP2 complex is still unknown, this form of regulation makes expression of HAP2-responding respiratory genes sensitive to the availability of environmental oxygen which is required for respiration (Olesen and Guarente, 1990).

The HAP2 complex has been well studied and has many interesting aspects. All four genes encoding subunits in the complex have been cloned. *HAP2* encodes a 265 amino acid protein which has a basic carboxy terminus and has a centrally-located (residues 120-133) polyglutamine tract (Pinkham *et al.*, 1987). The protein was found to localize to the nucleus (Olesen *et al.*, 1987). The *HAP3* locus encodes two divergently transcribed overlapping transcripts, one of 570 nucleotides (nt) and another of 3000 nt. Disruption analysis of the *HAP3* DNA sequence indicated the 570 nt transcript encodes the 144 amino acid protein that was essential for UAS2 acitivity, in addition to growth on nonfermentable carbon sources (Hahn *et al.*, 1988). It was demonstrated that both HAP2 and HAP3 bind UAS2, and HAP2 required the presence of HAP3 in order to bind to DNA even when *HAP2* is overexpressed. The HAP2- and HAP3-dependent

DNA contacts were shown to be the same, indicative of interdependent binding (Olesen *et al.*, 1987). However, *HAP2* transcription or nuclear localization does not require a functional HAP3 (Pinkham *et al.*, 1987). The association of HAP2 and HAP3 can occur in the absence of DNA, since they can be co-purified over four chromatographic steps (Hahn and Guarente, 1988).

HAP4, a third component of the complex was identified as a protein of 554 amino acids, the amino terminus of which (residues 1-327) interacts with the HAP2 and HAP3 proteins and the carboxy terminus of which (residues 424-554) contains a potential "acid blob" transcription activation domain. Deletion of the carboxy terminus inactivates the protein activity. This activity can be restored by fusing a heterologous activation domain from the GAL4 protein. The *HAP4* transcript contains a long leader sequence with the two upstream ATG's initiating two small open reading frames (ORFs) of nine and three amino acid residues, suggesting that there may be translational regulation analogous to GCN4 (Forsburg and Guarente, 1989). A *hap4* deletion mutant phenotype can be suppressed by fusing the activation domain of GAL4 to HAP2, demonstrating that HAP4 is not essential for the DNA binding activity of HAP2 and HAP3 to CCAAT sequences but may provide the transcription activation domain (Olesen and Guarente, 1990).

HAP5 encodes a protein of 216 amino acids which was found to be required for HAP2 and HAP3 binding to DNA. Purified bacterially-produced HAP2 and HAP3 could not bind to CCAAT sequences *in vitro* unless purified bacterially-produced HAP5 was also added to the binding reaction (McNabb *et al.*, 1995). Thus, this complex represents the first heterotrimeric DNA-binding factor in which three subunits are absolutely critical for DNA-binding activity.

The regulation of expression of most of the components of this complex has been examined in detail. *HAP2* was found to be expressed in low levels, and

derepressed in cells grown on a nonfermentable carbon source. The level of *HAP2* mRNA was unaffected in a *HAP3* mutant strain (Pinkham and Guarente, 1985). *HAP3* mRNA levels are not affected by carbon source nor by mutations in *HAP2* or *HAP3* (Hahn *et al.*, 1988). *HAP4* is very poorly expressed in repressing conditions, and shows a five-fold induction in mRNA levels when shifted to lactate media (Olesen and Guarente, 1990). The expression of *HAP5* has not yet been studied in great detail.

The functional domains of the HAP2 and HAP3 proteins have been further examined and localized. HAP2 contains an essential core of 65 amino acids (154-218) which is necessary and sufficient for the assembly and DNA binding activity of the complex. This core can be further subdivided into a 44 amino acid domain sufficient for subunit association and a 21 amino acid domain required for DNA sequence recognition and binding (Olesen and Guarente, 1990). The 21 amino acid DNA binding domain of HAP2 contains three histidine and three arginine residues which are critical for binding activity (Xing *et al.*, 1993). The subunit association domain of HAP2 has an α-helix structure with eight amino acids on one face of the helix dictating the specificity and the stability of the interaction (Xing *et al.*, 1994). Based on the mutational analysis of the HAP2 core, the region required for subunit association is determined to comprise two adjacent subdomains of 18 amino acids (McNabb *et al.*, 1995).

The HAP3 DNA binding domain is comprised of seven amino acids. It is interesting to note that the effects of three different mutations in the HAP2 DNA binding domain are suppressed by the N45H mutation (an asparagine at position 45 is changed to histidine) in the HAP3 DNA binding domain. In addition, this mutation in HAP3 can also suppress a mutation in the subunit assembly domain of HAP2. This suggests that short regions of HAP2 and HAP3 comprise a hybrid

DNA-binding domain and this domain holds the two subunits together in the CCAAT binding complex (Xing *et al.*, 1993).

Homologs of both HAP2 and HAP3 have been found in *Schizosaccharomyces pombe*, and in rat and human cells. All the HAP2 homologs contain a strongly conserved region of 60 amino acids which has been shown to be the functional part of the protein (Olesen *et al.*, 1991; Sinha *et al.*, 1995; Chodosh *et al.*, 1988). In *S. pombe*, HAP2 (Php2) is also involved in mitochondrial function (Olesen *et al.*, 1991). However, in mammals the homologous CCAAT binding complex has evolved into a global transcription factor. Chromatographic fractionation of human cell extracts has identified a multicomponent CCAAT binding complex, and cooperative binding between yeast and human subunits of this complex has been demonstrated. These studies revealed that both protein-protein interactions between the subunits and protein-DNA interactions allowing for site-specific binding have been conserved between the CCAAT binding complexes in yeast and man (Chodosh *et al.*, 1988). Corresponding experiments have also been performed with the rat components of the CCAAT binding complex with similar results and which interestingly, also show the requirement of three subunits in CCAAT DNA binding (Sinha *et al.*, 1995).

Genes regulated by HAP2/ 3/ 4/ 5 complex

Many of the genes discussed in previous sections of this introduction have been shown to be under HAP2/3/4/5 complex control, here refered to as the HAP2 complex. Not surprisingly, many of the TCA cycle enzymes are regulated by this complex. Citrate synthase (*CIT1*), aconitase (*ACO1*), and the KGDC complex (*KGD1*, *KGD2* and *LPD1*) have all been shown to be regulated by the HAP2 complex (Rosenkrantz *et al.*, 1994; Repetto and Tzagoloff, 1989; 1990; Bowman *et al.*, 1992). In addition, the succinate ubiquinone reductase subunits

(*SDH 1, 3, 4*) are also co-regulated with the enzymes in the TCA cycle (Daignon-Fournier *et al.*, 1994). Other mitochondrial respiratory enzymes are also regulated in the same manner as enzymes in the TCA cycle and TCA cycle-associated enzymes. Genes encoding several subunits of cytochrome *c* oxidase are regulated by the HAP2 complex. They include *COX4, COX5a* and *COX6* (Trueblood *et al.*, 1988; Trawick *et al.*, 1989). Expression of genes encoding cytochromes including iso-1-cytochrome *c* (*CYC1*), cytochrome c_1 (*CYT1*), and cytochrome *b* (*CYB3*) are also under the same control (Guarente *et al.*, 1984; Schneider and Guarente, 1991; Abraham *et al.*, 1994). Transcription of the gene encoding one of the ATP-ADP carriers (*AAC2*) is also regulated by the HAP2 complex. Interestingly, the regulatory sequences of *AAC2* contain an ABF1-factor binding site adjacent to the HAP2 complex binding site (Betina *et al.*, 1995). ABF1 is a phosphoprotein which exists in four different phosphorylation states, and the ratio of one state to another differs according to environmental conditions. It has been proposed that ABF1 may play a role in the coordination of gene expression with DNA synthesis and with cell division (Silve *et al.*, 1992). The presence of an ABF1 binding site adjacent to a HAP2 complex binding site has also been noted for genes encoding cytochrome *c* oxidase subunit 6 (*COX6*) and subunits 2 and 8 of ubiquinol cytochrome *c* oxidoreductase (*COR2* and *QCR8*, respectively) (Trawick *et al.*, 1992; Dorsman and Grivell, 1990; de Winde and Grivell, 1992). It has been suggested that ABF1 plays a role in the regulation of basal levels of transcription of genes encoding mitochondrial proteins, and that this factor is not absolutely required for carbon-source-regulated expression.

There are other genes under control of the HAP2 complex whose functions are still not known. A screen designed to isolate genes specifically regulated by the HAP2 complex resulted in the identification of *CYT1*, which has already been shown to be HAP2-regulated, *PTP1*, encoding a protein involved in

protein import and phosphate transport, *RPM2*, a gene involved in the maturation of mitochrondrial tRNA, and 22 other genes with unknown functions, suggesting HAP2 may play a general role in regulating mitochondrial processes (Dang *et al.*, 1994). Another HAP2-regulated gene whose function is unclear is *MRF1*, which encodes a sequence-specific single-strand DNA binding protein essential for the respiratory function of the mitochondria. It has been suggested that MRF1 is a transcriptional regulatory protein of gene products involved in the assembly of the mitochondrial respiratory protein complex (Yamazoe *et al.*, 1994). Lastly, the yeast flavohemoglobin, whose function is also unclear, has also been shown to be regulated by the HAP2 complex (Crawford *et al.*, 1995).

Heme induced genes

HAP1

The second class of heme-regulated genes includes genes whose expression is heme-dependent. These genes are under the control of the regulatory factor responsible for heme-induced regulation, HAP1. *HAP1* was initially characterized as a mutant that did not allow for iso-1-cytochrome *c* (*CYC1*) expression in the presence of glucose and heme (Guarente *et al.*, 1984). A *hap1-1* mutant shows a decrease in levels of all cytochromes and the accumulation of a pigment (possibly zinc porphyrin) due to the fact that apocytochrome and heme biosynthesis have become uncoordinated (there is not a proportional decrease in heme synthesis) (Mattoon *et al.*, 1990). The HAP1 protein is a 1483 amino acid protein of about 164 kDa which is not essential for cell viability and is expressed at a low constitutive level in the cell. It is a hydrophilic protein with a basic amino terminus and an acidic carboxy terminus.

The HAP1 protein can be subdivided into different functional domains. Residues 64-84, near the amino terminus show a strong homology to the zinc

finger motif found in the GAL4 DNA-binding regulatory factor (Creusot *et al.*, 1988). This DNA binding motif has some unusual properties. There are two isoforms of cytochrome *c* in yeast, iso-1-cytochrome *c*, which accounts for 95% of cytochrome *c* in the cell, and iso-2-cytochrome *c*, accounting for the remaining 5% of cytochrome *c* (Verdiere *et al.*, 1986). This difference in the level of the two isoforms is partially due to the differential level of HAP1 activation at the *CYC1* and *CYC7* promoters. Expression of *CYC1* is primarily due to HAP1 binding to regulatory sequences in *CYC1* termed UAS1. HAP1 also binds to regulatory sequences at *CYC7*. The HAP1 binding sequences at these promoters display remarkable dissimilarities. In addition, the orientation of the HAP1 site in *CYC1* relative to the TATA box is opposite to that found in *CYC7*. Despite the sequence differences, the pattern of HAP1 binding at these sites is similar, and the *CYC7*-HAP1 complex shows the same mobility as the *CYC1* UAS1-HAP1 complex. In addition, the affinities of HAP1 to the two different binding sites appear to be the same (Pfiefer *et al.*, 1987b). Closer inspection of the binding sequence shows the presence of a GC base pair at the same position in *CYC7* as a CG base pair in *CYC1;* changing the GC base pair of *CYC7* to a CG results in a higher level of expression (as is found for *CYC1*) (Cerdan and Zitomer, 1988). It was also demonstrated that the HAP1 zinc finger recognizes a CGG triplet, as has been found for the GAL4 protein (Zhang and Guarente, 1994a). Therefore, a certain degree of flexibility must exist in the HAP1 DNA binding domain which allows HAP1 to bind dissimilar sequences with the same relative affinity.

The *HAP1-18* allele encodes a variant of HAP1 that results in increased expression of *CYC7* and decreased expression of *CYC1*. It was demonstrated that while HAP1-18 is unable to bind to UAS1 of *CYC1*, it still retains the ability to bind to its binding site in *CYC7* (Pfiefer *et al.*, 1987b). The mutation in HAP1-18 consists of a substitution of a serine residue with an arginine residue at

position 63, immediately adjacent to the zinc finger domain. Further analysis of HAP1-18 found that the increase in activation of *CYC7* expression was not due to an altered binding affinity of HAP1-18 to *CYC7* binding sequences. Substitution of other amino acids at position 63 and expression of these constructs *in vivo* did not allow for increased expression from the *CYC7* UAS, thus showing that the increased transcription activity from *CYC7* in a *HAP1-18* mutant strain is not due to abolished competition from *CYC1* UAS1. Therefore, it appears that HAP1-18 is a better transcriptional activator than wild-type HAP1 when bound at the *CYC7* UAS and that the DNA-binding domain of HAP1 participates in transcriptional activation (Kim and Guarente, 1989).

Truncated versions of HAP1 also exhibit differences in binding to UAS1 of *CYC1*. A HAP1 derivative consisting of amino acids 1-99 or 1-115 is able to bind specifically at *CYC1* UAS1 but not at *CYC7* UAS. A derivative consisting of amino acids 1-148 is able to bind to the *CYC7* UAS. Mutagenesis of the cysteine residues at position 64 (to either alanine, threonine, or serine), and position 81 (to serine, threonine or proline) in the zinc finger abolished specific binding to both *CYC1* and *CYC7*, demonstrating that these cysteines are required for binding at these sites (Pfiefer *et al.*, 1989). It is interesting to note that mutagenesis of the cysteine at position 93 to either alanine, proline or arginine also destroys the regulatory property of HAP1. Mutagenesis of the histidine residues at position 91 (to tyrosine) and position 94 (to leucine) in the zinc finger showed that they are not essential for transcriptional activation (Defranoux *et al.*, 1994). It is thus likely that the metal ions (either zinc or cadmium) necessary for the zinc finger structure are associated with the cysteine residues (Timmerman *et al.*, 1994).

