Characterization of an Upstream Activation Sequence and Two Rox1p-responsive Sites Controlling the Induction of the Yeast HEM13 Gene by Oxygen and Heme Deficiency*

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The Saccharomyces cerevisiae HEM13 gene codes for coproporphyrinogen oxidase, an oxygen-requiring enzyme catalyzing the sixth step in heme biosynthesis. Its transcription has been shown to be induced 40–50-fold in response to oxygen or heme deficiency, in part through relief of repression exerted by Rox1p and in part by activation mediated by an upstream activation sequence (UAS). This report describes an analysis of HEM13 UAS and of the Rox1p-responsive sites by electrophoretic mobility shift assays, DNase I footprinting, and mutational mapping. HEM13 UAS is composed of two subelements: a 16-base pair sequence binding a constitutive factor acting as a transcriptional activator, and a 5′-flanking 20-base pair GC-rich region. Both subelements were required additively for transcription, but each element alone was sufficient for almost normal control by oxygen/heme deficiency. Mutations in both elements decreased the induction ratio 3–4-fold. HEM13 UAS conferred a 2–4-fold oxygen/heme control on a heterologous reporter gene. Two Rox1p-responsive sites, R1 and R3, were identified, which accounted for the 6–7-fold repression by Rox1p. A factor bound to a sequence close to site R3. This DNA-binding activity was only detected in protein extracts of aerobic heme-sufficient ROX1TUP1 cells, suggesting a possible role in site R3 function.

Genetic adaptive responses to hypoxic stress occur in many biological systems. Several systems for controlling these responses have been described in prokaryotes and eukaryotes. They involve the sensing of oxygen tension and the signal-transduction pathway leading to an alteration of gene expression. In bacteria, the sensor-regulator systems FixL, Fnr, and ArcB/ArcA are converted into active forms by oxygen deprivation (1–5). The rhizobial oxygen sensor FixL is a hemoprotein kinase whose activity is blocked by oxygen binding to the heme (1). In mammalian cells, a DNA-binding factor HIF-1 induced by hypoxia plays a central role in hypoxic gene activation, and there is some evidence that oxygen tension is sensed by a hemoprotein (Ref. 6 and references therein).

In the yeast Saccharomyces cerevisiae, oxygen control of gene expression is mediated essentially by two factors, which are active or synthesized only under aerobic conditions, the activator Cyp1p (Hap1) and the repressor Rox1p (reviewed in Refs. 7, 8). Heme is believed to serve as an effector molecule; its biosynthesis has an absolute requirement for oxygen (9), and heme-deprivation appears to mimic the effects of oxygen deficiency in most of the cases tested (7, 8); but the mechanism by which heme is implicated is not yet clear. Rox1p is a protein with a high mobility group motif that bends DNA when binding to a specific hypoxic consensus sequence and requires the general repressor complex Ssn6p/Tup1p to repress transcription of the target genes (10, 11). The expression of the ROX1 gene is oxygen/heme-dependent via a complex regulatory network that includes the transcriptional activator Cyp1p, whose activity is heme-dependent (12–15). The Rox1p concentration is therefore greatly reduced in the absence of oxygen or heme, and hypoxic gene expression is derepressed. The regulation by oxygen of the hypoxic genes studied so far in some detail, such as ANB1 (TIF51B) (isoform of translation initiation factor eIF-5A) (16, 17), COX5b (isoform of cytochrome oxidase subunit Vb) (18, 19), AAC3 (isoform of ADP/ATP translocator) (20), and ERI1 (1a-nosterol 14α-demethylase) (21), is entirely accounted for by the repression by Rox1p in aerobicosis.

