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Isolation, Sequence, and Regulation by Oxygen of the Yeast *HEM13* Gene Coding for Coproporphyrinogen Oxidase*

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The *HEM13* gene of *Saccharomyces cerevisiae* codes for coproporphyrinogen oxidase (EC 1.3.3.3) catalyzing the sixth enzymic step in the heme biosynthetic pathway. Its expression has been previously shown to be regulated negatively by heme and oxygen. We have isolated the *HEM13* gene by functional complementation of a *hem13* mutant and determined its nucleotide sequence. The open reading frame encodes a protein of 328 amino acids. Its calculated molecular weight (37,673), amino acid composition and amino-terminal sequence predicted from the DNA sequence are in agreement with those determined for the native enzyme (Camadro, J. M., Chambon, H., Jolles, J., and Labbe, P. (1986) *Eur. J. Biochem.* 156, 579–587). The 5′ ends of the *HEM13* transcripts were identified by nuclease S1 mapping; induction of *HEM13* resulted in an equivalent increase of the level of all the transcripts. 5′ deletion analysis revealed that DNA sequence located upstream of 409 nucleotides from the translational initiation codon was needed for depression under oxygen limitation. The loss of induction of coproporphyrinogen oxidase activity by anaerobiosis caused a considerable decrease of heme formation in anaerobic cells.

Coproporphyrinogen III oxidase (EC 1.3.3.3) is the sixth enzymic step in the heme biosynthetic pathway, catalyzing the sequential oxidative decarboxylations of the 2- and 4-carboxyethyl side chains in coproporphyrinogen III to yield the two vinyl groups in protoporphyrinogen. The two ordered decarboxylations most probably occur at the same catalytic site in the mammalian enzyme (1–3), but the exact mechanism for formation of the vinyl group is still uncertain (4 and references therein). The enzyme, located in the mitochondrial intermembrane space in rat liver (5, 6), has been purified from beef liver and shown to be a monomer (M_r = 71,600), devoid of any detectable cofactor and with a tyrosine residue possibly implicated in the oxidation process at the active site (7, 8). Surprisingly, in the facultative anaerobic yeast *Saccharomyces cerevisiae*, coproporphyrinogen oxidase is located in the cytosol and the purified enzyme is an homodimer, with a subunit M_r = 35,000. It contains two iron atoms per molecule of native protein and requires sulfhydryl group(s) for its activity; however, the involvement of iron in the oxidative catalytic activity of the enzyme has not been demonstrated (9). Both the mammalian and the yeast enzymes require oxygen for their activity. No anaerobic activity could be detected, even in the presence of the alternate electron acceptor described by Tait for the enzyme of *Rhodopseudomonas spheroides* (10). This poses the question of the formation of heme found in anaerobically grown yeast cells (11 and reference therein).

The synthesis of coproporphyrinogen oxidase in *S. cerevisiae* is subject to a negative control by oxygen and heme[1] and this regulation operates at the pretranslational level: the enzyme activity, together with the steady-state amounts of immunodetectable protein and of specific translatable mRNA, are increased 5–40-fold (depending upon the strains) in heme-deficient mutants and in wild type cells grown anaerobically (11). This places this enzyme within the family of proteins whose synthesis is transcriptionally controlled by heme and oxygen in yeast. This control is positive in the cases of iso-1-cytochrome *c* (12–17), catalases T and A (12, 18, 19), and Mn superoxide dismutase (14, 20, 21); it is negative in the case of a gene *ANB1*, adjacent to *CYC1* encoding iso-1-cytochrome *c*, whose transcript function is unknown (13, 14, 22). Both *cis*-acting upstream regulatory regions (13–16, 19) and *trans*-acting elements (14, 16) have been reported to mediate these regulations.

In this study, we report the isolation and nucleotide sequence of the yeast coproporphyrinogen oxidase gene. Deletion analysis of the 5′-flanking sequences revealed the presence of a regulatory region required for derepression of gene expression under anaerobiosis and, consequently, for maximal heme formation in anaerobic cells.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions—The *S. cerevisiae* heme-deficient mutant strains G204 (*hem1-5*), G216 (*hem13-1*) (23), and G225 (*hem13-4*)[2] were isolated from the wild type strain FL200 (*α his4*); the mutants H11 (*hem13-2*) and H12 (*hem13-3*) (24) were derived from the wild type strain SP4 (*α leu1 arg4*); the mutant 25B (*hem13-7*)[3] was isolated from the wild type strain BWG1-7A (*a leu2-*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03873.

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[1] The abbreviations used are: heme, ferrous-iron protoporphyrin IX; hemin, ferric-iron chloride protoporphyrin IX; bp, base pair(s); kb, kilobase pair(s).

[2] D. Urban-Grimal, unpublished results.

[3] L. Guarente, unpublished results.

3 leu2-112 his4-519 ade1-100 ura3-52). Standard yeast genetic methods (25) of mating, sporulation, and tetrad analysis were used for complementation test, linkage studies, and constructions of strains carrying appropriate mutations. The strain MZ4-15B (*α his3-15 leu2-3 leu2-112 trp1-1 ura3-251 ura3-373 hem13-1*) was used as the host strain for HEM13 plasmids.