Saturation mutagenesis of the DNA binding domain of HAP1 resulted in two classes of mutants: one with reduced activity at both *CYC1* UAS1 and *CYC7*

UAS, and the second which selectively eliminated activity at *CYC7* UAS. Interestingly, some mutants in both classes still displayed wild-type levels of DNA binding, indicating that they were specifically defective in transcription activation even though the altered residues were adjacent to the zinc finger (Turcotte and Guarente, 1992). Based on these experiments, specific amino acids at the base of the zinc finger and in the region immediately adjacent to the zinc finger (toward the carboxy terminus of the protein) were found to be involved in specific recognition of DNA sequences in UAS1 of *CYC1* and in *CYC7*.

Immediately adjacent to the zinc finger region of HAP1 is a dimerization domain (residues 123-148). HAP1 binds and activates transcription as a dimer. The dimerization domain has a coiled-coil structure similar to the structure of the dimerization domains of GAL4 and the myosin heavy chain (Zhang *et al.*, 1993).

HAP1 also contains a stretch of 12 glutamine residues (residues 127-189) which has been found in other regulatory proteins, and it is postulated that this region could function as a "hinge" separating two structural domains of the protein (Creusot *et al.*, 1988). The adjacent region (residues 245-444) contains six repeats of the KCPVDH motif. This motif is remarkably similar to HRM (heme regulatory motif) that is found in the mammalian δ-aminolevulinate synthase (ALAS) and HRI kinase, which inactivates translational initiation factor eIF2α. Although the HRM is not the same as the heme binding sequences of either cytochromes or globins which bind heme very tightly, it was shown to be able to associate with heme. A HRM-containing peptide of HAP1 binds heme directly and binding results in changes in both its physical and chemical properties. The cysteine residue in HRM is absolutely essential for heme binding and the conserved adjacent proline increases the affinity of heme binding (Zhang and Guarente, 1995). Deletion of this region results in constitutive transcriptional activation by HAP1 in the absence of heme or heme analogs. A deletion of

residues 447-1308 results in a HAP1 derivative with increased activity at *CYC1*-UAS1. The heme response of this protein remains intact. However, a larger deletion of residues 247-1308 results in a HAP1 derivative that can activate transcription constitutively (Creusot *et al.*, 1988; Pfiefer *et al.*, 1989). It is interesting to note that internal deletions result in HAP1 proteins with increased activity at *CYC1* UAS1 but decreased activity from the *CYC7* UAS, despite the fact that binding is normal (Kim *et al.*, 1990).

In addition to the six HRM repeats contained within residues 245-444, there is a seventh HRM repeat found at residues 1192-1197. This HRM is postulated to increase HAP1 activation in the presence of heme, since it is able to confer heme regulation when fused to a heterologous activation domain (Zhang and Guarente, 1995).

The activation domain of HAP1 lies within residues 1308-1483, at the carboxy terminus of the protein. Within this region, there is an increased concentration of acidic amino acids which is a conserved feature of activation domains from yeast to mammals (Guarente and Birmingham-McDonogh, 1992). Deletion of this domain destroys all the transcriptional activation by HAP1 (Pfiefer *et al.*, 1989).

Heme and the HRM domain are involved in the modulation of HAP1 activity on several levels. *HAP1* is constitutively expressed at a low level and HAP1 is localized to the nucleus in growth conditions containing or lacking heme (Zhang and Guarente, 1994b). It has been shown that the addition of heme increases the specific DNA binding activity of HAP1 *in vitro* (Pfiefer *et al.*, 1987a), possibly by promoting dimer formation (Zhang *et al.*, 1993). Under certain experimental conditions (overexpression of *HAP1* and low temperature gel retardation assay) a higher order protein-DNA complex can be detected containing the *CYC1* UAS1 and HAP1. Addition of heme to this reaction

reduces the level of the higher order complex and increases the formation of the HAP1 dimer complex. A HAP1 derivative containing a deletion of the HRM (residues 244-444 deleted) does not form this high molecular weight complex *in vitro* but does activate transcription constitutively, suggesting that the heme regulatory domain exerts negative control on both DNA-binding activity and the ability of HAP1 to activate transcription (Fytlovich *et al.*, 1993; Zhang and Guarente, 1994b).

HAP1 activity is also modulated by other general transcription factors. SIN3 is a non-DNA binding regulatory protein that negatively regulates expression of the yeast *HO* gene. SIN3 is a large 175 kDa protein which was found to positively regulate, via an indirect mechanism, the activities of GAL4 and HAP1 proteins (Nawaz *et al.*, 1994). HAP1 activity is also positively regulated by the TUP1/SSN6 complex. TUP1 is a β-transducin repeat protein and SSN6 is a tetratricopeptide (TPR) protein. The two proteins form a complex involved in the general repression of transcription by interacting with promoter-specific DNA binding proteins (Williams *et al.*, 1991; Keleher *et al.*, 1992; Herschbach *et al.*, 1994). HAP1 activity is decreased in either a *Δtup1* or a *Δssn6* strain. Addition of heme analogs to these strains does not restore the activity of HAP1, indicating that the reduction of HAP1 activity is not due to the lack of heme synthesis. Interestingly, deletion of the heme responsive domain alleviated the requirement for TUP1/SSN6. The deletion of *TUP1* or *SSN6* has no effect in the formation of either the high molecular weight complex or the dimeric complex, suggesting the effects exerted by the TUP1/SSN6 complex are more indirect. TUP1/SSN6 may be involved in the repression of a gene that encodes an inhibitor of HAP1 activity that functions via the heme responsive domain (Zhang and Guarente, 1994c).

Genes regulated by HAP1

HAP1 regulates genes in response to the availability of oxygen. There is a group of genes that are regulated by both the HAP2 complex and HAP1. These genes encode components of the electron transport chain. *CYC1* is regulated by these two regulatory complexes (Guarente *et al.*, 1984). *COR2* (QCR2) and *CYT1* are also both regulated in this manner (Dorsman and Grivell, 1990; Schneider and Guarente, 1991). *CYB2*, encoding cytochrome b_2 has been shown to be regulated by HAP1 and is induced by a nonfermentable carbon source (Lodi and Guiard, 1991). Yeast flavohemoglobin is also subject to this dual regulation although the significance of this is not apparent (Crawford *et al.*, 1995).

There is another group of HAP1-regulated genes whose function either directly or indirectly requires the presence of oxygen. In this group are the genes involved in sterol biosynthesis: *HMG1*, encoding HMG-CoA reductase, and *ERG11*, encoding lanosterol 14α–demethylase, both of which are induced by HAP1 (Thorsness *et al.*, 1989; Turi and Loper, 1992).

Heme repressed genes

ROX1

The last class of heme-regulated genes are the heme-repressed genes. One of the factors responsible for heme repression is the ROX1 repression factor. *ROX1* was originally identified during a search for mutants causing constitutive expression of *ANB1* which is normally repressed by heme and oxygen. Expression of *ANB1* is constitutively high in a *rox1* mutant (Lowry and Zitomer, 1984). *ROX1* expression was found to be induced by heme in a HAP1-dependent manner (Lowry and Lieber, 1986; Keng, 1992). Thus, the level of the ROX1 repressor increases in the presence of heme and expression of *ROX1*-regulated genes is repressed. Heme is not required for the function of ROX1 as a repressor (Keng, 1992). ROX1 was also shown to repress its own expression

(Deckert *et al.*, 1995). A *rox1* mutation was found not to disrupt heme metabolism and disruption of the *ROX1* locus did not result in a respiratory deficiency (Lowry and Lieber, 1986).

ROX1 encodes a 368 amino acid protein. The amino terminus of the protein contains a HMG motif found in other DNA binding proteins. The ROX1 binding site or operator consensus was identified in *ANB1* and other *ROX1*-regulated genes. The levels of repression of these genes varied with the number and the fidelity of the sites. Deletion of both *ROX1* operator sites in *ANB1* resulted in levels of expression of *ANB1* equivalent to that found in a *rox1* mutant (Lowry *et al.*, 1990). It was shown directly that ROX1 binds to the consensus operator sequence (Balasubramanian *et al.*, 1993).

Genes regulated by ROX1

Many ROX1-regulated genes are found in gene pairs which are inversely regulated by oxygen. ROX1 has been shown to repress the transcription of *COX5B* (subunit 5b of cytochrome *c* oxidase), *CYC7* (iso-2-cytochrome *c*), *ANB1* (eIF5A isoform), and *AAC3* (mitochondrial ADP/ATP translocator) (Trueblood and Poyton, 1988; Lowry and Zitomer, 1988; Lowry and Zitomer, 1984; Sabova *et al.*, 1993). Each one of these genes has a counterpart, whose expression is induced by oxygen (*COX5A, CYC1, TIF51A, AAC2*) (Trueblood *et al.*, 1988; Guarente *et al.*, 1984; Mehta *et al.*, 1990; Betina *et al.*, 1995).

Other genes regulated by the ROX1 repressor encode functions that help cells utilize limiting amounts of oxygen and include genes coding for enzymes that utilize oxygen as a substrate. *ERG11* (lanosterol 14α-demethylase) and *CPR1* (NADPH-cytochrome P_{450} reductase) are also controlled by ROX1 repression (Turi and Loper, 1992; Balasubramanian *et al.*, 1993). *OLE1* (Δ9 fatty acid desaturase), involved in fatty acid synthesis, is also regulated by ROX1

(Balasubramanian *et al.*, 1993). Lastly, *HEM13* (coproporphyrinogen oxidase) is also repressed by ROX1 in high heme and oxygen conditions (Keng, 1992).

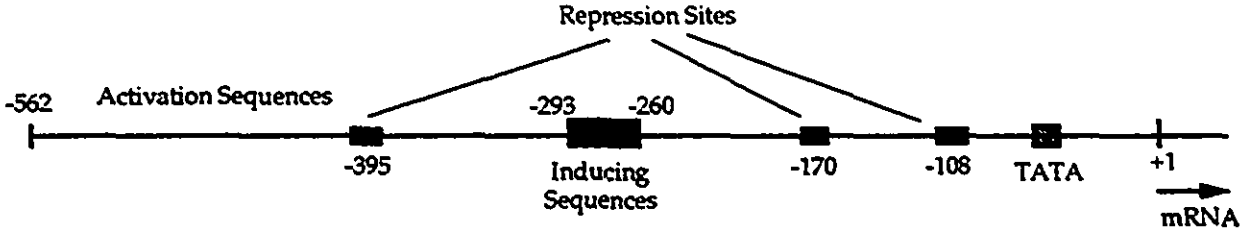
The TUP1/SSN6 complex is required for repression by ROX1. In a *Δssn6* or *Δtup1* strain, the expression of *ANB1, CYC7* and *HEM13* is increased (Balasubramanian *et al.*, 1993; C. DiFlumeri and T. Keng, unpublished results). However, the increased expression of these genes is not due to the decreased expression of *ROX1*. In fact, *ROX1* transcription is increased in a *TUP1* mutant (Zhang *et al.*, 1991). Therefore, ROX1 function requires the TUP1/SSN6 complex. This is similar to the TUP1/SSN6 requirement for repression of a-specific mating type genes via α2 (Hershbach *et al.*, 1994), glucose repression mediated by the MIG1 repressor (Treitel and Carlson, 1995), and haploid-specific gene repression mediated by a1/α2 (Komachi *et al.*, 1994).

HEM13

As mentioned previously, in yeast the heme biosynthetic pathway is primarily controlled by heme and oxygen levels, and the point where control is exerted is at the sixth step, which is catalyzed by coproporphyrinogen oxidase encoded by the *HEM13* gene. *HEM13* is repressed at the transcriptional level by heme and oxygen (Zagorec *et al.*, 1988) via the ROX1 repressor (Keng, 1992). HAP1 is involved in *HEM13* expression both in heme-proficient (+ heme) and heme-deficient (- heme) conditions. In the presence of heme, it serves to activate *ROX1* expression, which in turn represses *HEM13*. In the absence of heme, it fulfills an activating function, since expression under such conditions in a *hap1* mutant strain is significantly less that in a wild-type strain (Keng, 1992).

Deletion analysis of the *HEM13* promoter has identified 3 types of elements: activation, induction and repression elements (ROX1 consensus operator sequences) (Figure 4). Sequences responsible for the activation of

Figure 4. Regulatory sequences of *HEM13*. The activation sequences required for full level expression of *HEM13* in the absence of heme span a large region beginning at position -562 upstream of the transcription initiation site. The inducing sequences (-293 to -260) are required for induction of *HEM13* expression in the absence of heme. Three repression sites mediate repression of *HEM13* expression in the presence of heme.


Repression Sites
-562
Activation Sequences
-293
-260
-395
Inducing
Sequences
-170
-108
TATA
+1
mRNA

HEM13 expression are within a region spanning positions -562 to -259. Most of the regulation of expression by heme is mediated by three repression sites. Each repressor site was deleted individually and in combination and the effect on *HEM13* expression was examined. The three sites were each responsible for a 2.5-fold repression of *HEM13* expression. Deletion of two repression sites increased *HEM13* expression under repressing conditions eight-fold while deletion of all three sites resulted in a 20-fold increase in expression. The repression sites are homologous to the ROX1 repressor binding site consensus. Surprisingly, strong ROX1 binding was only detected for two of the three repression sites, suggesting ROX1 may bind in a cooperative manner (Keng *et al.*, 1993; Keng, Ushinsky, DiFlumeri and Richard, unpublished observations). The sequence from positions -293 to -260 is required for the induction of *HEM13* expression in the absence of heme. However, it does not function as an upstream activating sequence when placed in front of a heterologous gene. Hence, it is designated as an induction sequence.

Because of the complex nature of the regulatory region of *HEM13*, it was not likely that HAP1 and ROX1 represented all the factors needed to regulate *HEM13* expression. Thus the focus of this thesis is the identification of other *trans*-acting factors involved in *HEM13* expression. In addition, experiments examining the binding of factors to specific *HEM13* promoter fragments were also conducted.

MATERIALS AND METHODS

Strains and growth conditions: *Saccharomyces 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 100 μg/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 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-aminolevulinate synthase. The product of δ-aminolevulinate synthase is δ-aminolevulinate (ALA). *Δhem1* strains can grow only when the medium is supplemented with 50 μg/ml 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.

