Characterization of the Yeast HEM2 Gene and Transcriptional Regulation of COX5 and COR1 by Heme*

(Received for publication, December 22, 1986)

Alan M. Myers§, Mary D. Crivellone, T. J. Koerner§, and Alexander Tzagoloff
From the Department of Biological Sciences, Columbia University, New York, New York 10027

The respiratory deficiency of two noncomplementing mutants of Saccharomyces cerevisiae (C41 and N28) has been shown to be due to mutations in HEM2, the structural gene for δ-aminolevulinate dehydratase. The mutants are unable to convert δ-aminolevulinic acid to porphobilinogen and are not complemented by the hem2 mutant GL4 (Goldblatt, E. G., Liu, K.-P., Dagan, J., Adlersberg, M., and Sprinser, D. B. (1977) J. Biol. Chem. 252, 2846–2854). A gene capable of complementing the respiratory deficiency of C41 and N28 has been cloned by transformation of a hem2 mutant with a recombinant plasmid library of wild type yeast nuclear DNA. The sequence of the protein encoded by the cloned gene exhibits extensive homology to the recently reported sequence of human δ-aminolevulinate dehydratase (Wetmur, J. G., Bishop, D. F., Cantelmo, C., and Desnick, R. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7703–7707).

Several approaches were taken to study the effect of heme on transcription of PET genes known to code for subunit components of respiratory enzymes and of mitochondrial ATPase. The first involved measurements of the steady state levels of mRNAs for subunit 5 of cytochrome oxidase and the β subunit of F1 ATPase in wild type and in a hem2 mutant. Secondly, transcription of the genes coding for the cytochrome oxidase and ATPase subunits as well as of the COR1 gene coding for the 44-kDa core 1 subunit of cytochrome QH2-cytochrome c reductase was quantitated by fusing the 5′-flanking and the coding region of each gene to the lacZ gene of Escherichia coli in vectors capable of integrating into yeast chromosomal DNA. The different lacZ fusions were integrated into nuclear DNA of a wild type strain and of hem2 mutants allowing expression of β-galactosidase to be studied as a function of intracellular heme. These experiments indicate that the promoters of the genes for subunits of the respiratory complexes are regulated by heme. In contrast, the expression of the ATPase subunit appears to be heme-independent. Because neither subunit 5 of cytochrome oxidase nor the core 1 subunit of cytochrome QH2-cytochrome c reductase are hemoproteins, transcriptional regulation by heme may be a general mechanism for controlling the synthesis of mitochondrial proteins involved in respiration.

Heme is an important compound utilized in various forms as the prosthetic group of respiratory cytochromes a + a3, b, c and c1, of cytochrome b6, required for biosynthesis of unsaturated fatty acids, and of cytochrome P-450 utilized in steroid biosynthesis (1, 2). In yeast, heme also plays an important regulatory role in biogenesis of the respiratory chain enzymes. Heme is required for assembly of cytochrome oxidase (3) and is specifically required for transcription of the gene coding for apo-iso-1-cytochrome c (4, 5).

Yeast mutants defective in most of the steps of the heme biosynthetic pathway have been reported (2, 6–8). Such mutants will grow fermentatively if supplied with a source of sterol and unsaturated fatty acid and can grow respiratively on nonfermentable substrates if supplied with a source of heme. However, certain mutations in heme biosynthetic enzymes will support fermentative but not respiratory growth in the absence of supplements (8) and thus should be included among collections of nuclear respiratory-deficient mutants of yeast.