Yeast cells were grown for 16–24 h at 30 °C with vigorous shaking. For anaerobic growth, special air-tight flasks were used with nitrogen bubbling (26). Complete medium contained 1% yeast extract (Difco), 1% Bacto-peptone (Difco), and 2% glucose or 2% glycerol as carbon source. Minimal medium contained 0.67% yeast nitrogen base (without amino acids) (Difco), 2% glucose, and when required nutritional supplements at 20 mg/liter. Tween 80 (0.2%) and 30 mg/liter ergosterol were present in all media except when aerobic cultures were supplemented with 15 mg/liter hemin (made up freshly in 0.1 N NaOH). Cells were harvested in the logarithmic growth phase (1–5 × 10^7 cells/ml) and used immediately.

The *Escherichia coli* strains HB101 (27), JM105 and JM109 (28) were used for cloning, maintenance, and propagation of plasmids. They were grown in 1% Bacto-tryptone (Difco), 0.5% yeast extract, and 1% NaCl (pH 7.5). Ampicillin, when added, was 50 mg/liter.

Cytochrome and Heme Contents—Spectrophotometric analysis of cytochromes and porphyrins was performed on whole cells at liquid nitrogen temperature (23). Total heme content of whole cells was measured as described (23) from the spectrum of the pyridine hemochromes.

Coproporphyrinogen Oxidase—The enzyme, purified from yeast, and antisera to it raised in rabbits have been prepared by Camadro *et al.* (9). The activity was measured by a fluorometric coupled assay (29) on cell-free extracts prepared after breakage of the cells with glass beads. A more sensitive radiochemical assay (30), using [^{14}C] coproporphyrinogen (31), was used in the case of the *hem13* mutants. Measurements were done in triplicate and on cell extracts obtained from at least two separate experiments. The activity was expressed as nanomoles of protoporphyrinogen formed per h per mg of protein of the extract at 30 °C. Proteins were determined by the method of Lowry *et al.* (32) using bovine serum albumin as standard.

Transformation—*S. cerevisiae hem13* mutant strains, grown in glucose complete medium supplemented with hemin, were transformed by the spheroplast method (33) or by the lithium chloride procedure (34). The transformants, plated on minimal medium supplemented with hemin, were selected for uracil prototrophy; the ura$^+$ colonies were then assayed for respiratory competence on glycerol plates. *E. coli* transformations were performed as described (35).

DNA Isolation and Techniques—Large-scale preparations of bacterial plasmids were done by the method of Clewell and Helinski (36) and rapid small-scale preparations by a boiling method (37). Total DNA was prepared from yeast as described by Nasmyth and Reed (38). All enzymes used for DNA manipulations were obtained from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim, or Pharmacia LKB Biotechnology Inc.; they were used as recommended by the suppliers or according to Maniatis *et al.* (37). Gel electrophoresis, Southern blotting, and recovery of DNA fragments from agarose gels were accomplished by standard procedures (37).

Plasmids—Plasmid pools of partial *Sau*3A digests of yeast chromosomal DNA ligated into the high copy number vector YEp24 were obtained from D. Botstein (Massachusetts Institute of Technology) (39). They were used to isolate the plasmids pHEM13-1 and pHEM13-2. The low copy number vector pFL38 was used to study the expression of the gene *HEM13*. This vector, obtained from F. Lacroute (University of Strasbourg, France), is derived from pUC19 (28) and contains the gene *URA3* and a *ARS-CEN* cassette. Plasmid pS-S-Sp was constructed by ligating into the *Sst*I and *Sph*I sites of the multicloning site linker of pFL38 a 3.7-kb fragment obtained after partial *Sst*I and total *Sph*I digestions of pHEM13-1. Similarly, plasmids pS-Sp, pX-Sp, and pD-Sp were constructed by digesting pHEM13-1ΔB (Fig. 1*a*), respectively, with *Sst*I plus *Sph*I, *Xmn*I plus *Sph*I, and *Dra*I plus *Sph*I, purifying the fragments, and ligating into pFL38 cut with *Sst*I or *Sma*I and *Sph*I. These constructions were confirmed by restriction analysis.

DNA Sequencing—DNA sequencing was performed by the dideoxy chain terminator method (40), using a sequencing kit (Bethesda Research Laboratories) and 2′-deoxycytosine 5′-[^{35}S](α-thio) triphosphate (Amersham Corp.). The M13 phages tg130 and tg131 (Amersham Corp.) were used to generate single-stranded templates for the sequencing reactions (41). Subclones were prepared by two different methods: direct cloning of specific restriction fragments into RF M13 vectors; unidirectional deletions by exonuclease III (28) in the previous clones. All parts of the DNA sequenced were sequenced from at least two independent clones and each sequencing reaction was at least duplicated.