TABLE 1

Strains used in this study[a]

Strain	Genotype	Source
TKY22	*MATa Δtrp1::hisG*	T. Keng
TKY24	*MATa hap1::LEU2 Δtrp1::hisG*	T. Keng
TKY24α	*MATα hap1::LEU2 Δtrp1::hisG*	This study
SCI4	*MATa* UAS_{GAL} *HEM13::LEU2*	This study
SCI4αΔ1	*MATα* UAS_{GAL} *HEM13::LEU2 Δtrp1::his G*	This study
SCI4.4	*MATa* UAS_{GAL} *HEM13::LEU2 hap1-23*	This study
SCI4.3	*MATa* UAS_{GAL} *HEM13::LEU2 hap1-43*	This study
SCI4.2	*MATa* UAS_{GAL} *HEM13::LEU2 hem15- 2*	This study
SCI4.4 6d	*MATα* UAS_{GAL} *HEM13::LEU2 hap1-23 Δtrp1::hisG*	This study
SCI4.3 2b	*MATα* UAS_{GAL} *HEM13::LEU2 hap1-43 Δtrp1::hisG*	This study
TKY24HL	As for TKY24, *HEM13-lacZ::TRP1*	This study
SCI4αΔ1HL	As for SCI4αΔ1, *HEM13-lacZ::TRP1*	This study
SCI4.4Δ1HL	As for SCI4.4Δ1, *HEM13-lacZ::TRP1*	This study
SCI4.32bHL	As for SCI4.3 2b, *HEM13-lacZ::TRP1*	This study
CDY1	*MATa Δtrp1::hisG rox1::LEU2*	C. Di Flumeri

[a] All strains were derived from TKY18 *MATa ade1 ura3-52 leu2-3 leu2-112 Δhem1* (T. Keng).

Plasmids, transformation and DNA techniques: Plasmids containing various fusions used in these experiments are listed in Table 2A. 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 Dr. M. Haldi 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 respectively contain the entire upstream regulatory and coding sequences of the *HEM15* and *HAP1* genes. 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 *Δhem1* 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

TABLE 2A

Plasmids used in this study [a]

Plasmid	Fusion or Gene	Source
YCp13Z	*HEM13-lacZ*	C. Richard
YCpSD5	UAS_{GAL}-*CYC1-lacZ*	T. Keng
pBL101	*CYC1-lacZ*	L. Guarente
pTP101	*CYC7-lacZ*	M. Haldi
pTKACZ	*ANB1-CYC1-lacZ*	T. Keng
pBIO1	*CYC1-lacZ*	B. Osbourne
YIp13Z-T	*HEM13-lacZ*	T. Keng
YEpHEM15	*HEM15*	T. Keng
YCpHAP1	*HAP1*	K. Pfeifer

[a] Plasmids YCp13Z, YCpSD5, pBL101, pTKACZ and YCpHAP1 are centromeric plasmids with the *ARS4-CEN1* region. pTP101 and YEpHEM15 have the two-micron origin of replication, while pBIO1 and YIp13Z-T are integrative plasmids. Both plasmids pBIO1 and YIp13Z-T contain the *TRP1* marker. All other plasmids contain the *URA3* marker.

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, Fink and Hicks (1986). SCI4 was first grown at 30°C on YEPD plates supplemented with ALA for two to three days. The colonies were collected and resuspended in 10 mM potassium phosphate, pH 7.0. The suspension was pipeted into sterile screw cap tubes into which 0, 20, 40 or 60 µl ethyl methanesulfonate was added. The tubes were incubated at 30°C for 60 minutes 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°C for four 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°C for two to three 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 Dr. J. Pinkham) and growth on galactose. The mating type of segregants were determined by mating with *MATa met13* and *MATα met13* tester strains. A *trp1* deletion was then introduced into SCI4a using a *Eco*RI-*Bgl*II fragment from pNKY1009 (a gift of Dr. N. Kleckner) which contains a *trp1* deletion disrupted by *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 fluoro-orotic acid (FOA) plates, the FOA resistant colonies were tested for both uracil and tryptophan auxotrophies (Boeke *et al.*, 1984). This resulted in SCI4α Δ1, a *MATα Δtrp1::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 with 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 had 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 OD_{600} 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 μg/ml ALA or with Tween, ergosterol and methionine (Guarente *et al.*, 1984). The RNA samples were separated on 1% agarose gels run in MOPS-formaldelyde buffer as described (Sambrook *et al.*, 1989) and transferred onto FLASH membranes (Stratagene). Anti-sense 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 *Dra*I-*Sph*I fragment of *HEM13* cloned into the *Hin*cII and *Sph*I sites of pAM18. The *ROX1* probe used was transcribed from the plasmid pAMROX1-XH which contains a 2.0 kb *Xba*I-*Hin*dIII fragment from YCpROX1 (kindly provided by Dr. R. Zitomer) subcloned into the vector pAM18. Actin message was probed with anti-sense RNA synthesized from pSPactin which contains a 3.3 kb *Eco*RI-*Bam*HI 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'-ACTGGTTGTCTATCATGAAA-3' and 5'-GCGGGATGCGAACGAGCATG-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 Dr. D. Y. Thomas). Single-stranded DNA was isolated and sequenced using Sequenase (USB) 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 Dr. B. Turcotte). This plasmid was digested with *Sca*I, treated with shrimp alkaline phosphatase (USB) and ligated to the 1.7 kb *Sca*I 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 *Hin*dIII and *Bst*EII 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 *Hin*dIII and *Bst*EII 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*, YIp13Z-T, an integrative plasmid with a *HEM13-lacZ* fusion and a *TRP1* marker was linearized with *Xba*I 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 one hour at -70°C. They were then washed with 1/10 volume extraction buffer (200 mM Tris-HCl pH 8.0, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 7 mM 2-mercaptoethanol). The cell pellet was resuspended in extraction buffer and was disrupted by agitation at 4°C with a vortex mixer in the presence of 2/3 volume of acid washed glass beads (0.45 mm diameter). Extracts were incubated for 30 minutes on ice and then centrifuged for 45 minutes at 12 krpm. The supernatant was collected and the proteins were precipitated by the addition of 100% $(NH_4)_2SO_4$ in 20 mM HEPES pH 8.0, 5 mM EDTA to a final $(NH_4)_2SO_4$ 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.

Preparation of *CYC1* fragment for DNA binding: The 86 bp probe containing UAS1 of *CYC1* was isolated from pLG669ZΔ229-178 by digestion with *Xho*I and *Sma*I and purification from a 5% polyacrylamide gel. The fragment was labelled using Klenow fragment and α-^{32}P-TTP (ICN Biomedicals). The Klenow enzyme was inactivated by incubation at 75°C for 10 minutes and the labelled DNA was purified using a Nick-Spin column (Pharmacia) as per manufacturer's instructions.

Preparation of *HEM13* fragments for DNA binding: Specific DNA fragments were amplified by the polymerase chain reaction. Plasmid YCp13Z-TRP1 or pCRΔ3-425 were used as templates in amplification reactions using

primers listed in Table 2B. Plasmid pLG669ZΔ229-178 was used for amplification of the 86 bp control fragment containing UAS1 of *CYC1* using oligos ΔA/X5' and ΔA/X3'. Each fragment was amplified using Taq DNA polymerase (Bio/Can or Appligene) as per the manufacturer's instructions, purified from a 5% polyacrylamide gel and digested with the appropriate restriction endonuclease. After digestion, the DNA fragments were isolated from a 5% polyacrylamide gel that had been run in TBE buffer (90 mM Tris, 90 mM Boric acid, 2.5 mM EDTA).

The 153 bp fragment PCR1, from position -639 to -486 upstream of the transcription initiation site of *HEM13*, was generated by *Hin*fI digestion of a larger fragment (-639 to +1) obtained by amplification of YCp13ZTRP1 with primers OL13-9 and OLI3T. Fragment PCR2 was a 139 bp fragment containing sequences from position -562 to -424 and was amplified from pCRΔ3-424 as a template, using primers OL13-7 and CYCX1. The amplified DNA was digested with *Xho*I and the fragment PCR2 containing *HEM13* regulatory region was then further purified. Fragment PCR3 represents a region from position -469 to -333 and was generated by amplification of the region between -469 and +1 from YCp13Z with primers OL13-4 and OL13T, digestion with *Sac*I, followed by isolation of the particular fragment. Fragment PCR4 corresponds to a 121 bp fragment from position -378 to -259. It was obtained by *Dra*I digestion of the amplification product of a reaction with oligonucleotides OL13-380 and OL13-T from a YCp13Z-TRP1 template. PCR5a was derived from a DNA fragment amplified from YCp13Z-TRP1 using oligonucleotides OL13-5 and OL13T. Digestion of the fragment with *Hin*dIII yielded the 208 bp fragment from position -326 to -118. PCR5b was derived from the same amplification product, but resulted from digestion with *Eco*47III instead and represents the 145 bp fragment from -145 to +1.

Table 2B

Oligonucleotides used in this study[a]

NAME	SEQUENCE (5' TO 3')	POSITION
CYC1-lacZ		
lacI'	CGGGCAACAGCCAAGCTC	+102 to +85 (R)
CYCX1	CACGCCTGGCGGATCTGCTCGAG	-155 to -177 (R)
ΔA/X5'	AATTCCCGGGAGCAAGATCA	-319 to -298
ΔA/X3'	GCGGGATCCCGCCTGGCGGATCTG	-157 to-171 (R)
HEM13		
OL13-9	GCCTTGCCCTTTTGTTG	-639 to-623
OL13-7	TCACAGCCCGCAAATCCTCTGGAGTTTC	-562 to -539
OL13-4	GGGCGGAGTTTTTACTGTGTG	-469 to -452
OL13-1	CTATAGGCACGGTATTTT	-421 to -404
OL13-380	GGGTCACACCATTAGCCCCCT	-378 to -362
OL13-5	GGGTGCGAGATTTCTGCGCTGAAAG	-326 to -305
OL13T	GGAATTCCGCTCAAAAAAAGAAAACCCGG	+1 to -20 (R)
OL3	ACGAGTCGCAGGAAGAGA	-256 to-239
OL5	GCCTTTTCTGGTTCTCCC	-209 to -192
OL7	GCATAAGACTGCAACCAA	-165 to -148
OL12	GCGCGTCCCCGGGCCCTT	-284 to -267
OL14	TCAAAGAACGCTACGCTT	-229 to -212
OLFL5	TCTTTCACAGCGCGTCCGCG	-293 to -274
OL2'	GCGGGATCCCTGTTCCTGCGACTC	-240 to -254 (R)
OL4a	GCGGGATCCGAGCGTTATTGGTGG	-190 to -204 (R)
OL6'	GCGGGATCCTTGGTTGCAGTCTTA	-148 to -162 (R)
OL8'	GCGGGATCCTAGCCTTTCGAAACG	-92 to -106 (R)
OL13'	GCGGGATCCGCGTAGCGTTCTTTG	-214 to -229 (R)
OL15'	GCGGGATCCCAAGCTGGAGCGTTA	-172 to -186 (R)

[a] Underlined sequence is present in the template used for amplification reactions. pLG669ZΔ229-178 or pCRΔ3-425 were templates in *CYC1-lacZ* reactions and YCp 13Z-T was the template used for *HEM13* amplification reactions. Bold lettering inidicates the *Bam*HI site in the primer. The oligonucleotides were used in pairwise combinations generating the fragments shown in Figures 10 and 12.

Fragments PCR2, PCR3, PCR4, PCR5b as well as the control fragment from *CYC1* were labelled using α-32P TTP (ICN Biomedicals), while fragments PCR1 and PCR5a were labelled with α-32P dATP. The Klenow fragment of DNA polymerase (New England Biolabs) together with the appropriate mixtures of unlabelled deoxynucleoside triphosphates were present in each labelling reaction. Reactions were incubated at 30°C for 30 minutes, the Klenow fragment was inactivated by incubation at 75°C for 10 minutes, and the labelled fragments were purified on Nick-Spin Columns (Pharmacia), following the manufacturer's directions.

PCR5a was further subdivided into small overlapping fragments. A series of 14 primers were used in amplification reactions using YCp13Z-TRP1 as template (Table 2B). A *Bam*HI site was engineered into the sequence of each reverse primer. After amplification, each fragment was isolated from a 5% polyacrylamide gel and eluted in 400µl of 0.5 M ammonium acetate, 1mM EDTA at 37°C overnight. The DNA was then precipitated in 2 volumes of ethanol at -20°C for 30 minutes, spun down in microfuge for 10 minutes, dried and resuspended in water. The fragments were then cleaved with *Bam*HI and purified, using the Nucleotrap DNA Isolation Kit (Machery-Nagel) following the manufacturer's directions. The absorbance at 260 nm of each purified fragment was then determined. 200 ng of each fragment was used in a labelling reaction using the Klenow fragment of DNA polymerase I and α-32P dATP, α-32P TTP, α-32P dGTP, α-32P dCTP, and a mixture of unlabelled deoxynucleoside triphosphates excluding dGTP. The reaction was incubated for 15 min at 37°C, at which time an excess of unlabelled dGTP was added and the reaction was incubated for an additional 15 min at 37°C. The reaction was then terminated by incubation at 75°C for 10 min, and the labelled fragment purified using

Microspin columns (S200, S300, S400, Pharmacia) following the manufacturer's directions.