The HEM13 gene encodes the enzyme coproporphyrinogen oxidase that catalyzes the sixth step in the heme biosynthetic pathway. The enzyme uses only oxygen as an electron acceptor for the oxidative decarboxylation of coproporphyrinogen (9). HEM13 is the only gene in the pathway that is closely regulated (8, 9); its transcription is induced 40–50-fold under conditions of oxygen or heme deficiency (22, 23). Previous work has shown that coproporphyrinogen oxidase activity becomes rate-limiting for heme production when its substrate oxygen is limiting; the cells respond to oxygen limitation by increasing the amount of the enzyme (23). Hence, coproporphyrinogen oxidase may play a crucial role in linking heme synthesis to the oxygen/heme-dependent control of gene expression, and it is important to understand how the HEM13 gene is regulated. Cyp1p has been found to interfere with this regulation (24), mainly by its role on the synthesis of Rox1p that represses HEM13 under aerobic heme-sufficient conditions (12, 13). But the repression of HEM13 by Rox1p is only partial, and a preliminary deletion-mutant analysis of the HEM13 promoter has revealed the presence of a region acting as an upstream activation sequence (UAS)† that is required for full induction under oxygen/heme deprivation (15). Such a UAS that responds positively to the absence of oxygen/heme is a novel regulatory element for yeast genes regulated by oxygen/heme. The present study describes the cis- and trans-elements of this UAS by directed mutagenesis and electrophoretic mobility shift assays. We have also identified the Rox1p-responsive sites and an

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† The abbreviations used are: UAS, upstream activation sequence; EMSA, electrophoretic mobility shift assays; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

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Fig. 1. Structure of the HEM13 promoter. A, diagram of the HEM13 upstream sequence in plasmid pH13Z showing the location of the restriction sites evoked in this work. A, ApaI; B, BamHI; D, DraI; E, EcoRV; Ec, Eco47III; H, HindIII; N, NruI; S, SacI; Sc, SacII; Sf, SfiI; X, XmnI. The regulatory elements analyzed here, R1, R2, R3, and UAS, and the putative TATA element are indicated. The arrows mark the initiation sites of transcription. B, nucleotide sequence of the HEM13 promoter showing the probes used in EMSA: the SfiI-SacI and SacI-Eco47III fragments and the four PCR-amplified (1–4) fragments.

MATERIALS AND METHODS

Yeast Strains and Methods—The Saccharomyces cerevisiae strains used in this study were BY101-28 (Mata ur3-32 his3Δ1 leu2-3,112 trp1-289) and its derivatives apr1 (aro1: HIS3), Δup1 (cypl: LEU2), Δup1 (tap1: LEU2), ΔH1 (hem1Δ), and ΔcyplΔH1 (cypl: LEU2 hem1Δ) constructed in this laboratory (15); strain FY62420 (Mata ur3-32 his3Δ200 leu2-3,112 trp1-Δ901 [ys2–801 suc2–39 MEL1]) and the derived (up1-Δ1: HIS3) strain SM10 from W. S. Moye-Rowley (25); strain Y328 (Mata ur3-32 leu2-3,112 gcn4–2) and the derived (bas1–2) strain Y329 from B. Daigean-Fornier (26). Yeast cells were grown routinely in rich glucose medium (YPD) (28). Tween 80 (0.2%) and ergosterol (30 mg/liter) (Te) were added for culturing heme-deficient cells and for anaerobic growth (15); pure nitrogen was bubbled for 30 min after inoculating the medium. Cells were collected in the logarithmic growth phase and used immediately.

DNA Manipulations and Site-directed Mutagenesis—Enzymes were obtained from New England BioLabs, Life Technologies, Inc., Boehringer Mannheim, and Appligene. Routine DNA manipulations, including the polymerase chain reaction (PCR), followed standard procedures (29). The Escherichia coli strain DH15a was used for cloning and propagating plasmids. Mutagenesis was carried out using the Altered Sites in vitro Mutagenesis System (Promega). The single-stranded DNA template was prepared from phagemid pALTER-1 (Promega) containing the 1.18-kb EcoRI-BamHI HEM13 fragment from pH13Z. The oligonucleotides used for mutagenesis, PCR, and sequencing were synthesized.

on an Applied Biosystem DNA Synthesizer at the Service de Synthèse d'Oligonucleotides, Institut Jacques Monod, and purified by polyacrylamide gel electrophoresis. All mutations were verified by sequencing on double-stranded plasmids using the Sequenase kit (U. S. Biochemical Corp.), HEM13-specific oligonucleotide primers, and [32P]dATP.