RNA Preparation and Northern Blotting—Total RNA was prepared from glass bead broken cells as previously described (11) and checked quantitatively by ethidium bromide fluorescence after agarose gel electrophoresis. Samples (10 μg) were electrophoresed on 1.5% agarose gels in the presence of 6% formaldehyde. Transfer procedures to GeneScreen membranes and hybridization conditions were those recommended by the manufacturer (Du Pont-New England Nuclear). A 2.3-kb *Dra*I-*Dra*-I fragment (Fig. 1*b*) inserted in M13tg130, radioactively labeled with ^{32}P by nick translation (using a nick translation kit from Amersham Corp.), was used as the *HEM13* hybridization probe. The *URA3* gene was used as an internal standard. The Northern blots were quantified by scanning densitometry.

S1 Nuclease Mapping—The 5′ ends of the *HEM13* transcripts were mapped as described by Gallwitz *et al.* (42) using S1 nuclease. The double-stranded DNA probe was the *Dra*I-*Cla*I fragment from the 5′ end of the *HEM13* gene (Fig. 1*b*) labeled at the 5′ end of the *Cla*I site with [γ-^{32}P]dATP by phage T4 polynucleotide kinase after dephosphorylation of the 5′-P end (37). Total cellular RNA (100 μg) was hybridized with the denatured probe in 50 μl of hybridization buffer at 42 °C for 12 h. The hybridization mixture was then diluted 10-fold into S1 nuclease buffer and aliquots were incubated for 30 min at 25 °C or 37 °C with various amounts of S1 nuclease. S1-resistant hybrids were ethanol-precipitated and electrophoresed on a 6% polyacrylamide, 8.3 M urea sequencing gel along with the products of the Maxam and Gilbert (43) cleavage reactions performed on the *Dra*I-*Cla*I fragment.

Miscellaneous—Established procedures, referenced in Ref. 11, were used for *in vitro* translation of total RNA; immunoprecipitation; preparation of cell extracts for protein electrophoresis, blotting, and immunodetection using ^{125}I-protein A. Computer analyses were performed using the system B.I.S.A.N.C.E. (Base Informatique sur les Sequences d'Acides Nucleiques pour les Chercheurs Europeens) at the Centre Inter-Universitaire de Traitement de l'Information, Paris.

RESULTS

Assignment of the Structural Gene of Coproporphyrinogen Oxidase to the HEM13 Locus—In preparation for cloning the coproporphyrinogen oxidase gene by functional complementation, five mutants defective in coproporphyrinogen oxidase activity were analyzed in detail both genetically and biochemically: G216 (23), H11 and H12 (24) already described, G225 and 25B recently isolated. They were totally heme-deficient, therefore unable to grow respiratively on glycerol, except H12 which showed residual heme synthesis; all accumulated and excreted coproporphyrin III. This phenotype was dependent on a single recessive nuclear mutation at the locus *hem13*. Furthermore, complementation test revealed interallelic complementation between the mutants G225 and H11; although the diploid synthesized cytochromes normally, no recombinant wild type spores were detected among six tetrads. This result is consistent with the finding that the purified enzyme is a homodimer (9). *hem13* was mapped on chromosome IV, at a map distance of 23 cM from *trp1*: among 39 tetrads from a cross between G216 and a *trp1* strain, 21 tetrads were parental ditype and 18 were tetratype. To determine which chromosome arm carried *hem13*, G216 mutant was crossed to strain 382 (from the Yeast Genetic Stock Center, Berkeley, CA) carrying the thermosensitive mutation *rna11-1* located on the left arm of chromosome IV at a map distance of 17.8 cM from *trp1* (44). Although only a few tetrads were analyzed due to poor sporulation and viability of the spores, the data showed no linkage between *hem13-1* and *rna11-1* (9 tetratype, 1 parental ditype, 1 nonparental ditype). *HEM13* is thus located on the right arm of chromosome IV.

The five *hem13* mutants and their respective parental strains were assayed for coproporphyrinogen oxidase activity. The steady-state amounts of coproporphyrinogen oxidase protein were determined in cell-free extracts by immunoblotting

using an antiserum raised against yeast coproporphyrinogen oxidase. The steady-state concentrations of specific coproporphyrinogen oxidase mRNA were estimated by *in vitro* translation of total RNA and immunoprecipitation of the protein product. The results presented in Table I show that the mutant G216 made no immunodetectable protein, *in vivo* or *in vitro*. Furthermore the mutant phenotype of G216 was found to be suppressed by paromomycin (data not shown), an aminoglycoside antibiotic shown to cause phenotypic suppression of nonsense mutants in yeast (45). Therefore it is likely that the *hem13-1* allele carries a nonsense mutation. The other mutants synthesized an inactive protein of the same relative molecular mass as the normal coproporphyrinogen oxidase. But the amounts of protein and of functional mRNA were much higher in the mutants G225, H11, and 25B than in their respective wild type parental strains. This is not surprising since we have previously reported that heme deficiency leads to an increased synthesis of coproporphyrinogen oxidase (11). It is likely that the residual synthesis of heme in H12 (24) prevents that increase.

In sum, these results demonstrate that the locus *HEM13* represents the structural gene coding for coproporphyrinogen oxidase.