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 μl volumes containing 4 mM Tris-HCl pH 8.0, 60 mM KCl, 2 mM $MgCl_2$, 4% glycerol, 1 mM DTT, 100 μg/ml BSA, 1 μg sonicated salmon sperm DNA and 10 μg protein. The concentration of radioactive *CYC1* probe used was 0.5 ng and the concentration of radioactive *HEM13* probe used was 2 ng. Hemin was added to a final concentration of 40 μM when used. Reaction mixes were incubated 20 minutes at room temperature and loaded immediately onto 4% polyacrylamide gel (acrylamide to bisacrylamide weight ratio 40:1) in 1 x TBE buffer. Each gel was pre-run for 30 minutes at 25 mA in 1 x 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: Strain SCI4 containing a *HEM13-lacZ* fusion on plasmid YCp13Z was mutagenized with ethyl methanesulfonate as described in Materials and Methods. 31,500 independent colonies were screened for altered expression of *HEM13* on X-Gal plates. Ninety-four of these still showed a mutant phenotype when re-tested on X-Gal plates and were assayed for *HEM13-lacZ* expression in liquid assays. Of these, ten 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 re-transformed 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 five units in the presence of heme. This level of expression remained low at twelve units in the absence of heme. Expression of *HEM13-lacZ*

TABLE 3

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

		Units of β-Galactosidase Activity[b]		
Strain[a]	Relevant genotype	ALA	TEM	+DP
SCI4	*HAP1 HEM15*	6	228	6
TKY24	*hap::LEU2 HEM15*	33	48	33
SCI4.4	*hap1-23 HEM15*	30	75	25
SCI4.3	*hap1-43 HEM15*	5	12	7
SCI4.2	*HAP1 hem15-2*	34	342	255
SCI4.2/ YEpHEM15	*HAP1 hem15-2/HEM15*	2	239	n.d.

[a]All strains contained 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 media supplemented with δ-aminolevulinate (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. n.d. = not determined.

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 analogue deuteroporphyrin IX. Addition of deuteroporphyrin IX to the *Δhem1* 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 may be a leaky mutation in *HEM15*, the structural gene for ferrochelatase (Guarente *et al.*, 1984). In order to examine this possibility, SCI4.2 was transformed with YEpHEM15, a two micron 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 its 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 5). 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 indicates that it was approximately ten-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 SCI4αΔ1 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 was clearly recessive; the level of *HEM13-lacZ* expression of two units under repressing and 156 units under derepressing conditions in the heterozygous diploids was identical to that found with the wild type homozygous diploid (SCI4 X SCI4αΔ1). However, the heterozygous diploid formed when SCI4.3, the mutant strain which exhibited an uninducible level of expression of *HEM13-lacZ*,

Figure 5. *HEM13* RNA levels in the mutant strains SCI4.4 and SCI4.3. Total RNA was isolated from the wild-type strain SCI4, and the two mutant strains SCI4.4 (*hap1-23*) and SCI4.3 (*hap1-43*) grown in the presence of ALA (+ALA) or Tween 80, ergosterol and methionine (-ALA). 30 μg of total RNA was electrophoresed 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 radio-labelled *HEM13* RNA probes. The filter was then stripped and reprobed with the *ACT1* probe.

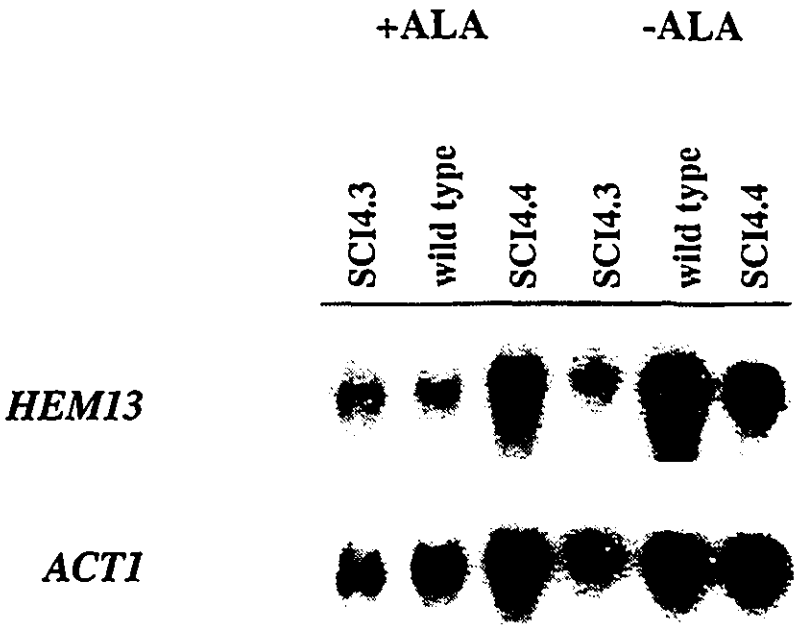

+ALA
-ALA
SCI4.3
wild type
SCI4.4
SCI4.3
wild type
SCI4.4
HEM13
ACT1

TABLE 4

Diploid analysis of mutant strains

Parental strains [a]	β-galactosidase activity [b] ALA	TEM
SCI4 x SCI4αΔ1	2	177
SCI4 x TKY24α (*hap1::LEU2*)	2	143
SCI4.4 x SCI4αΔ1	2	156
SCI4.3 x SCI4αΔ1	1	64

[a] Each mutant strain harboring YCp13Z, a CEN plasmid that carries a *HEM13-lacZ* fusion with a *URA3* marker, was mated with the wild type strain SCIαΔ1. Diploids were selected on plates without tryptophan and uracil.
[b] Assays were performed on strains grown in synthetic glucose media supplemented with 50 μg/ml δ-aminolevulinate (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.

was crossed with the wild type strain exhibited 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 were 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 diploid. 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 TKY24α. The results 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 (TKY24α X SCI4.4) was 10 units in the presence of heme and 28 units under inducing conditions, and was identical to expression in a *hap1::LEU2* homozygous diploid (TKY24 X TKY24α). 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 X SCI4.3 and TKY24α X SCI4.3 diploids is extremely low and similar to that observed in SCI4.3

TABLE 5

Complementation analysis of mutant strains

Parental strains [a]	Units of β-galactosidase activity [b] +ALA	+TEM
SCI4 x SCI4αΔ1	2	177
TKY24 x TKY24α	12	34
SCI4.4 6d x SCI4.4	11	33
SCI4.3 2b x SCI4.3	2	7
TKY24α x SCI4.4	10	28
TKY24α x SCI4.3	2	7
SCI4.4 6d x 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 *Δtrp1::hisG* marker. Diploids were selected on synthetic glucose plates lacking tryptophan and uracil. The diploids were grown in synthetic glucose media supplemented with 50 μg/ml δ-aminolevulinate (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.

2b X SCI4.3 diploids. This suggests that the mutation present in SCI4.3 may be a mutation of *HAP1*.

SCI4.3 is a unique allele of *hap1*: To determine whether SCI4.3 contained a mutation in *HAP1*, diploids from the SCI4.3 X TKY24α cross were induced to sporulate and the resulting tetrads were analyzed for the pattern of expression of *HEM13-lacZ* (Table 6). In each of the six tetrads examined, two spores showed elevated expression of *HEM13 -lacZ* of 20 to 30 units in the presence of heme and 25 to 51 units in the 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. This 2:2 segregation of phenotypes 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 Dr. K. Pfeifer). The results are summarized in Table 7. The presence of the vector YCp50 in either strain has little effect on *HEM13-lacZ* expression. Normal regulation of *HEM13-lacZ* expression in strain SCI4.4Δ1HL was completely restored when transformed with YCpHAP1, such that expression is now three units in the presence of heme and induced to 280 units in the absence of heme. However, in strain SCI4.32bHL 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*.

TABLE 6

The mutation in SCI4.3 is a novel allele of *hap1*.

Parental strains[b]	Tetrad	ALA	β-galactosidase activity[a] Spore a	b	c	d
SCI4.3 x TKY24α	1	+	7	30	31	5
		-	8	24	46	13
	2	+	23	29	4	5
		-	49	27	15	9
	3	+	5	30	29	6
		-	16	51	25	7
	5	+	4	30	28	5
		-	12	45	22	9
	6	+	3	22	4	26
		-	7	53	7	43
	8	+	4	5	28	26
		-	13	7	51	29

[a] The numbers represent β-galactosidase activity expressed from YCp13Z, a plasmid containing a *HEM13-lacZ* fusion present in all spores. The four spores from each tetrad were grown in the synthetic glucose media supplemented with 50 μg/ml δ-aminolevulinate (+) or with Tween 80, ergosterol and methionine (-). Activity is expressed in Miller units.

[b] See Table 1 for genotypes of parental strains.

TABLE 7

Complementation with a plasmid copy of *HAP1*

Strains [a]	Plasmid	β-galactosidase activity [b] ALA	TEM
SCI4αΔ1HL	none	2	239
SCI4.4Δ1HL	YCp50	18	35
SCI4.4Δ1HL	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 media supplemented with 50 μg/ml δ-aminolevulinate (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.

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 eIF5A, which is negatively regulated by heme (Lowry and Lieber, 1986). The results are summarized in Table 8. 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* expression 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

TABLE 8

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

	Units of β-galactosidase activity,[a]					
	CYC1-lacZ		*ANB1-CYC1-lacZ*		*CYC7-lacZ*	
Strain (*HAP1* allele)	ALA	TEM	ALA	TEM	ALA	TEM
SCI4 (*HAP1*)	80	<1	2	238	12	22
TKY24 (*hap1::LEU2*)	3	3	10	3	2	<1
SCI4.4 (*hap1-23*)	6	2	11	7	4	2
SCI4.3 (*hap1-43*)	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 or in the presence of Tween 80, ergosterol and methionine (TEM). Results are averages of duplicate assays performed on at least two colonies from each transformation.

strain with wild type was transformed with the *CYC1-lacZ* plasmid pBL101, as well as YCp13Z which contains the *HEM13-lacZ* fusion. In each case, the mutation that affected *HEM13* expression co-segregated with that affecting *CYC1* expression (Table 9). 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 (Figure 6). Northern blot analysis and densitometry of these RNA samples show that *ROX1* transcript levels cannnot be detected in the *HAP1* strain in the absence of ALA. Addition 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 can be detected in the absence of ALA in the medium. This level is not significantly altered with ALA in the medium.

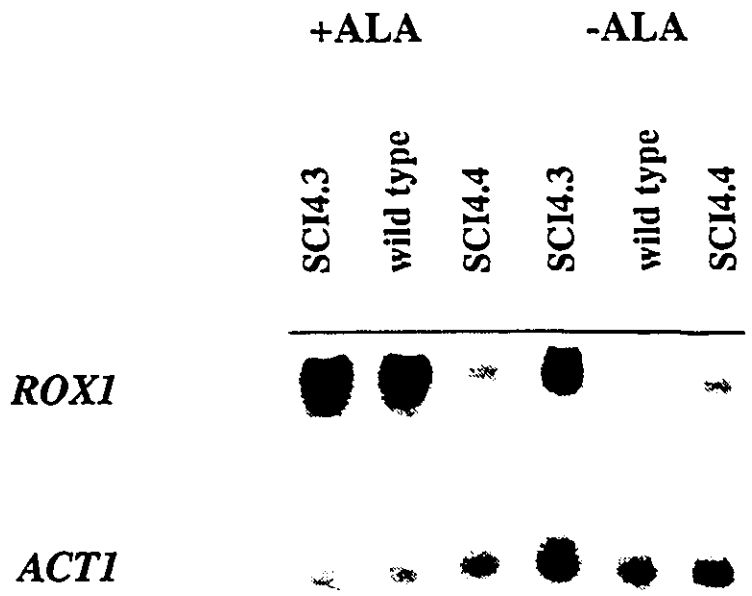
TABLE 9

Expression of *HEM13-lacZ* and *CYC1-lacZ* in tetrads.

		Units of β-galactosidase activity[a]			
		HEM13-lacZ		*CYC1-lacZ*	
Diploids	Spore	ALA	TEM	ALA	TEM
SCI4.4	1a	5.7	219	71.8	<1
X	1b	30.5	17.7	5.9	1.7
SCIαΔ1	1c	6.3	230	68.3	<1
	1d	28.5	17	6.2	1.6
SCI4.3	2a	6.0	225	30.8	<1
X	2b	5.5	176	35.4	<1
SCIαΔ1	2c	5.6	6.0	76.2	21
	2d	5.9	8.2	63	17.7
SCI4.4		30	18	6	2
SCI4.3		6	6	102	32
SCI4αΔ1		6	228	80	<1

[a] *HEM13-lacZ* was introduced as YCp13Z and *CYC1-lacZ* introduced as pBL101 into the individual spores. The numbers represent the averages of duplicate assays and are reported as Miller units.

Figure 6. *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). 30 μg of total RNA was electrophoresed on a 1% agarose gel in MOPS-formaldehyde buffer as described. RNA samples were transferred on to FLASH membranes and probed with radioactively labelled *ROX1* RNA probe. The filter was stripped and reprobed with *ACT1* probe.

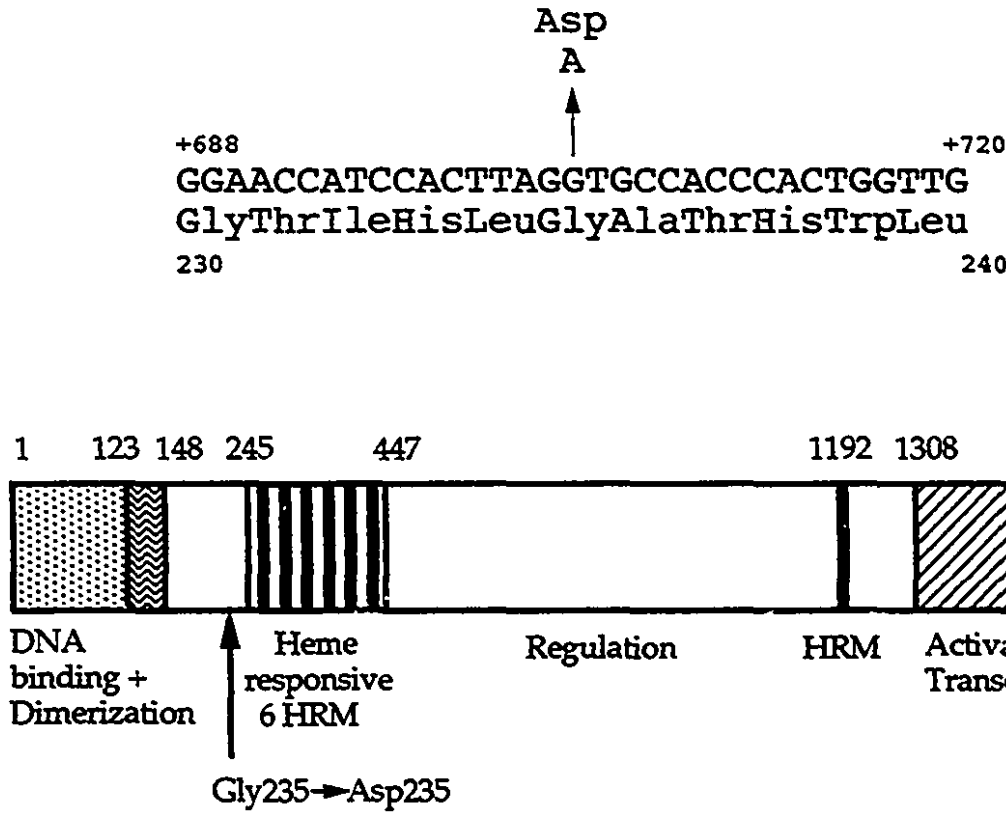

+ALA
-ALA
SCI4.3
wild type
SCI4.4
SCI4.3
wild type
SCI4.4
ROX1
ACT1

Conversely, in strain SCI4.3, expression of *ROX1* has become constitutively high and heme-independent.