Plasmids and β-Galactosidase Assay—Plasmid pH13Z (Fig. 1), containing the HEM13-lacZ fusion, was obtained by ligating 1076 nucleotides of the 5' untranslated region and the first 34 codons of HEM13 to the lacZ gene of the episomal plasmid YEp357 (30). The mutations generated in the HEM13 promoter were introduced into plasmid pH13Z by exchanging the 1.18-kb EcoRI-BamHI fragment, to yield plasmids pH13Z/m10 to pH13Z/m18 cut with EcoRI and SacI. Similarly, pH13Z/m11–18, pH13Z/m14–19, and pH13Z/m14–18 were obtained by replacing the 0.74-kb EcoRI-SacI fragment of pH13Z/m10 into pH13Z/m8 cut with EcoRI and SacI. Plasmid pH13Z/m11–18, pH13Z/m14–19, and pH13Z/m14–18 were obtained by replacing the 0.74-kb EcoRI-ApaI fragment from pH13Z/m11 and pH13Z/m14 into pH13Z/m18 and pH13Z/m19.

Plasmids pS8X-H13Z and pS8X-H13Z/m14–18 (Fig. 5) were constructed as follows. A 75-bp DNA fragment (fragment 4 in Fig. 1) corresponding to the region −384 to −310 was PCR-amplified using the two oligonucleotides 5'-CCGGGATCCCGAATCTGGAGAAATGAACTTTCT and 5'-CCGGGATTCCGCTTCTCGCTCGGCA, which carried the 1.5-kb Smal-XhoI fragment that had been deleted, pS8X, served as control. The amplified fragments cut with BamHI and EcoRI were first cloned into pVO10 (31). They were recovered by cutting with BamHI (blunt-ended) and XhoI and then ligated into the left-most Smal and right-most XhoI sites of the CYC1-lacZ fusion plasmid pLG669Z (32), in place of the CYC1 and ANB1 gene regulatory sequences. Plasmid pLG669Z, from which the 1.5-kb Smal-XhoI fragment had been deleted, pSX, served as control. Plasmids were transformed into the different yeast strains, and β-galactosidase activity was measured on SDS/chloroform-permeabilized cells (28) from cultures (Aₒ₆₆₀ ≈ 0.5; approximately 5 x 10⁶ cells/ml)
Regulatory Elements of Yeast HEM13

Fig. 2. Protein binding to the SacI-Eco47III fragment of the HEM13 promoter. A, the labeled 190-bp SacI-Eco47III fragment was incubated for 15 min at 4 °C with 1.0 μg of poly(dI-dC) plus crude protein extracts (30 μg) prepared from wild-type strain S150-2B (WT) and from its congenic cyp1Δ and rox1Δ mutant derivatives. O2, the cells were grown aerobically; N2, the cells were grown anaerobically; H2, the congenic heme-deficient hem1Δ mutant strains were used. Electrophoresis was carried out at 4 °C. First lane, probe incubated without protein extract. B, EMSA competition experiments with protein extracts from aerobic wild-type cells were carried out as above but with a molar excess (50- to 150-fold) of unlabeled PCR-amplified 75-bp fragment 4 (see Fig. 1), wild-type (WT), or carrying mutation m18 or m19. The sample in the first lane was incubated without added protein.

Protein extracts were prepared from yeast cells (33), and the final protein pellet was suspended in 20 mM Tris-HCl, pH 8, containing 0.1 mM EDTA, 1 mM dithiothreitol, 20%glycerol, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard.

Results

Binding of a Factor to the HEM13 UAS Element—Our deletion mutant analysis of the HEM13 promoter showed that the 190-bp SacI-Eco47III DNA fragment contains a UAS that is required for basal level expression of the HEM13 gene and its full induction by oxygen and heme deficiency (15). To locate the cis-acting element(s) within this DNA fragment and identify any putative factor(s) that may bind to it, we performed electrophoretic mobility shift assays (EMSA). The SacI-Eco47III DNA fragment yielded a single retarded band, complex A, with protein extracts prepared from wild-type cells grown under aerobic, anaerobic, and heme-deficient conditions. This band was also seen with protein extracts prepared from...
cyp1Δ, cyp1Δ hem1Δ, and rox1Δ mutant cells (Fig. 2A). The formation of complex A was completely competed out by a 100-fold excess of the unlabeled DNA fragment, and preliminary experiments with overlapping segments of the SacI-Eco47III fragment used as competitors suggested that the protein-binding DNA sequence was located around position −333 (DraI restriction site) (results not shown). This was confirmed by determining the protected DNA region in complex A by indirect DNase I footprinting experiments (Fig. 3). No extended footprint was detected, but highly reproducible single base protections were observed at positions −328 and −342, together with sites more sensitive to cleavage at positions −340, −337, and −333. Site-directed mutations were made which changed 5–6 nucleotides around each of the protected sites; mutation m19 changed the sequence TCTACT to GAGATC, and mutation m18 changed the sequence AC-GAG to GATCT (see Fig. 4). The ability of the factor(s) to bind to the resulting altered sites was assessed by competition experiments. EMSAs were carried out with the labeled SacI-Eco47III fragment in the presence of the 75-bp PCR-amplified DNA fragments (fragment 4 in Fig. 1) carrying mutation m18 or m19 as competitors. A 150-fold molar excess of the mutated DNA fragment m18 did not prevent the formation of complex A, whereas the mutated DNA fragment m19 significantly impaired its formation, although not as completely as did the nonmutated wild-type fragment (Fig. 2B). These results indicate that the two sites contribute to complex formation but to different degrees. The fact that no other retarded band appeared in these competition experiments suggests, but does not prove, that a single factor binds to this approximately 17-bp region (−342 to −326). The nucleotide sequence of the 3′-end of this region (CGACTC on the noncoding strand), whose mutation m18 abolished complex formation, showed some similarity to the recognition sequences of the Gcn4p, Bas1p, Yap1p yeast transcriptional activators (TGACTC, see Ref. 34 for references). But none of these factors is implicated in complex A formation, since a normal complex was formed in EMSA using protein extracts prepared from gcn4Δ bas1Δ and yap1Δ mutant cells (data not shown).