Isolation of the HEM13 Gene—A yeast genomic library in the vector YEp24 was screened for recombinant DNA plasmids that complemented the respiratory defect of the strain 25B carrying the missense allele *hem13-7*. Two such plasmids were isolated: pHEM13-1 and pHEM13-2. They were used to transform the strain MZ4-15B carrying the nonsense allele *hem13-1*. All transformants showing uracil prototrophy were respiratory competent, indicating that the complementation of the respiratory defect by these plasmids was not allele-specific. Further support for the presence of *HEM13* in these plasmids came from the measurement of coproporphyrinogen oxidase activity. Compared to the wild type nontransformed strains (*cf.* Table I), the transformed cells showed an increased activity which could be further increased 15-fold by growing the cells anaerobically (Fig. 1*a*). This indicated that the regulatory regions had also been cloned.

Restriction mapping of the yeast DNA fragments present in the two plasmids revealed that the 12-kb insert of pHEM13-1 was entirely included in the 15-kb insert of pHEM13-2. Therefore only pHEM13-1 was further analyzed. The minimum complementing portion of this insert was determined after *in vitro* deletions and analysis of the capability of the resulting plasmids to complement the *hem13-1* mutation, as outlined in Fig. 1*a*. From these experiments, the 2.8-kb *Sst*I-*Bam*HI/*Sau*3A fragment was defined as essential for complementation. Its detailed restriction map is given in Fig. 1*b*. However, as seen in Fig. 1*a*, it was not sufficient to ensure normal regulation of *HEM13* by oxygen: the coproporphyrinogen oxidase activity was increased only 3–4-fold under anaerobiosis when directed by the plasmid pHEM13-1ΔS.

TABLE I

Biochemical analysis of hem13 mutant strains

Coproporphyrinogen oxidase activity (nanomoles/h/mg of protein) was measured in acellular extracts by a sensitive radiochemical assay (30). Coproporphyrinogen oxidase protein and functional mRNA were determined and quantified by immunoblotting and *in vitro* translation/immunoprecipitation, respectively, as described previously (11). The wild type parental strain was assigned a value (+); ++ and +++ mean a 4–6- and 20–40-fold increase, respectively, in the corresponding mutants; −, not detectable.

Strain	Relevant genotype	Coproporphyrinogen oxidase		
		Activity	Protein	mRNA
FL200	*HEM13*	1.85 ± 0.20	+	+
G216	*hem13-1*	0.004 ± 0.002	−	−
G225	*hem13-4*	0.002 ± 0.001	++	++
SP4	*HEM13*	0.145 ± 0.015	+	+
H11	*hem13-2*	0.002 ± 0.001	+++	+++
H12	*hem13-3*	0.009 ± 0.003	+	+
BWG1-7A	*HEM13*	0.45 ± 0.05	+	ND[a]
25B	*hem13-7*	0.002 ± 0.001	+++	ND

[a] Not determined.

Nucleotide Sequence and Organization of the HEM13 Gene and Adjacent Regions—The sequencing strategy employed is illustrated in Fig. 1*b*. The nucleotide sequence (1951 bp) of the DNA fragment containing the *HEM13* gene and its 5′- and 3′-flanking regions is shown in Fig. 2. It contained a single large open reading frame, starting at the ATG codon at position 1–3, ending with an ochre codon at position 985–987, and coding for a protein of 328 amino acids with a calculated M_r = 37,673. This agreed with the molecular weight, 35,000, of the enzyme subunit estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The predicted amino acid composition also agreed with that determined from hydrolysis of the protein (9). Moreover, the first 10 amino acids of this open reading frame matched exactly the 9-residue amino-terminal sequence determined for the native yeast coproporphyrinogen oxidase, with the exception of the initiating methionine (9). It is likely that this methionine is removed post-translationally by an aminopeptidase which cleaves the amino-terminal methionine when it precedes a proline residue (46).

The codon usage in the *HEM13* gene is that of a moderately expressed gene. The codon bias index calculated after Bennetzen and Hall (47) is 0.47. Of the 61 codons, 51 are used, but the 25 preferred codons are used in majority. It is interesting that this codon usage can accommodate dramatic differences in the expression of *HEM13*: coproporphyrinogen oxidase can represent from 0.005 to 4% of total protein, in SP4 strain, and in anaerobic cells carrying *HEM13* on a multicopy plasmid, respectively.

The analysis of the 5′-flanking region revealed the presence of a single potential, canonical "TATA-like" sequence at position −161, with respect to the initiating ATG. Tracts of poly(T/A) are found downstream of that TATA element, but before the transcription starts. Such tracts have been described in many yeast genes and might play a role for constitutive transcription (48).

The initiation sites of transcription have been determined by S1 nuclease mapping analysis of the mRNA synthesized in a wild type strain (FL200) and in an isogenic heme-deficient mutant (G204) producing increased amounts of coproporphyrinogen oxidase mRNA (11). The labeled DNA probe extended from the *Dra*I site at position −333, to the *Cla*I at position +100. Two major protected fragments of similar intensity and a minor one were detected, which mapped at positions −75 −74, −47 −46, and −52 −51, respectively (Fig. 3). The 5′ termini are located within or near the DNA sequence PyAAPu, often found at the transcription initiation sites in yeast genes (49). As seen also in Fig. 3, heme deficiency stimulated the *HEM13* transcription by increasing the levels of the same set of transcripts as that observed in noninduced cells, indicating that the same initiation sites are used upon induction.