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 to 444, and the transcription activation domain from amino acid 1308 to 1483. An internal region from amino acid 445 to 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, in addition to 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 indicated that amongst the three domains, there was only one change, a G to A substitution, at position +704 of the *HAP1* coding region (Figure 7). This resulted 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 *Hin*dIII-*Bst*EII 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

Figure 7. 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; Zhang *et al.*, 1993; Zhang and Guarente, 1995) and the location of the *hap1-43* mutation with respect to these domains.


Asp
A
+688
+720
GGAACCATCCACTTAGGTGCCACCCACTGGTTG
GlyThrIleHisLeuGlyAlaThrHisTrpLeu
230
240
1
123
148
245
447
1192
1308
1483
DNA
binding +
Dimerization
Heme
responsive
6 HRM
Regulation
HRM
Activation of
Transcription
Gly235→Asp235

to replace the corresponding fragment in 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 10) indicate that expression of *HEM13-lacZ* in strain TKY24HL was not affected by introduction of the vector pRS316. However, regulation was 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 was increased to 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.

***In vitro* binding to *CYC1* promoter sequences:** 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 protein made from the *hap1-43* allele may be heme-independent. To investigate this possibility, we examined the DNA binding activity of the various mutant HAP1 proteins. Figure 8 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

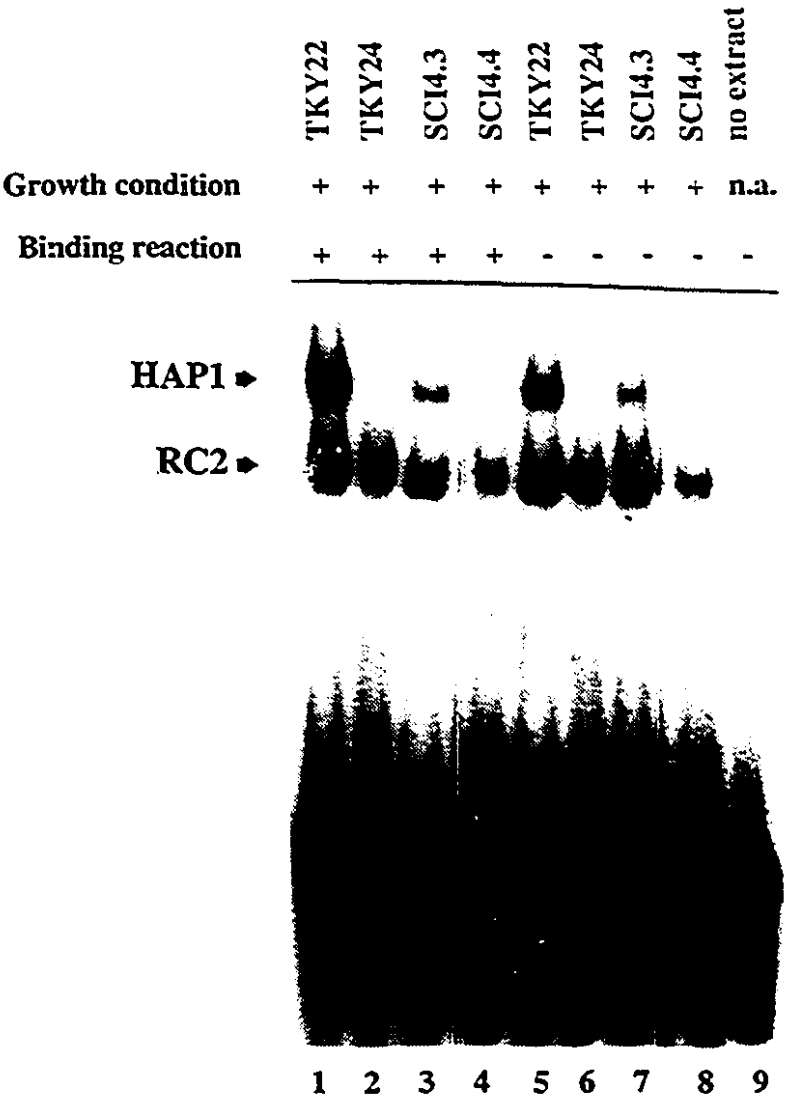
TABLE 10

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

Plasmid	*HAP1* allele	Units of β-galactosidase activity [a]	
		ALA	TEM
pRS316	*hap1::LEU2*	17	37
pHAP1-S	*hap1::LEU2/HAP1*	2	210
pHAP1ARSCEN	*hap1::LEU2/HAP1*	2	184
pHAP1-43	*hap1::LEU2/hap1-43*	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.

Figure 8. Binding of HAP1 variants to *CYC1* UAS1. 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 presence of heme (ALA). The extracts were incubated with an 86 base pair probe containing the UAS1 of *CYC1* at room temperature for 20 minutes. The protein-DNA complexes were then resolved on a 4% high ionic strength polyacrylamide gel. Lanes 1-4 also contained 40 μM hemin added directly to the binding reaction. In lanes 5-9, no hemin was added to the binding reactions.

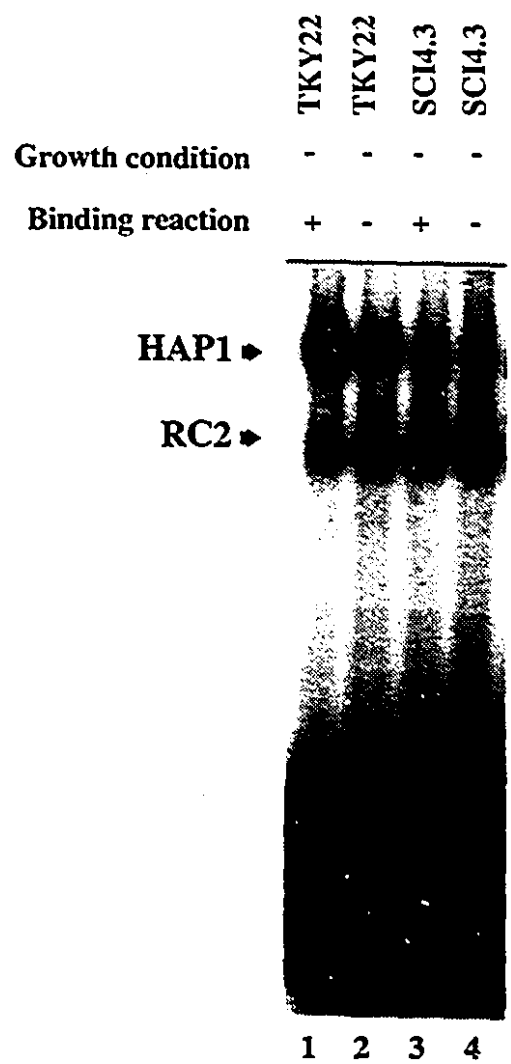

TKY22
TKY24
SCI4.3
SCI4.4
TKY22
TKY24
SCI4.3
SCI4.4
no extract
Growth condition
+ + + + + + + + n.a.
Binding reaction
+ + + + - - - - -
HAP1
RC2
1 2 3 4 5 6 7 8 9

slower mobility contains HAP1 protein. Addition of heme to the binding reactions stimulated HAP1 binding to the fragment two-fold 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, 8). Interestingly, the HAP1-43 protein made in strain SCI4.3 still retained the ability to bind DNA. However, unlike the wild type HAP1 protein, the binding activity of this altered protein was not stimulated by the presence of heme in the binding reaction, as confirmed by densitometric analysis (lanes 3 and 7).

Figure 9 shows the binding assays performed using extracts prepared from cells grown in the absence of ALA. Interestingly, HAP1 protein made in strain TKY22 grown under heme-deficient conditions and in the absence of heme in the binding reactions was still able to bind to DNA (see lane 2). Addition of heme to the binding reaction enhanced the DNA binding activity approximately two-fold (compare lanes 1 and 2). The HAP1-43 protein made under heme-deficient conditions was also able to bind DNA. However, densitometry of the autoradiograms revealed that this binding activity was not stimulated by heme in the binding reaction (lanes 3 and 4).

***In vitro* binding to *HEM13* promoter sequences:** The results from analysis of the *HEM13* promoter suggests that the region involved in expression of *HEM13* is quite complex and involves both positive and negative regulatory elements (See Figure 4). In an attempt to understand the complex interactions that result in the repression of *HEM13* expression by heme, *in vitro* protein-DNA binding assays were performed. In addition, we also wanted to test if any of the complexes that were observed were due to the ROX1 or HAP1 proteins.

Figure 9. Binding of HAP1 and HAP1-43 to *CYC1* UAS1. The extracts were prepared from *HAP1* (TKY22) and *hap1-43* (SCI4.3) strains grown in the absence of heme (TEM). The extracts were incubated with an 86 base pair probe containing the UAS1 of *CYC1* at room temperature for 20 minutes. The protein-DNA complexes were then resolved on a 4% high ionic strength polyacrylamide gel. Reactions in lanes 1 and 3 contained 40 μM hemin in the binding reactions and the reactions in lanes 2 and 4 did not contain any additional hemin.


TKY22
TKY22
SCI4.3
SCI4.3
Growth condition - - - -
Binding reaction + - + -
HAP1
RC2
1 2 3 4

Previous analysis has indicated that both of these proteins play a role in the regulation of *HEM13* expression; ROX1 functions as a repressor for *HEM13* expression while HAP1 is required for the full activation of *HEM13* expression in the absence of heme (Keng, 1992). Initially, the entire *HEM13* promoter from positions -640 to +1 was subdivided into six overlapping fragments 1-5b (Figure 10) . Each fragment was individually tested for its ability to bind proteins in extracts prepared from the *hem1* mutant strain (TKY22) grown in the presence and in the absence of heme (data not shown). Fragment 5a that spans the region from position -326 to position -121 displayed the strongest protein-DNA interactions (Figure 11). In all, five different protein-DNA complexes (complexes A-E) were detected in the DNA binding assays, using extracts prepared from cells grown both in the absence and presence of heme. Two of the complexes, C and D, were formed in higher levels than the other three complexes. Addition of heme to the binding reactions caused a decrease in formation of all five complexes (Figure 11, compare lanes 1 and 2 and lanes 3 and 4). In particular, formation of complexes A and B, the two complexes that migrate with the slowest mobility, was dramatically decreased and became barely detectable. The same five complexes could be detected when binding reactions were performed using extracts prepared from a *rox1::LEU2* mutant strain (Figure 11, lane 8), a *hap1* mutant strain (Figure 11, lane 6) as well as the *hap1-43* mutant strain (Figure 11, lanes 10 and 12). Formation of complex E, the complex with the fastest mobility was higher in binding reactions with extracts prepared from *rox1::LEU2* and *hap1-43* mutant strains (Figure 11, lanes 7-12). Addition of heme to the binding reactions with these alternate extracts also resulted in a decrease in complex formation (Figure 11, lanes 7, 9 and 11).

Figure 10. A series of overlapping *HEM13* promoter fragments from -639 to +1 used in the *in vitro* binding experiments with TKY22 (wild-type) whole cell extracts grown in the presence and absence of heme. See Materials and Methods for details.