**Mutational Analysis of the HEM13 UAS**—The effects of the two mutations m18 and m19 on HEM13 gene expression were then assessed in vivo. The functional significance of a 5′-flanking GC-rich sequence (−363 to −344) was also explored by generating extensive mutations, m11 to m19, in this sequence,
and analyzing their effects, individually or in combination with mutations m18 and m19, on HEM13 expression and regulation. The mutations were introduced into plasmid pH13Z (Fig. 1) carrying a gene fusion between HEM13 and E. coli lacZ, and the resulting plasmids were transformed into the wild-type strain S150-2B and its hem1Δ derivative deficient in heme synthesis. HEM13 expression was analyzed by measuring β-galactosidase activity in the S150-2B transformants grown under aerobic (O2) and anaerobic (N2) conditions. These results demonstrate that HEM13 UAS acts as a UAS element in a heterologous context and confers heme and oxygen control on the reporter gene to the same extent as it does in its native context.

Identification of the Rox1p-responsive Sites—HEM13 expression is repressed by the repressor Rox1p under aerobic heme-sufficient conditions (11–13, 15). The HEM13 upstream sequence contains three sequences that are identical or similar to the hypoxic consensus sequence for Rox1p recognition, YYYY-TGTTCT (10, 17). R1 (−185 CCCATTGTTCTC −174) centered about 20 nucleotides upstream from the TATA element, R2 (on the noncoding strand, −238 TGGTTTACAA −249), and R3 (−475 TCAATTAGTT −464) (Fig. 1). They were destroyed individually and in combination by introducing multiple base pair substitutions within the consensus core sequence ATTGTT to explore the relative role of these presumptive Rox1p-mediated control elements (Fig. 6). Derivatives of plasmid pH13Z carrying these various mutations were transformed into the wild-type strain S150-2B and an isogenic deletion mutant strain, and the transformants were assayed for β-galactosidase activity (Fig. 6). Mutating R2 did not affect the expression, whereas gene expression was increased 1.3–1.5-fold after mutating R1 and 3.6–3.8-fold after destroying R3. Mutating both R1 and R3 had an additive effect and led to an expression activity similar to that measured with the wild-type plasmid in a rox1Δ background. Since incapacitating these two cis elements R1 and R3 had the same effect as genetic elimination of the trans-acting factor, we conclude that they are the only functional Rox1p-responsive elements in the HEM13 promoter. The 6–7-fold repression by Rox1p observed with these multicopy gene fusion constructs correlates well with the 5–6-fold ROX1-dependent repression measured for the chromosomal HEM13 gene (15).