The region downstream of the *HEM13* stop codon contained various nucleotide sequences that are found in the 3′-flanking region of many (but not all) yeast genes and which might represent signals controlling transcription termination. The tripartite sequence TAG.....TATGAT.....TTT....., analogous to that described by Zaret and Sherman (50), is found at positions 1000, 1014, and 1037. The sequence

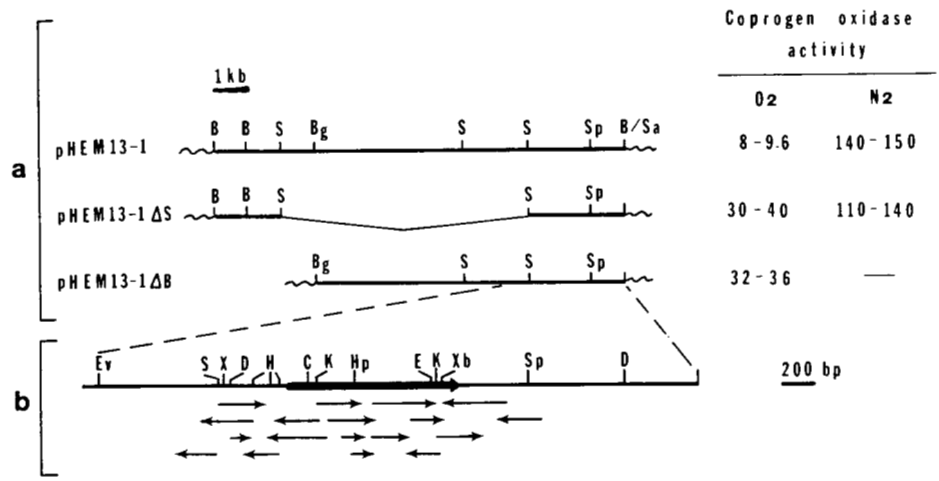

FIG. 1. **Subcloning, restriction map, and sequencing strategy for the HEM13 gene.** *a*, internal deletions of the *Sst*I-*Sst*I or the *Bam*HI-*Bgl*II fragments were realized in the original plasmid pHEM13-1. The resulting plasmids were introduced in the mutant MZ4-15B (*hem13-1*) by transformation and coproporphyrinogen oxidase activity (nanomoles of protoporphyrinogen/h/mg of protein) was measured in acellular extracts obtained from the transformed cells grown aerobically (O_2) or anaerobically (N_2). *Wavy lines* represent vector YEp24. The *insert* is indicated by a *straight line*. *b*, restriction map of the fragment containing the *HEM13* gene. The position of the protein-coding sequence is indicated by a *thick line*. The abbreviations used are: *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *D*, *Dra*I; *E*, *Eco*RI; *Ev*, *Eco*RV; *H*, *Hin*dIII; *Hp*, *Hpa*I; *K*, *Kpn*I; *S*, *Sst*I; *Sa*, *Sau*3A; *Sp*, *Sph*I; *X*, *Xmn*I; *Xb*, *Xba*I. The *arrows* indicate the direction and the length of DNA sequenced in the individual clones.

TTATTTATA, similar to the sequence TTTTTATA described by Henikoff *et al.* (51), is present at nucleotide 1055.

The general organization of the *HEM13* gene fits well with the length of its transcript estimated to be 1.15 kb from RNA blotting analysis (Fig. 4). In addition, we have established by Southern blot analysis (data not shown) that the DNA sequence of the isolated fragment is co-linear with a single copy of the *HEM13* gene in the yeast genome.

Regulation of HEM13 Expression—We have shown previously that the expression of the *HEM13* gene is controlled by heme and oxygen at a pretranslational level in a negative fashion (11). The isolation of *HEM13* provided us with a new tool to investigate further that regulation and its physiological significance.

Northern hybridization experiments, illustrated in Fig. 4, confirmed previous findings obtained with a less direct approach (11). The transcripts were much more abundant in hemeless mutants (*lanes 2* and *6*) and in anaerobically grown wild type cells (*lane 9*): the relative increase was 3–5-fold and 20–40-fold in the FL200 and SP4 genetic backgrounds, respectively. The growth of the heme-deficient mutant G204 under anaerobic conditions (*lane 3*) did not cause a further increase in the level of *HEM13* transcript. Exogenous hemin added to anaerobic cultures of wild type cells (*lane 11*) did not reverse the effect of anaerobiosis, whereas the addition of hemin to aerobic cultures of the *hem13* mutant H11 (*lane 8*) led to a substantial decrease in the level of the *HEM13* transcript. However, as already pointed out (11), these experiments are hampered by possible problems in hemin uptake, especially under anaerobic conditions, which preclude any conclusion to be drawn as to whether the oxygen and heme act independently or not.