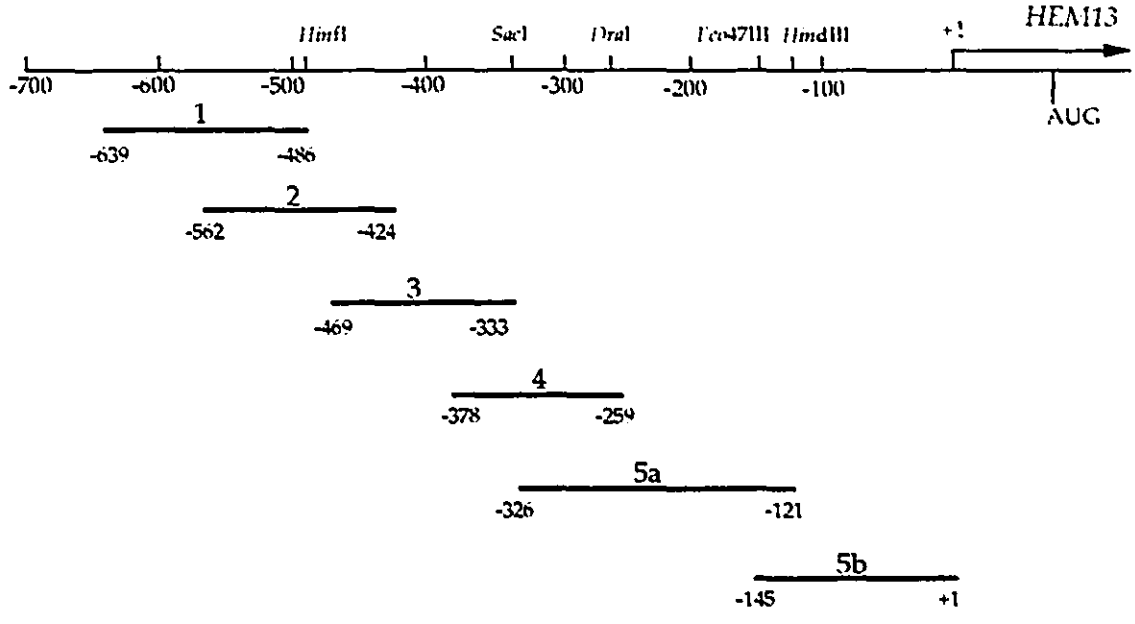
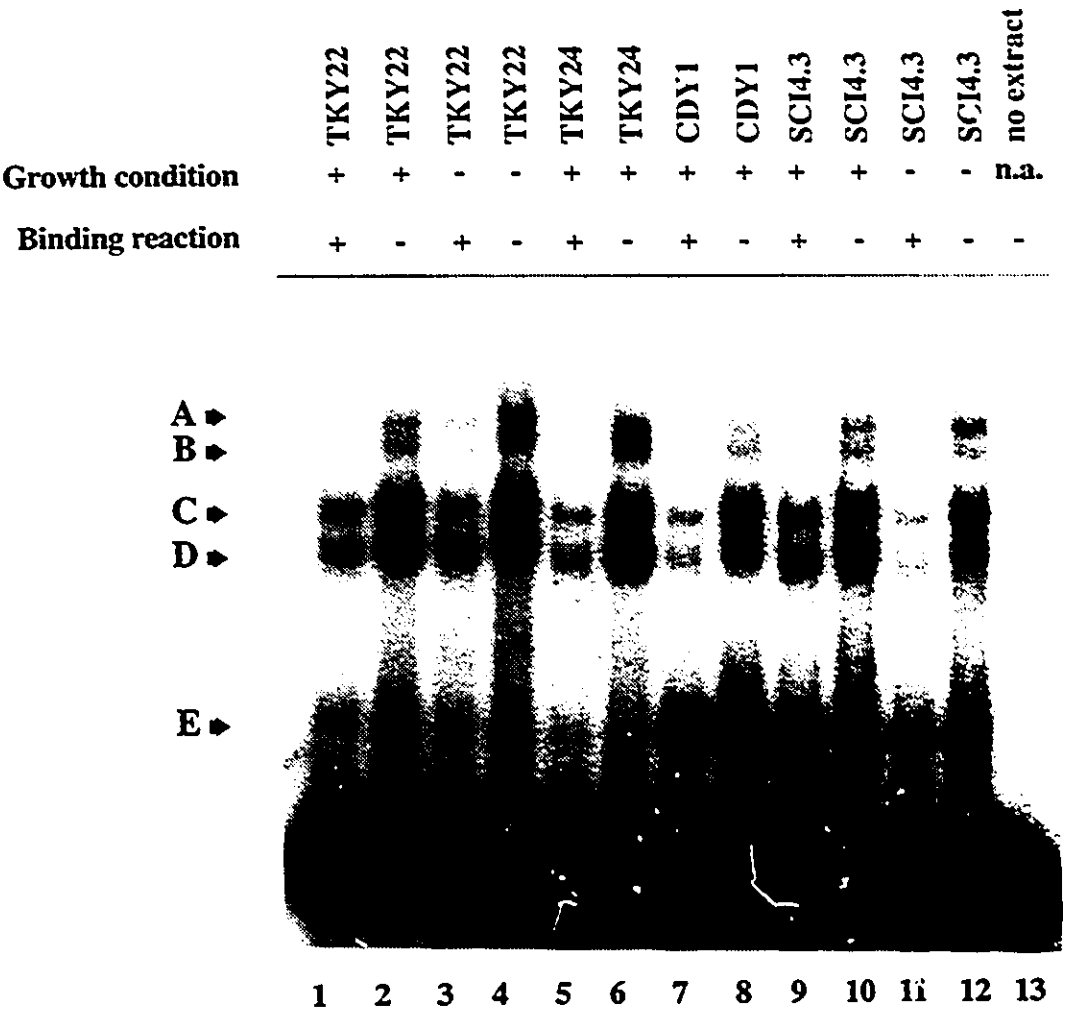

HEM13
HinfI
SacI
OraI
Eco47III
HindIII
+1
-700
-600
-500
-400
-300
-200
-100
AUG
1
-639
-486
2
-562
-424
3
-469
-333
4
-378
-259
5a
-326
-121
5b
-145
+1

Figure 11. Gel electrophoresis DNA binding assays with *HEM13* promoter fragment 5a. Whole cell extracts were prepared from TKY22 (wild-type), TKY24 (*hap1::LEU2*), CDY1 (*rox1::LEU2*) and SCI4.3 (*hap1-43*) strains grown in the presence of heme (+) or in the absence of heme (-). The cell extracts were incubated with radiolabelled *HEM13* promoter fragment 5a (-326 to -121) at room temperature for 20 minutes. The protein-DNA complexes were resolved in a 4% high ionic strength polyacrylamide gel. Lanes 1, 3, 5, 7, 9, 11 contained 40 μM hemin. No hemin was added to reactions in lanes 2, 4, 6, 8, 10, 12, 13.


+ TKY22
TKY22
TKY22
TKY22
TKY24
TKY24
CDY1
CDY1
SCI4.3
SCI4.3
SCI4.3
SCI4.3
no extract
Growth condition
+ + - - + + + + + + - - n.a.
Binding reaction
+ - + - + - + - + - + - -
A
B
C
D
E
1 2 3 4 5 6 7 8 9 10 11 12 13

To further investigate protein binding to fragment 5a, the fragment was subdivided into smaller overlapping fragments (Figure 12). Figures 13-19 show the complexes formed in binding reactions with some of the fragments tested. Addition of extracts to labelled fragments E (-326 to -190) and H (-326 to -148) respectively also allowed the formation of five complexes similar to what was seen with fragment 5a (Figure 13, lanes 5 and 7 and Figure 14, lanes 5-8). The formation of these complexes was also decreased with the addition of heme to the binding reaction. The shortest fragment that allows formation of complexes A-E is fragment A_L (-326 to -214) (Figure 15, lane 2).

Complexes A and B, the two complexes with the slowest mobility were formed when extracts were added to fragment A (-326 to -240) but not when extracts were incubated with fragment AEH (-284 to -214). (Compare Figure 13, lane 1 to lane 3 and Figure 16, lanes 1 and 3.) Moreover, no complex A or B formation could be detected with fragment FL (-293 to -148) (data not shown). Thus, complexes A and B are deduced to be formed by protein factors binding to sequences from -326 to -293. It has been demonstrated that deletion of this region causes a decrease of *HEM13* expression both in the presence and absence of heme (Keng, Ushinsky, DiFlumeri and Richard, unpublished observations).

We have noted that complexes C and D, of intermediate mobility, were formed when extracts were incubated with fragments E (-326 to -190) and H (-326 to -148) respectively (Figure 13, lanes 5 and 7). However, only one of these two complexes could be detected in binding reactions with fragments A (-326 to -240) and AEH (-284 to -214) (Figure 13, lanes 1 and 3 and Figure 14, lanes 1-4). Interestingly, the extent of complex D formation increased when fragment A was incubated with extracts in the absence of heme (Figure 14, lanes 1 and 3 and Figure 16, lane 2). In addition to an increase in complex D formation, another complex can be detected in extracts made from cells grown

Figure 12. Subdivision of *HEM13* promoter fragment 5a. A series of oligonucleotides (listed in Table 2B) were used in various combinations to yield these fragments. Each fragment was gel purified, digested with *Bam*HI, repurified and treated with Klenow fragment of DNA polymerase I with radiolabelled nucleotides. See Materials and Methods for details.

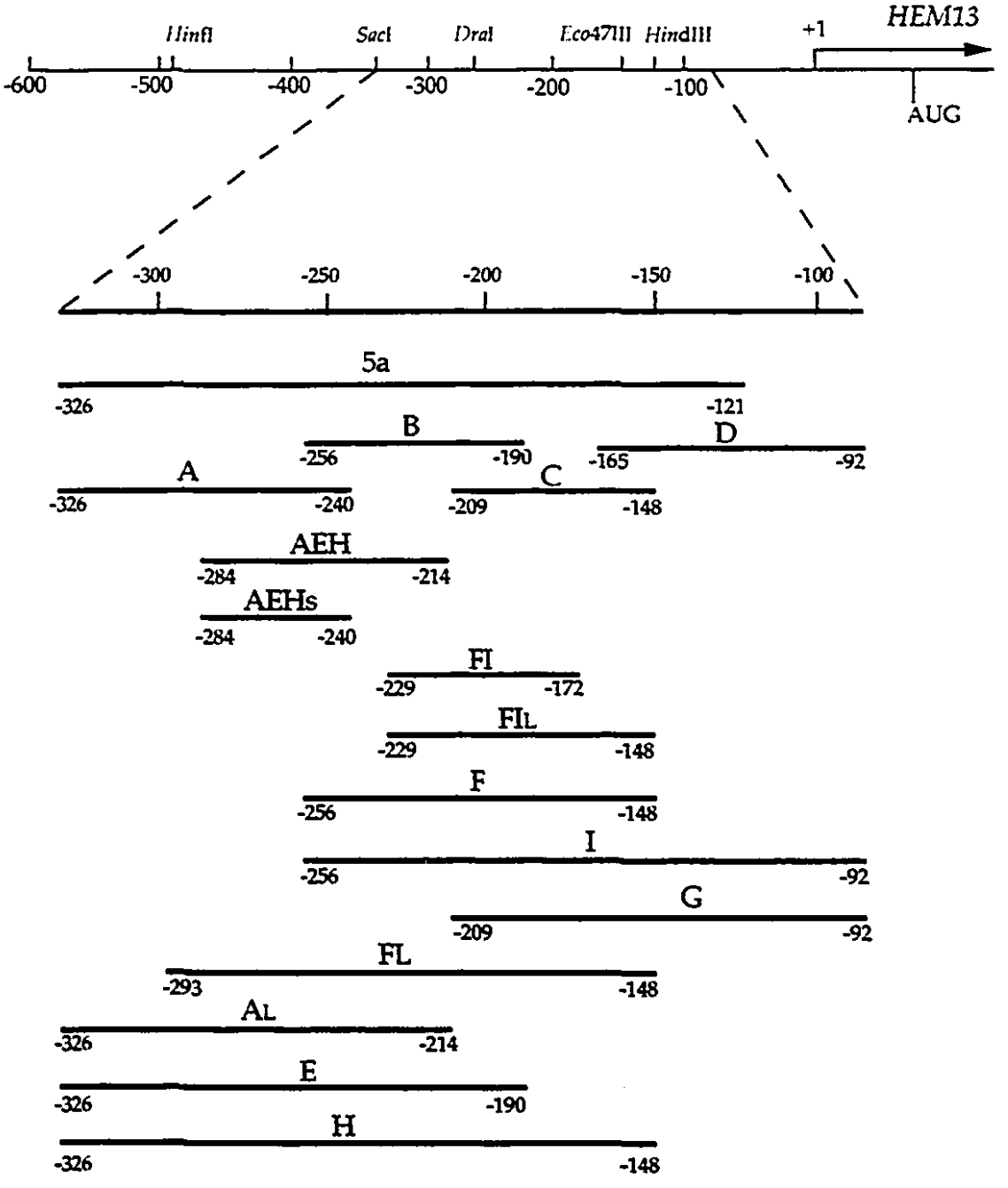

HEM13
HinfI
SacI
DraI
Eco47III
HindIII
+1
-600
-500
-400
-300
-200
-100
AUG
-300
-250
-200
-150
-100
5a
-326
-121
B
-256
-190
D
-165
-92
A
-326
-240
C
-209
-148
AEH
-284
-214
AEHs
-284
-240
FI
-229
-172
FIL
-229
-148
F
-256
-148
I
-256
-92
G
-209
-92
FL
-293
-148
AL
-326
-214
E
-326
-190
H
-326
-148

Figure 13. Gel electrophoresis DNA binding assay. Fragments A (-326 to -240), AEH (-284 to -214), E (-326 to -190) and H (-326 to -148) from the *HEM13* promoter were incubated for 20 mintues at room temperature with whole cell extracts made from TKY22 (wild-type) cells grown in the presence of heme. No heme was added to the binding reactions. NE= no extract was added to the binding reactions.

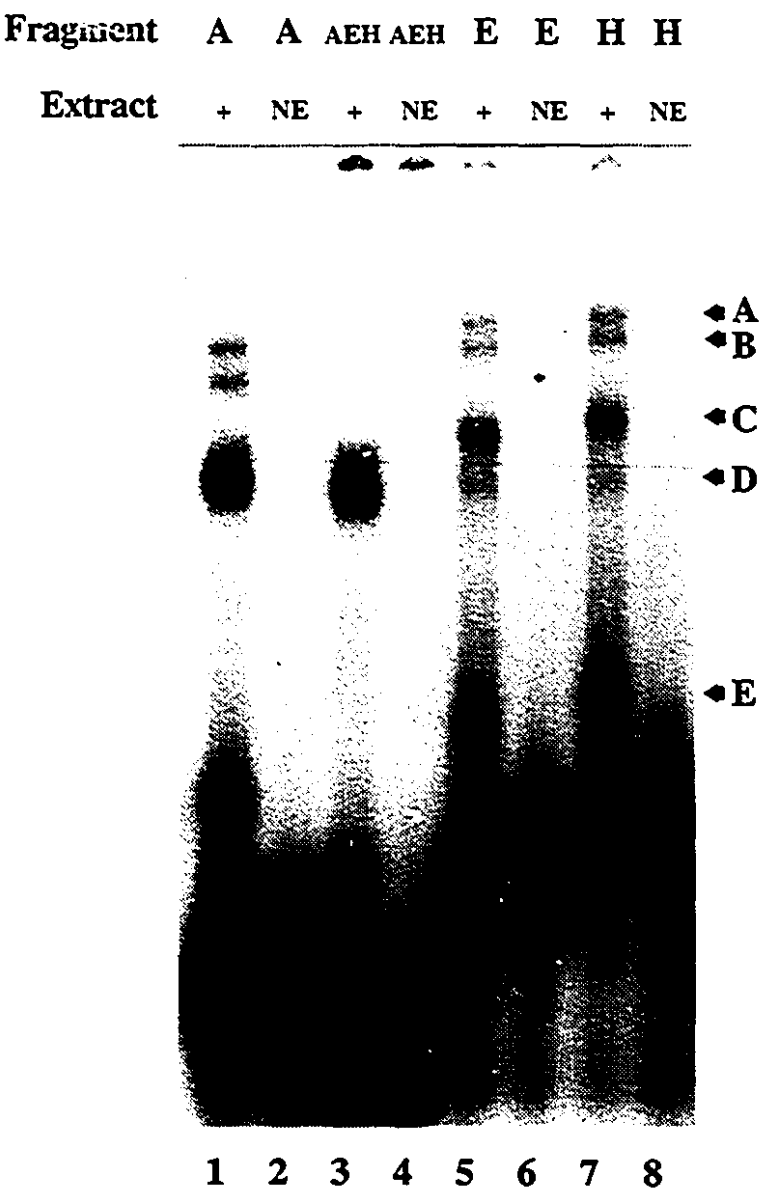

Fragment A A AEH AEH E E H H
Extract + NE + NE + NE + NE
A
B
C
D
E
1 2 3 4 5 6 7 8

Figure 14. Gel electrophoresis DNA binding assay. Fragments A (-326 to -240) and H (-326 to -148) from the *HEM13* upstream noncoding sequences were incubated for 20 minutes at room temperature with whole cell extracts made from TKY22 cells (wild-type) grown either in the presence (+) or absence (-) of heme. Lanes 1, 3, 5, 7 have no hemin added to the binding reactions while lanes 2, 4, 6, 8, contained 40 μM hemin in the binding reactions.