As part of our efforts to characterize the genetic lesions of a collection of (pet)1 mutants of Saccharomyces cerevisiae, we have identified four separate complementation groups to be defective in heme biosynthesis. In the present communication we present evidence that members of one such group are blocked in the second step in heme biosynthesis due to mutations in δ-aminolevulinate (ALA) dehydratase, the enzyme encoded by HEM2 (7). The HEM2 gene has been cloned and its nucleotide sequence determined. In addition, hem2 mutants have been used to study the heme-dependent expression of three unrelated genes required for respiratory function. Two of the genes code for subunit polypeptides of respiratory enzymes, and the third codes for a subunit of the mitochondrial ATPase. Both COX5 and COR1, coding for subunits of cytochrome oxidase and coenzyme QH2-cytochrome c reductase (9–11), respectively, are dependent on heme for their expression, although the protein products do not bind heme. In contrast, expression of the ATP2 gene coding for the β subunit of F1 ATPase (12) is not affected in the hem2 mutants.

* This research was supported by Research Grant GM 25250 and a Research Service Award GM 11429 (to M. D. C.) from the National Institutes of Health, United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Biochemistry and Biophysics, Iowa State University, Ames, IA  50011.
§ Present address: Dept. of Pathology, Duke University, Durham, NC  27710.

‡ To whom correspondence should be addressed.

1 The abbreviations used are: pet, nuclear mutations in yeast causing a respiratory-defective phenotype; kh, kilobase pairs; bp, base pairs; X-Gal, β-galactosidase-3-indolyl β-d-galactopyranoside; ALA dehydratase, δ-aminolevulinate dehydratase; ρ, cytoplasmic petite mutant lacking mitochondrial DNA.
MATERIALS AND METHODS

Yeast Strains and Growth Media—The yeast strains used in this study are listed in Table I. The pet mutants C41 and N28 were isolated following mutagenesis of wild type strain D273-10B/A1 with ethylmethane sulfonate or nitrosoguanidine (14). Rich media contained 1% yeast extract, 2% peptone, and 2% glucose (YPD), galactose (YPG4), or glycerol (YPEG). Glucose-repressed cultures were grown on the same medium containing 8% glucose. Minimal medium (WO) contained 0.67% yeast nitrogen base minus amino acids (Difco), 0.05% glucose, and was supplemented as required with adenine, histidine, leucine, uracil, tryptophan, and methionine at 20 μg/ml. Sporulation medium contained 0.1% yeast extract, 0.06% glucose, and 1% potassium acetate. Solid media for yeast contained 2% agar. Hemin chloride, dissolved in 50% ethanol, 20 mM NaOH at 3.75 mg/ml, was added to a final concentration of 13 μg/ml. Exoperotel, Tween 80, and deuteroporphyrin IX were added to growth media at final concentrations of 20 pg/ml, 5 mg/ml, and 2.5 pg/ml, respectively.

Cloning of HEM2—The hem2 mutant C41/U1 was transformed with a genomic library consisting of partial Sau3A fragments of yeast nuclear DNA averaging 7–10 kb digested to the BamHI site of the shuttle vector YEp24 (15). This library was generously provided by Dr. Marian Carlson (Department of Human Genetics, College of Physicians and Surgeons, Columbia University, New York). Approximately 5 × 10^6 plasmid cells grown in YPGal were transformed with 1 μg of the recombinant library as described previously (16). Transformants were selected on minimal glycerol medium (0.67% yeast nitrogen base minus amino acids, 0.05% glucose, 2% glycerol, 1.2 mg sorbitol) for uracil prototrophy and respiratory competence. The respiratory-competent phenotype of the clone C41/U1/T4 was verified by segregation tests to be plasmid-inherited.

Assay of ALA Dehydratase Activity—Yeast cells were grown to stationary phase in YPD supplemented with heme as required. Approximately 5 g wet weight of cells were washed once in 0.1 M glycine/NaOH, pH 9.0, resuspended in 20 ml of the same buffer, and homogenized with glass beads for 1 min in a Braun homogenizer. The suspension was centrifuged at 30,000 rpm for 30 min in a Spincos Type 30 rotor, and the supernatant fraction was collected. The protein content in each extract was measured by the method of Lowry et al. (17). ALA dehydratase activity was measured as described by De Barreiro (18). Assay mixtures (0.5 ml) contained 0.1 M glycine/NaOH, pH 9.0, 1–5 mg of protein, and 2.5 μmol of δ-aminolevulinic acid. After incubation for 30 min at 50 °C, the reactions were terminated by addition of an equal volume of 5% trichloroacetic acid. Protein was removed by brief centrifugation at 2,000 rpm, and the amount of porphobilinogen in the supernatant solution was measured with modified Hg-Ehrlich's reagent as described by Urata and Granick (19).