To determine regions upstream of the *HEM13* gene required for its regulation, a series of plasmids were constructed that had different amounts of the 5′-flanking sequence of *HEM13*. They all carried the same 3′ end from the stop codon to the *Sph*I site (Fig. 1*b*). The "wild type" plasmid, pS-S-Sp, contained approximately 2310 bp of DNA upstream of the initiating ATG. The "deleted" plasmids, pS-Sp, p*X*-Sp, and pD-Sp, had 409, 367, and 333 bp of upstream sequences, respectively (*Sst*I, *Xmn*I, and *Dra*I sites in Fig. 2). The inserts of all of these plasmids were joined at their 3′ ends to the *Sph*I site, and at their 5′ ends to the *Sst*I or *Sma*I sites of the multicloning site linker of the vector. The vector used in this analysis was pFL38, a derivative of pUC19, which carries the yeast *URA3* gene, a yeast centromere and a yeast *ARS* sequence, and is present in only a few copies per cell. These plasmids were introduced into the strain MZ4-15B (*ura3, hem13-1*) by transformation. Two randomly selected transformants by each plasmid were grown aerobically and anaerobically and the cells were assayed for total heme content and for coproporphyrinogen oxidase activity. The results are presented in Table II. The level of coproporphyrinogen oxidase activity directed in aerobic cells by the different plasmids varied slightly, probably reflecting changes in plasmid copy number (52). Anaerobiosis increased the activity 44-fold in MZ4-15B/pS-S-Sp cells, which compared well with wild type cells (11). The deletions of the DNA sequences upstream to the *Sst*I, *Xmn*I, or *Dra*I sites resulted in an almost complete loss of control of *HEM13* expression by oxygen; the cause of the residual 2–3-fold induction upon anaerobiosis is unclear at present. It must be noted that there exists a discrepancy between these results and those reported in Fig. 1*a*, where the deletion of the DNA sequence upstream to the *Sst*I site (pHEM13-1ΔS) led to an increase in aerobic expression while anaerobic expression did not change significantly. But the B/Bg deletion (pHEM13-1ΔB), far away from the *Sst*I site, also increased the aerobic expression. We think that the data of Table II, obtained with smaller centromeric plasmids, are more reliable than the results of Fig. 1*a* obtained with large DNA inserts borne on a high copy vector. Therefore, we conclude that anaerobic derepression is likely to be mediated by positive activation acting through sequence(s) located upstream from −409.

An important result, reported also in Table II, is that a good correlation exists between the level of coproporphyrinogen oxidase activity and the amount of heme synthesized by the different transformants grown anaerobically. This means that under anaerobiosis coproporphyrinogen oxidase was the rate-limiting step for heme production. It is likely