Fragment	A	A	A	A	H	H	H	H
Growth condition	+	+	-	-	+	+	-	-
Binding reaction	-	+	-	+	-	+	-	+

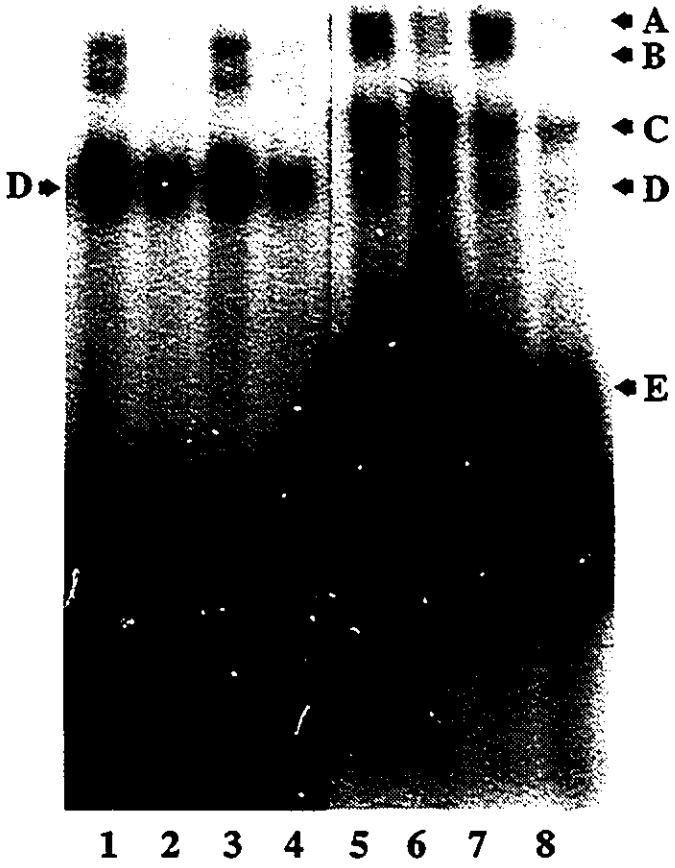


Figure 15. Gel electrophoresis DNA binding assays with fragments E and A_L. Fragments E (-326 to -190) and A_L (-326 to -214) from the *HEM13* promoter were incubated for 20 minutes at room temperature with whole cell extracts prepared from TKY22 (wild type) grown in the presence of heme. No hemin was added to the binding reactions.

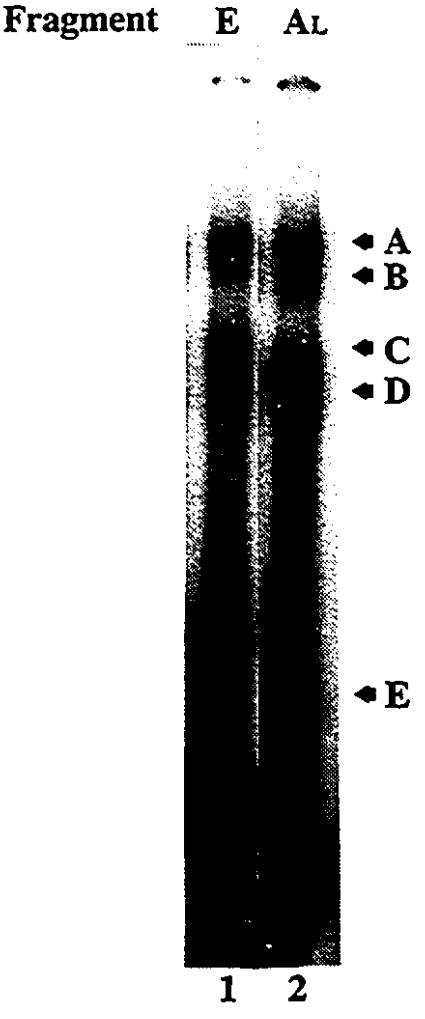
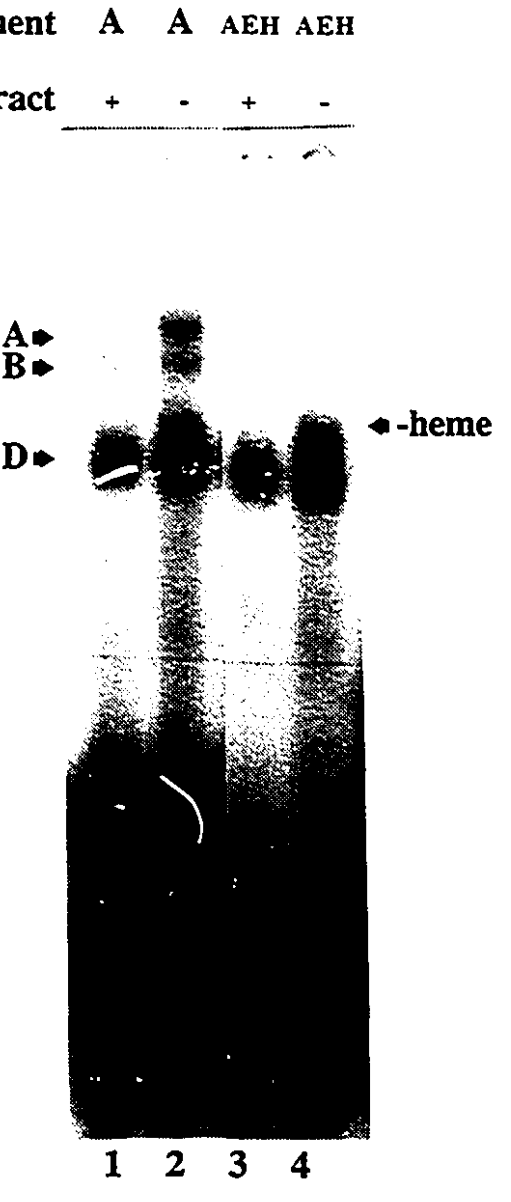

Fragment
E
AL
A
B
C
D
E
1
2

Figure 16. Gel electrophoresis DNA binding assay with *HEM13* promoter fragments A and AEH. Fragments A (-326 to -240) and AEH (-284 to -214) from the *HEM13* promoter were incubated for 20 minutes at room temperature with whole cell extracts made from TKY22 (wild-type) cells grown in the presence (+) or absence (-) of heme. No hemin was added to the binding reactions.


Fragment A A AEH AEH
Extract + - + -
A
B
D
-heme
1 2 3 4

in the absence of heme. Using the smaller AEH fragment (-284 to -214) in binding reactions with extracts made from cells grown in the absence of heme, the formation of another complex which migrated more slowly than complex D was strongly evident (Figure 16, lane 4). When the AEH fragment was mixed with extracts made from heme-sufficient cells, this complex was barely detectable (Compare Figure 16, lanes 3 and 4). This complex was also evident using fragment AEH_S (-284 to -240) suggesting the this complex is formed by interaction of protein factors with sequences between positions -284 and -240 upstream of the *HEM13* coding region (Figure 17 lanes 1 and 3). This region overlaps the sequences from -293 to -260 which have been shown to be needed for the induction of *HEM13* expression in the absence of heme (Keng, Ushinsky, DiFlumeri and Richard, unpublished observations).

It was also noted that a complex with high mobility could be detected both using fragment A (-326 to -240) and fragment C (-209 to -148) (Figure 13, lane 1 and Figure 18, lane 2). This suggests that complex E formed on fragment 5a (-326 to -121) is actually two different complexes that migrate in the same fashion. The fast migrating complex detected with Fragment C was demonstrated to be heme insensitive (Figure 19 compare lanes 3 and 4). It is interesting to note that no complex formation was detected with fragment B (-256 to -190) or with fragment D (-165 to -92) (data not shown). This allowed further delination of the binding sites of the complexes observed. This analysis of the *HEM13* promoter and the *trans*-acting factors involved confirms the complex nature of the regulatory pathway governing *HEM13* expression, supporting the idea that the sixth step of the heme biosynthetic pathway is an important control point in the heme and oxygen regulatory circuit.

Figure 17. Gel electrophoresis DNA binding assay with fragment AEHs. Fragment AEHs (-284 to -214) was incubated for 20 minutes at room temperature with whole cell extracts prepared from TKY22 (wild-type) cells grown in the presence (+) or absence (-) of heme. Lanes 2 and 4 contain 40 μM hemin which was added directly to the binding reactions. In lanes 1 and 3, no hemin was added to the binding reactions.

Fragment	AEHs	AEHs	AEHs	AEHs
Growth condition	+	+	-	-
Binding reaction	-	+	-	+

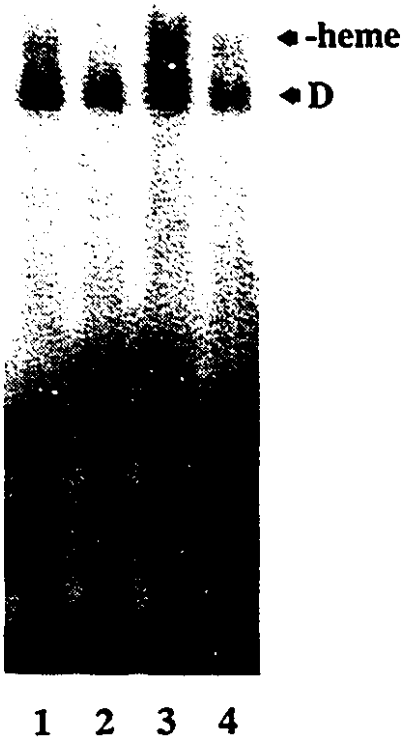
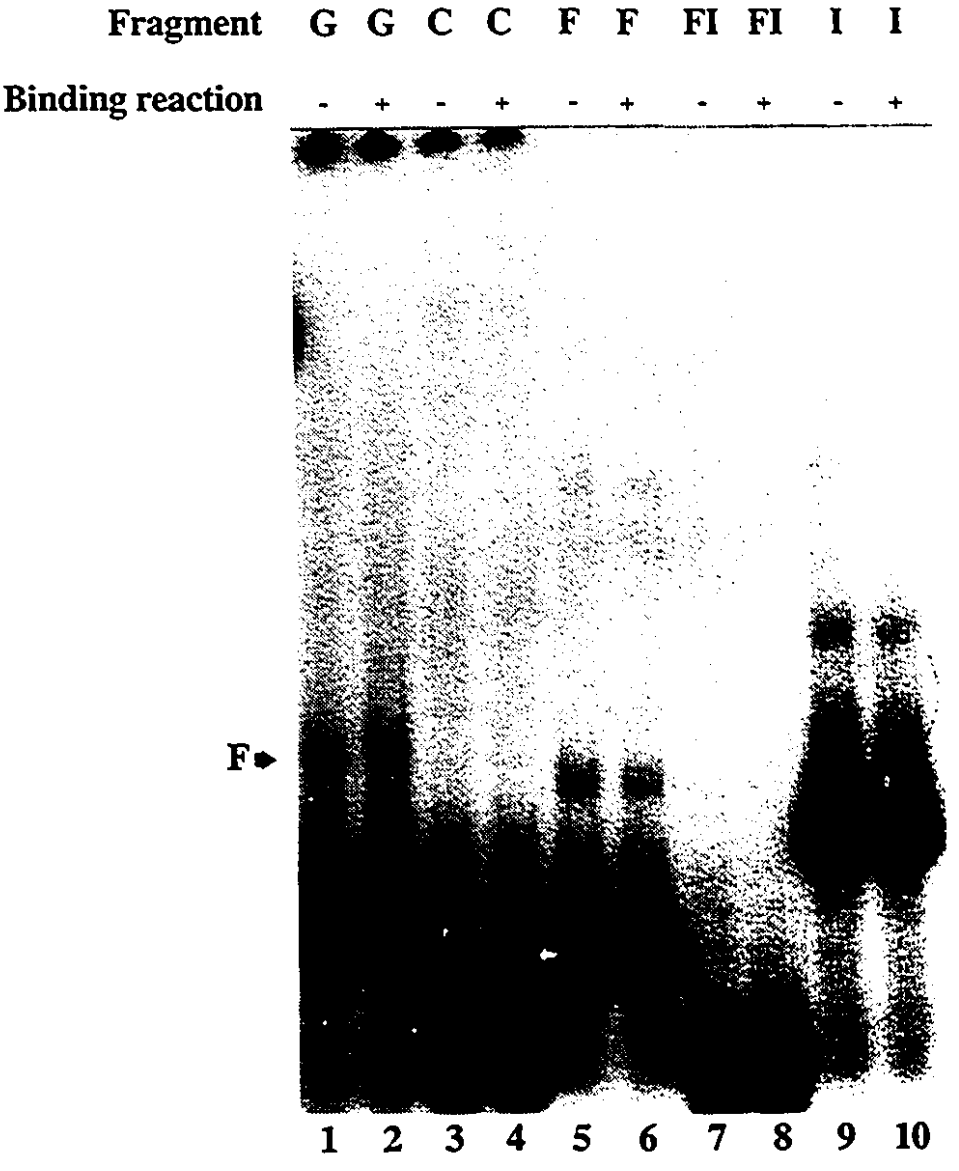


Figure 18. Gel electrophoresis DNA binding assay with fragments from the *HEM13* promoter. Fragments G (-209 to -92), C (-209 to -148), I (-256 to -92), F (-256 to -148) and FI_L (-229 to -148) from the *HEM13* promoter were incubated for 20 minutes at room temperature with whole cell extracts prepared from TKY22 (wild-type) cells grown in the absence of heme. No heme was added to the binding reactions.