 Manipulation and Sequence Determination of Nucleic Acids—Standard techniques were used for preparation of recombinant plasmids from Escherichia coli, restriction endonuclease analysis, isolation and ligation of restriction fragments, transformation of E. coli, and screening of E. coli transformants (20). E. coli strain RR1 (proA leuB6 lacY galK2 xyl-5 mtl-1 ara-14 rpsL20 supE44 hsdS2 X- lacZΔM15) grown in LB broth was used as the host. The sequence of the HEM2 gene and flanking regions was determined by the method of Maxam and Gilbert (21) by using 5'-end-labeled single stranded restriction fragments. All restriction sites used for 5' end labeling were cloned from the plasmid site, and most of the nucleotide sequence was confirmed from the complementary strand.

Hybridization and Immunochemical Analyses—The conditions of hybridization of nick-translated probes to Southern blots of yeast genomic DNA have been described previously (22). Polyadenylated RNA was isolated from total yeast RNA by affinity chromatography on poly(U)-Sepharose (23). The polyadenylated RNA was separated by electrophoresis in nondenaturing agarose gels, transferred to diazobenzyloxymethyl paper, and probed with nick-translated restriction fragments as described by Alwine et al. (24). Western blot analysis was performed as described by Schmidt et al. (25).

Construction and Expression of lacZ Gene Fusions—PET genes were fused to the lacZ gene of E. coli in the vectors YIp533, YIp356, and YIp356R (26). Expression of β-galactosidase activity was observed on solid medium containing X-Gal and quantitated in peroxidase. The results were visualized under light microscopy with modified Hg-Ehrlich's reagent as described by Urata and Granick (19).

RESULTS

Growth Properties of Respiratory-deficient Mutants C41, N28, and aW303HEM2—Respiratory-deficient mutants C41 and N28 are two members of complementation group G32. Mutant strain aW303HEM2 is a third noncomplementing mutant of this group created by in situ disruption of the HEM2 gene (see below). C41, N28, and aW303HEM2 form respiratory-competent diploid cells when mated to a rho0 tester strain lacking mitochondrial DNA, indicating that each of the three strains contains a recessive mutation in nuclear DNA. Spontaneous reversion of C41 and N28 to respiratory competence occurs with a frequency of less than 10^-4.

| Table 1 | Genotypes and sources of S. cerevisiae strains |
|---|---|
| Strain | Genotype | Source |
| D273-10B/A1 | a, met6 | Ref. 13 |
| W303-1A | a, leu2, his3, his4, ura3, ade2, trp1 | R. Rothstein* |
| W303-1B | a, leu2, his3, ade2, trp1 | R. Rothstein* |
| C41 | a, met6, hem2-15 | This study |
| N28 | a, met6, hem2-16 | This study |
| C41/U1 | a, ura3-255 | C41 × W303-1A |
| N28/U2 | a, ura3-255 | N28 × W303-1A |
| W303/HEM2 | a, leu2, his3, ura3, ade2, trp1, HEM2::LEU2 | This study |
| aW303/HEM2 | a, leu2, his3, ura3, ade2, trp1, HEM2::LEU2 | This study |
| GL1 | a, gal2, hem1-3 | Ref. 7 |
| GL3 | a, gal2, hem2-4 | Ref. 7 |
| GL5 | a, gal2, hem5-5 | Ref. 7 |
| GL6 | a, gal2, hem4-1 | Ref. 7 |
| GL9 | a, gal2, hem5-1 | Ref. 7 |
| WCZ | a, leu2, his3, ura3, ade2, trp1, CYC1-lacZ (URA3) | This study |
| WBZ | a, leu2, his3, ura3, ade2, trp1, COX6-lacZ (URA3) | This study |
| W44Z | a, leu2, his3, ura3, ade2, trp1, COR1-lacZ (URA3) | This study |
| C41CZ | a, ura3, hem2-15, CYC1-lacZ (URA3), aux4 | C41/U1 × WCZ |
| C41BZ | a, ura3, hem2-15, ATP2-lacZ (URA3), aux4 | C41/U1 × WBZ |
| C41VY7 | a, ura3, hem2-15, COX5-lacZ (URA3), aux4 | C41/U1 × WBZ |
| N2844Z | a, ura3, hem2-16, COR1-lacZ (URA3), aux4 | N28/U2 × W44Z |