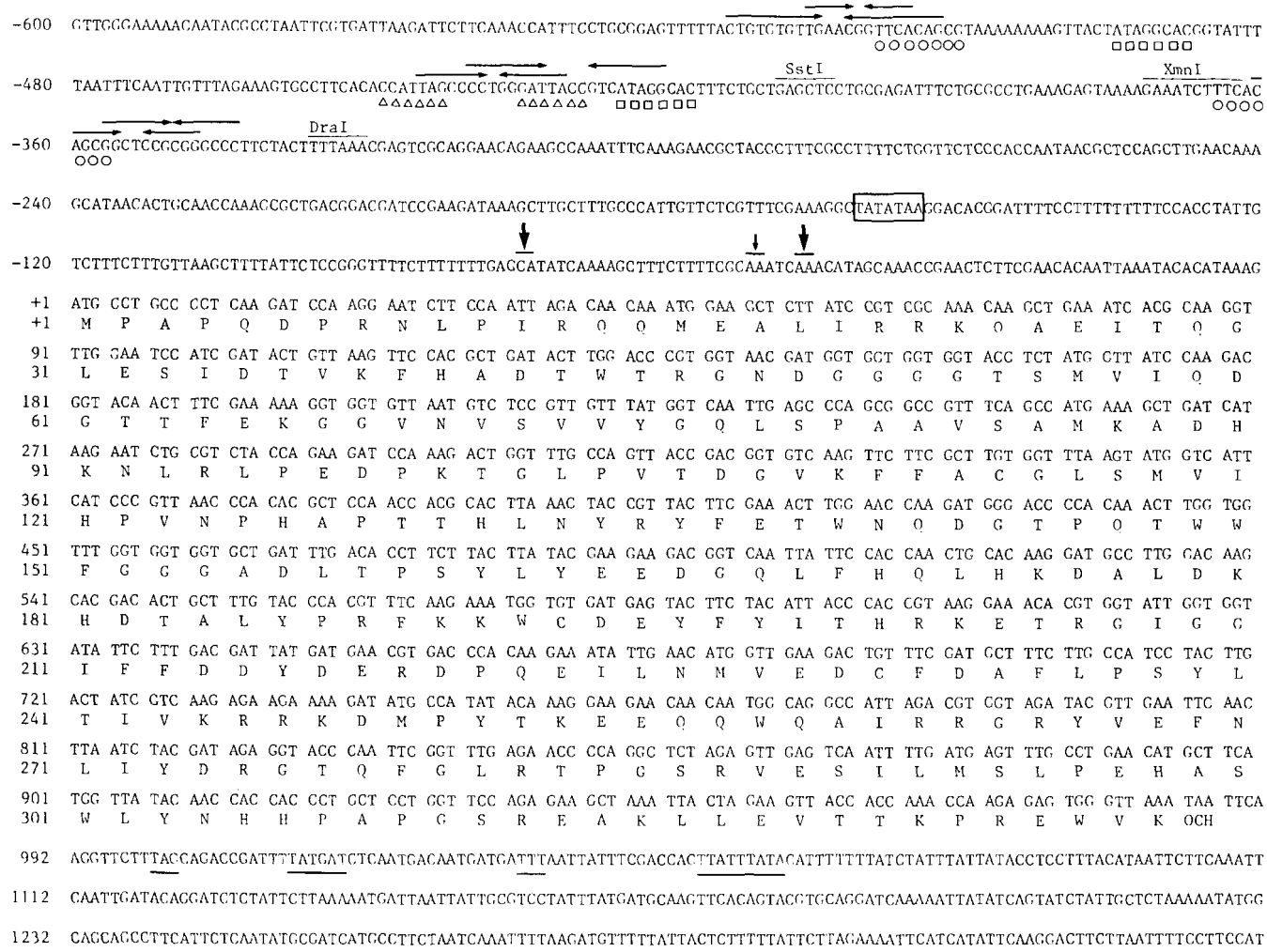

FIG. 2. **Nucleotide sequence of the HEM13 gene with its flanking regions.** The 1951-bp sequence with the deduced amino acid sequence encoded by the 987-bp open reading frame is shown. The putative "TATA" element is *boxed*. The *vertical arrows* indicate the transcription initiation sites. The three restriction sites, *SstI*, *XmnI*, and *DraI*, used for 5′ deletion studies are indicated. Repeat sequences are *underlined* with different symbols (○, □, △). Palindromic sequences are represented by *arrows* pointing at the point of symmetry. Motifs present in the 3′ region of many yeast genes have been *underlined*.

that this is a consequence of the very low concentration of oxygen present in anaerobic culture since oxygen is required for coproporphyrinogen oxidase activity (9). The cells respond to oxygen limitation by increasing the amount of enzyme. The enzyme following coproporphyrinogen oxidase in the heme biosynthetic pathway, protoporphyrinogen oxidase, is also an oxidase requiring oxygen for its activity. But its activity is not increased upon anaerobiosis, probably because it is already high in aerobic cells (about 20 times the activity of coproporphyrinogen oxidase) and is far from functioning at its maximal velocity *in vivo* (53). The formation of heme by anaerobically growing yeast cells can therefore be explained by (i) the high activity of these two oxidases, induced for coproporphyrinogen oxidase and constitutive for protoporphyrinogen oxidase, (ii) their high affinity for oxygen ($\leq$ 0.1 μM),[4] (iii) the presence of trace amounts of oxygen in the cultures due, presumably, to its diffusion through the tubings (54).

DISCUSSION

The isolation and sequencing of the yeast *HEM13* gene encoding coproporphyrinogen oxidase provides further information to analyze the structure of the enzyme and understand the regulation of its synthesis.

The sequence data confirmed the high content of aromatic amino acid residues previously reported for the yeast (9) and the bovine liver (7) enzymes: it amounts to 11.3% (15% if histidine is taken in account), which is significantly higher than what is usually found (5–7%). They are often found clustered and close to the amino acid residues known as potential iron-ligands: cysteine, methionine, glutamate, aspartate, histidine, and tyrosine. These aromatic residues might be important in providing the proper (hydrophobic ?) environment to the substrate molecule, or to the iron atom and its ligands. The secondary structure analysis (55) of coproporphyrinogen oxidase predicted a large number (19–21) of β turns, in agreement with the unusually high proline content (6.7%). The determination of the nature of the mutations leading to the loss of activity of the proteins made by the mutant strains should help to define the amino acids contributing to the active site of the enzyme or important for its secondary structure.

The synthesis of coproporphyrinogen oxidase is transcriptionally induced in response to oxygen or heme deficiency.

[4] J. M. Camadro and P. Labbe, unpublished results.

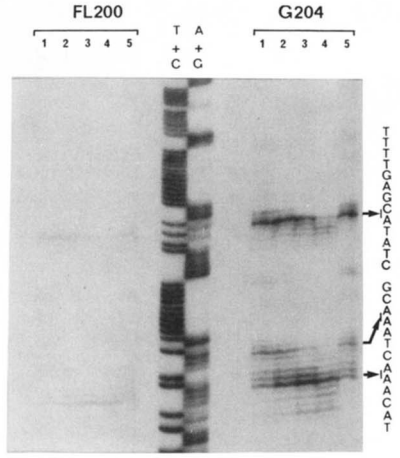

FIG. 3. **Nuclease S1 mapping of the 5′ termini of the HEM13 transcripts.** Total cellular RNA (100 μg) isolated from the wild type strain FL200 or the isogenic heme-deficient mutant G204 was hybridized with the 5′ end-labeled *Dra*I-*Cla*I fragment. Hybrids were digested at 37 °C with 1 (*lane 1*), 5 (*lane 2*), 25 (*lane 3*), or 100 (*lane 4*) units of nuclease S1, or at 25 °C with 5 units of nuclease S1 (*lane 5*). They were run on a sequencing gel along with the Maxam and Gilbert specific cleavage reactions performed on the *Dra*I-*Cla*I DNA probe. *Arrows* indicate the sequence positions of the 5′ termini.