Fragment G C I F FIL

1 2 3 4 5

Figure 19. Gel electrophoresis DNA binding assay. Fragments G (-209 to -92), C (-209 to -148), F (-256 to -148), FI (-229 to -172) and I (-256 to -92) from the *HEM13* promoter were incubated for 20 mintues at room temperature with whole cell extracts prepared from TKY22 (wild-type) cells grown in the absence of heme. Lanes 2, 4, 6, 8, 10 contained 40 μM hemin added directly to the binding reactions. No hemin was added to reactions in lanes 1, 3, 5, 7, and 9.

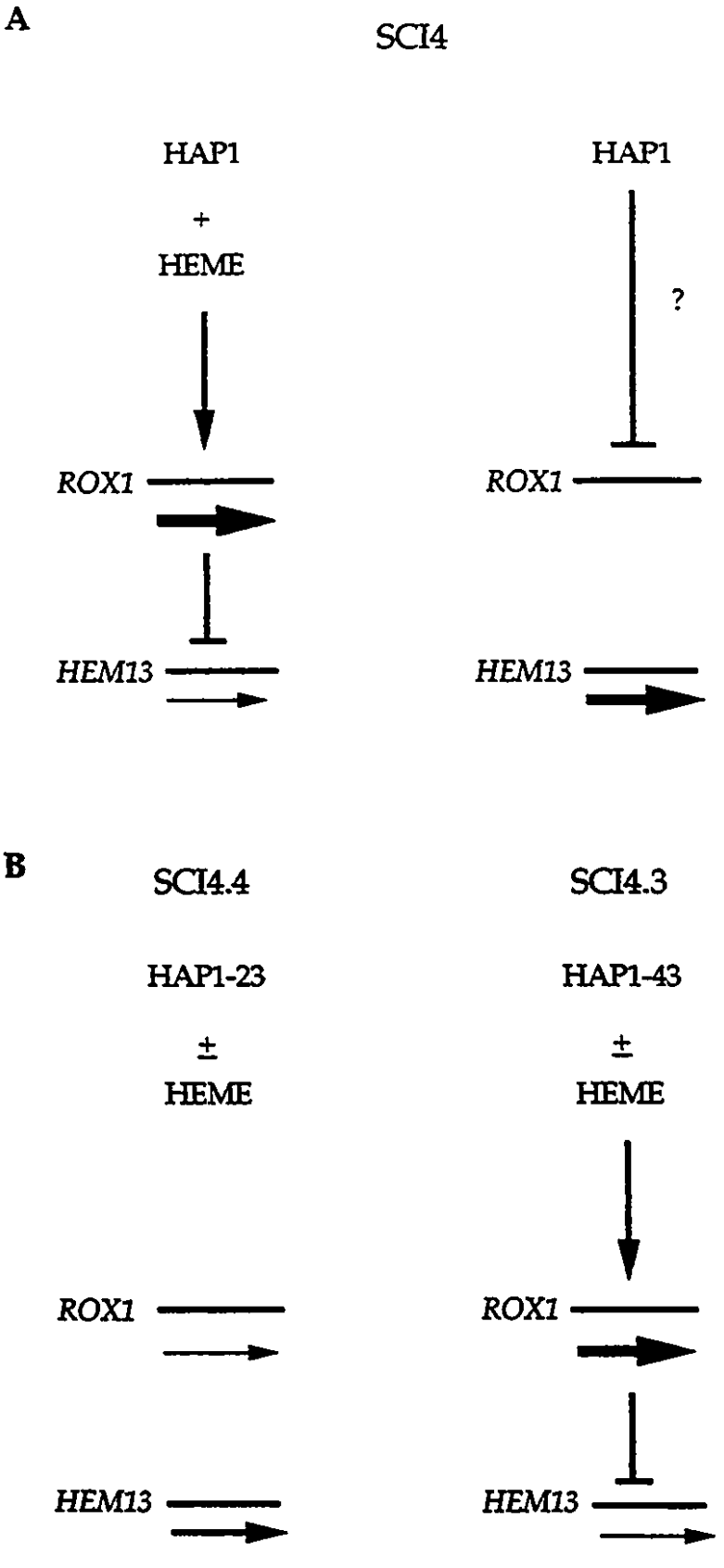

Fragment G G C C F F FI FI I I
Binding reaction - + - + - + - + - +
F
1 2 3 4 5 6 7 8 9 10

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; Thorsness *et al.*, 1989). Regulation of *HEM13* expression involves the HAP1 and ROX1 proteins (Figure 20A). 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 expression approximately forty-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* mRNA 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 was seen previously in a *hap1::LEU2* mutant strain (Keng, 1992) and suggests a mechanism by which HAP1 can play a role in regulating expression of *HEM13* under

Figure 20. Regulation of *HEM13* expression in strains SCI4, SCI4.4, and SCI4.3. In panel A, 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 panel B, 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. Under these conditions, *HEM13* transcription is repressed.


A
SCI4
HAP1
+
HEME
ROX1
HEM13
HAP1
?
ROX1
HEM13
B
SCI4.4
HAP1-23
±
HEME
ROX1
HEM13
SCI4.3
HAP1-43
±
HEME
ROX1
HEM13

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. 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 20B).

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 20B). 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 transcription.

The *HAP1* gene product is a protein of 1483 amino acids which has heme-dependent DNA binding activity (Pfeifer *et al.*, 1987b; Pfeifer *et al.*, 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 six repeats of the KCPVDH motif and is postulated to be responsible for the heme response. Deletion of this region in the *HAP1ΔK* allele results in a HAP1 protein that is able to exhibit a high degree of DNA binding activity *in vitro* and activate expression *in vivo* even in the absence of heme (Pfeifer *et al.*, 1989; Fytlovich *et al.*, 1993). Between these two domains is a polyglutamine-rich region between amino acids 177 and 189. The carboxy terminal 176 amino acids are required for transcription activation while the internal region from residues 445 to 1308 is involved in regulating the activity of the HAP1 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 7).

Another independent *hap1* mutant allele, *hap1-132* has been found. This mutation is a cysteine to tyrosine substitution at amino acid 1048. A strain with the *hap1-132* mutant allele has high levels of heme-independent expression of *CYC1* and therefore displays a phenotype similar to that of the *HAP1ΔK* allele that is deleted for the heme-responsive domain between amino acids 247 and 444 (Haldi and Guarente, 1995).

An interesting comparison can be made between the phenotypes conferred by the *hap1-43* allele, the *hap1-132* allele and the *HAP1ΔK* allele. Although all the alleles allow for heme-independent expression of *CYC1* and other HAP1-regulated genes, the mutations associated with these alleles fall within different regions of the HAP1 coding region. In addition, unlike *hap1-132* and *HAP1ΔK* which allow a large 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, Haldi and Guarente, 1995), the *hap1-43* allele is not associated with significantly elevated levels of *CYC1-lacZ* expression under such conditions.

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 directed against the amino terminus of HAP1 bypasses the heme requirement for binding. Heme binds to the KCPVDH motifs that are found within the heme responsive domain between residues 247 and 444 (Zhang and Guarente, 1995). 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 of low mobility, and that was only detected when the binding reactions were conducted at 5°C. Addition of heme to the reaction changed this pattern to a well-defined band shift of higher mobility. Overproduction of a modified allele of HAP1 which contained a deletion of the heme-responsive domain from amino acid 247 to 444 resulted in the formation of the well-defined band shift of 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 was unable to activate transcription because it was found in a complex with as yet unidentified X factor(s) which gave the diffuse pattern in gel electrophoresis DNA binding assays. Addition of heme to the binding reaction allowed factor X to dissociate

from the complex and the bound HAP1 was able 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-*CYC1* UAS1 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. Surprisingly, we were able to detect a significant level of wild type HAP1-*CYC1* UAS1 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. This is supported by earlier work on saturation mutagenesis of the HAP1 DNA binding domain. In this work, mutants were found that were able to bind DNA but were unable to transactivate (Turcotte and Guarente, 1992). This indicates that the transactivation ability of HAP1 is not directly proportional to the level of HAP1 binding to DNA.

The *hap1-43* allele gave rise to a protein that bound to *CYC1* UAS1. The mobility of the complex was identical to that formed with extracts prepared from the *HAP1* strain. However, binding of the HAP1-43 protein to DNA was not stimulated by addition of heme to the binding reaction. Since the HAP1-43 protein was 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 also 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 with 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 7). 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. Interestingly, a similar observation was also made for the *hap1-132* allele (Haldi and Guarente, 1995).

In another approach to gain further understanding of regulation of *HEM13* expression, a detailed promoter analysis was carried out. Deletion studies identified three different types of elements involved in *HEM13* expression (Figure 4). These elements included the upstream activation sequences involved in the constitutive expression of *HEM13*, and upstream induction sequences involved in derepression of *HEM13* in the absence of heme, and a series of three elements homologous to the ROX1 hypoxic operator sequence, which is responsible for repression of *HEM13* expression in the presence of heme (Lowry *et al.*, 1990; Keng, Ushinsky, DiFlumeri and Richard, unpublished observations).

Protein binding to DNA fragments containing sequences from the *HEM13* promoter was also examined. The entire promoter region was first subdivided

into overlapping fragments and binding to each fragment was determined. The DNA fragment which displayed the strongest DNA-protein interaction was fragment 5a, containing sequences from position -326 to -121. Protein binding to fragment 5a was assessed using whole cell extracts prepared from wild-type (TKY22), *hap1::LEU2* mutant (TKY24), *rox1::LEU2* mutant (CDY1), and *hap1-43* mutant (SCI4.3) strains (Figure 11). Five protein-DNA complexes (A-E) were detected in binding reactions with each of the mutant extracts, indicating that in this experiment, neither ROX1 nor HAP1 interactions with *HEM13* regulatory sequences could be found. It is not surprising that no ROX1-dependent complexes were observed under the experimental conditions used as it was reported that the levels of ROX1 in yeast extracts is too low for DNA binding activity to be detected (Balasubramanian *et al.*, 1993; DiFlumeri, Liston, Acheson and Keng, unpublished observations). Although a sequence with homology to the HAP1-binding site consensus can be found within the upstream noncoding region of *HEM13*, this sequence does not play a role in the regulation of *HEM13* expression as a deletion that removed this region had no effect on *HEM13* expression (Keng, Ushinsky, DiFlumeri and Richard, unpublished observations). Therefore, the absence of a HAP1-dependent complex is not unexpected and further suggests that the role of HAP1 in regulation of *HEM13* expression is indirect (Figure 20A). It is noteworthy that the use of extracts prepared from strains grown in the presence of heme, under conditions when expression of *HEM13* is repressed, or the addition of heme to the binding reactions, or both, resulted in decreased formation of all complexes, especially complexes A and B (Figure 13). This observation suggests that these proteins represent factors that are involved in activation of *HEM13* expression.

Fragment 5a was further subdivided (Figure 12) into smaller fragments and binding to each subfragment was tested. The shorter fragments allowed

formation of complexes that migrated with a similar mobility as complexes formed with the full-length fragment 5a. In addition, the formation of the complexes was also inhibited by the presence of heme (Figure 14). Complexes A and B were formed by protein factor(s) binding to sequences from position -326 to -293. The results of deletion analysis suggest that these sequences are within the extensive region required for full level expression of *HEM13* in the absence of heme. Interestingly, the formation of these two complexes was significantly decreased in the presence of heme whether it was present in the growth medium or added to the binding reactions. This observation suggests that these factors are always present in the cell and that their DNA binding activities are functionally regulated by intracellular heme levels. Such a mechanism of regulation would allow for a faster response to changing environmental conditions.

Formation of complex E was deduced to require sequences from position -295 to -280. The formation of this complex was not significantly altered by the addition of heme to the binding reaction (Figure 16, lanes 1 and 2). Deletion analysis indicated that this region contained the inducing sequences required for the increase in *HEM13* expression in the absence of heme (Figure 4). Also binding to this region encompassing the inducing sequences is the complex that can only be detected in extracts prepared from cells grown in the absence of heme. Its formation was found to require sequences from position -284 to -240. (See Figure 16, lanes 3 and 4 and Figure 17, lanes 3 and 4.)

Other protein -DNA complexes were found in other regions of the *HEM13* upstream noncoding sequences. Careful examination of the *HEM13* upstream sequences did not reveal any homology to the binding sites of either the general transcription factor ABF1 or RAP1. Complex C formation required sequences from position -230 to -214 while that of complex D required sequences from

position -284 to -240. The formation of these complexes was not as strongly affected by addition of heme to the binding reaction (Figure 11, compare lanes 1 and 2). The sequences required for formation of complex F were localized to the region spanning position -175 to -160.

These binding studies reveal the formation of multiple protein-DNA complexes with DNA fragments containing sequences from the upstream noncoding region of *HEM13*. While the significance of this complex set of protein-DNA interactions is unclear at the moment, parallel analysis of the regulatory sequences of *HEM13* by deletion mutations have also indicated that the regulation of *HEM13* expression by heme is the result of interactions between multiple DNA elements. In the presence of heme, ROX1 is proposed to bind to the ROX1 consensus operator sites within the regulatory region of *HEM13* and repress its transcription. In the absence of heme, ROX1 levels decrease and *HEM13* is expressed. Interestingly, formation of the different complexes also increases in the absence of heme in the extracts and binding reactions, making it tempting to speculate that the proteins in these complexes may represent transcriptional activators. The identity and function of such proteins await further characterization.

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