Binding of a Factor Upstream of the R3 Site—In addition to
the Rox1p-responsive site R3, sequences related to the binding site for Cyp1p were found in the HEM13 promoter region upstream of the SacI restriction site (15). It was also reported that Cyp1p could act as a weak repressor (2-fold) of HEM13 in aerobiosis and a weak activator (2-fold) in the absence of heme(12,13,24), independently of its role on ROX1 expression (15). The possible binding of Cyp1p, and/or other potential trans-acting factor(s) to the region upstream of SacI, was evaluated using EMSAs with various labeled restriction fragments extending from the NruI to XmnI sites; shorter, overlapping, PCR-amplified DNA fragments were also used (probes 1–3 in Fig. 1). The 93-bp Sfc1-SacI DNA fragment (Fig. 7) and probes 1–3 (data not shown) yielded a DNA-protein complex B that was specific as judged by competition with a 50-fold molar excess of unlabeled DNA. Complex B was formed only with protein extracts prepared from aerobic cells; it was absent when proteins were prepared from anaerobic or heme-deficient cells or from rox1Δ and tup1Δ mutants. Competition experiments (see Fig. 7B, third lane for the 29-bp probe 3) localized the sequence involved in the formation of complex B to the DNA segment −500 to −472, immediately upstream of the R3 site. Further attempts to delineate the binding site within this 29-bp segment by footprint analysis and mutagenesis were unsuccessful.

### DISCUSSION

We have defined the cis and trans components of the two major regulatory pathways that control the induction of HEM13 expression in response to oxygen/heme deficiency: a 6–7-fold aerobic repression exerted by Rox1p and a 3–4-fold activation mediated by a novel UAS element. The two mechanisms provide a 20–30-fold induction to HEM13, which is slightly less than that observed experimentally (40–50-fold). The additional 2-fold activation to reach full induction level might be mediated by Cyp1p under heme deprivation and by unknown factor(s) under anaerobiosis (15). But Cyp1p probably acts indirectly since it apparently does not bind HEM13 promoter probes, even when using protein extracts enriched in Cyp1p and under conditions optimized for detecting DNA-Cyp1p complexes (33) (data not shown).

HEM13 UAS—The HEM13 UAS is about 40-bp long and is composed of two subelements, a 16–17-bp sequence binding a protein factor A and a 5′-flanking 20-bp GC-rich region. Both subelements are required additively for basal level of transcription, but each element alone appears to be sufficient for almost normal control of transcription by oxygen/heme. The protein-DNA complex A likely represents functionally important interactions, since different mutations affect both in vitro binding and in vivo promoter activity in a similar manner; this implicates protein factor A as a transcriptional activator. The DNA sequence of the factor A-binding site contains two blocks of dyad symmetry, CGAGTCG and TC-N11(AT-rich)-GA, which could be recognition motifs for factor A (see bottom of Fig. 3). Our results tend to favor the second one, but more work is needed to determine this with certainty. The role of the GC-rich region is not clear. It could bind a factor that was not detected in vitro in our EMSA conditions (perhaps via the rotationally
symmetric CGG triplets), and this factor might participate with factor A in the additive transcriptional effect observed. Or its intrinsic DNA structure could impose constraints on the neighboring factor A-binding site. Unusual structures have been described for d(CCGCGG) (35) and d(GGGCCC) (36) sequences, which are both present within the GC-rich region. Sequence comparisons revealed no obvious similarity of HEM13 UAS to the binding sites for known transcription factors (34) and to sequences in the upstream regions of other oxygen-regulated genes (7), including the hypoxic COX5b promoter (19, 39), COX5b (39), AAC3 genes (Fig. 8). Various levels of repression stringency have been reported for the Cox1p-responsive sites found in the ANB1, COX5b, and AAC3 genes (Fig. 8). It has been suggested that the poly(dT-dA) tracts are very long in the ANB1 and AAC3 genes, shorter in COX5b, and very short and imperfect in HEM13 (Fig. 8). In the case of COX5b, a specific factor, Ixr1p(Ord1p), is also implicated in the aerobic repression (42).
Ixr1p(Ord1p), a two high mobility group-box protein initially isolated as a protein that binds to and bends platinated DNA (43), recognizes a 44-bp COX5b DNA segment encompassing the Rox1p element, probably in a structure-specific rather than a sequence-specific manner (42). Poly(dT-dA) sequences, by virtue of their intrinsic structure, can alter chromatin structure so that transcription factors gain better access to their cognate binding sites located in the vicinity (44). In the absence of long poly(dT-dA) sequences, other factors could be required to provide similar functions. The function of factor B (and Ixr1p ?) may be to help Rox1p bind to its recognition sequence and anchor the general repressor complex Ssn6p Tup1p with the proper protein-protein interactions to ensure its repressor activity.

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