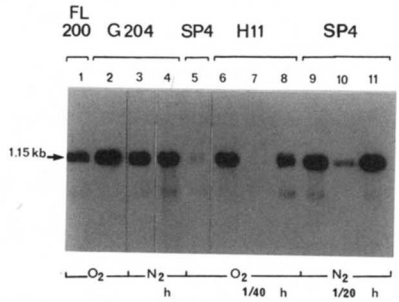

FIG. 4. **Northern blot analysis of the expression of the HEM13 gene.** Total cellular RNA (10 μg) was fractionated by electrophoresis, transferred to nitrocellulose sheet, and hybridized to the *HEM13* probe. RNA was isolated from wild type (FL200, SP4) or heme-deficient (G204 (*hem1-5*), H11 (*hem13-2*) strains grown aerobically (O_2) or anaerobically (N_2) in medium supplemented where indicated with hemin (*h*). *Lanes 7* and *10* contain RNA diluted 40- and 20-fold, respectively.

TABLE II

Influence of upstream DNA regions on regulation by oxygen of HEM13 gene

Plasmid	5′-flanking DNA	Coproporphyrinogen oxidase activity[a]			Heme[b] content N_2
		O_2	N_2	N_2/O_2	
	bp				
pS-S-Sp	2310	1.3	57	44	45
pS-Sp	409	2	5.3	2.6	25
		3.4	7.4	2.2	21
pX-Sp	367	1.1	2.5	2.3	3
		0.7	3.5	5	12
pD-Sp	333	1.15	2.5	2.2	4
		1.2	3.5	2.9	10

[a] Coproporphyrinogen oxidase activity (nanomoles/h/mg protein) was measured (with an error of ±10%) in acellular extracts of the strain MZ4-15B (*hem13-1*) carrying the indicated plasmids. The cells were grown aerobically (O_2) or anaerobically (N_2) in glucose complete medium. The data represent the values obtained in two independent transformants for each plasmid.

[b] Determined in whole cells by its pyridine hemochrome (23) and expressed in nanomoles/g of dry weight.

We have presented evidence that the depression by anaerobiosis is mediated by DNA sequence(s) located upstream of 409 nucleotides 5′ to the initiation codon. The analysis of the nucleotide sequence between −600 and −400 reveals the presence of two interesting features which might be relevant to gene regulation (Fig. 2). First, there exists between −531 and −514 a motif TGTG-N_{10}-CACA flanked by A/T-rich regions, analogous to the conserved sequence present in the upstream promoter element of the nitrogen fixation (*nif*) genes and which is required for activation by the oxygen-regulated (56) *nifA* gene product (57). This motif conforms to a consensus sequence found in many procaryotic genes at the sites of binding of the regulatory multimeric proteins (58). Second, a 15-bp sequence CCTGGGATTACCGTC, beginning at position −440, shows strong homology (11 nucleotides out of 15) with a region of the upstream activation site UAS1 of the gene *CYC1* encoding iso-1-cytochrome *c*. This region has been shown to be essential for the activity of UAS1 (59); it is involved in binding a protein factor present in extracts from normal cells but absent in extracts made from anaerobic or heme-deficient cells (60), as well as the product of the regulatory *HAP1* gene (59, 61) which activates transcription of *CYC1* via UAS1 in response to heme (16). Whether these two upstream sequences are of functional importance for the regulation of *HEM13* by oxygen, heme, or both, remains to be determined.

A number of genes have been described, in different organisms, whose transcription is activated under low oxygen concentration. This is the case, for example, of the *Adh1* gene encoding alcohol dehydrogenase 1 in maize (62 and references therein), and of the genes coding for light-harvesting and reaction center proteins in photosynthetic bacteria (63 and references therein). In *E. coli* the anaerobic induction of many genes encoding enzymes involved in anaerobic electron transport is under positive control of the *fnr* gene. The Fnr protein shares considerable homology with the catabolite activator protein CAP and its synthesis is independent of anaerobiosis (64 and references therein). It has been proposed that the mode of action of Fnr involves conformational change of the protein induced by redox effectors (65). A similar mechanism might operate in yeast and mediate the regulation by both oxygen and heme. In effect, the heme-deficient and anaerobic cells share some traits in common: same mode of energy metabolism, absence, or nonfunctioning of the electron transport chains (mitochondrial and microsomal) linked to oxygen, and a low redox potential. One can imagine that regulatory element(s) could sense the physiological state of these cells by changing its conformation via redox-linked modification(s) or via binding of an effector reflecting the redox status of the cells. These changes would then control the regulatory activity of such element(s).

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