*These strains contain additional auxotrophic markers which were not determined.

* Department of Human Genetics, College of Physicians and Surgeons, Columbia University, New York.
While C41 and N28 can grow on rich (YPD) or minimal (WO) glucose media, this was not true of aW303VHEM2, whose growth on YPD required Tween 80 and ergosterol supplements as a source of unsaturated fatty acid and sterol, respectively. aW303VHEM2 has an additional requirement of methionine for growth on minimal glucose medium. C41 and N28 show slight growth on rich glycerol medium (YEPG), while aW303VHEM2 shows no growth on this medium. Growth of the three mutant strains on glycerol medium supplemented with heme is indistinguishable from that of wild type yeast.

**G32 Mutants Are Deficient in ALA Dehydratase**—Strains carrying the hem2-15 or hem2-16 allele were tested for their ability to complement a series of mutants known to be deficient in specific steps in the heme biosynthetic pathway (7). The pet mutants formed respiratory-competent diploid cells when mated to tester strains carrying hem1, hem3, hem4, or hem5 mutations but failed to complement the tester strain with the hem2-4 allele. This mutant is known to be deficient in ALA dehydratase, the enzyme responsible for catalyzing the condensation of two molecules of 6-aminolevulinic acid to form one molecular of porphobilinogen (7). The hem2 mutants C41/U1 and aW303VHEM2 were also found to lack ALA dehydratase activity as evidenced by the absence of enzyme activity in soluble cell extracts (Table II). The absence of ALA dehydratase is consistent with the observation that aW303VHEM2 is a methionine auxotroph. Mutations in ALA dehydratase prevent formation of uroporphyrinogen III, a precursor of the siroheme cofactor of sulfite reductase required for methionine biosynthesis (28). Absorption spectra of mitochondria from C41 and N28 show a general decrease in cytochromes (Fig. 1), an additional indication of heme deficiency.

**Isolation of HEM2**—The wild type gene HEM2 was selected by transformation of the hem2-15 strain C41/U1 with a plasmid library of yeast genomic DNA consisting of partial Sau3A fragments of 7–10 kb ligated to the BamHI site of the URA3-bearing vector YEp24 (15). The library was used to transform C41/U1 to respiratory competence. Transformants were selected for growth on glycerol and uracil prototrophy. Transformant C41/U1/T4 was found to contain an autonomously replicating plasmid, because vegetative progeny of this transformant showed co-segregation of the respiratory-competent and uracil-independent phenotypes. The transforming plasmid pG32/T4, isolated from C41/U1/T4, was amplified in *E. coli* and analyzed by mapping of restriction enzyme recognition sites. The plasmid contains a genomic insert of approximately 11 kb (Fig. 2).

The genetically active region of the yeast DNA insert in pG32/T4 was localized further by subcloning segments into the shuttle vector YEp352 (29) and testing the ability of each recombinant plasmid to transform C41/U1 to respiratory competence (Fig. 2). The smallest subclone (pG32/ST7) capable of transforming the hem2-15 mutant contains a 1.3-kb HindIII fragment of yeast genomic DNA. This subclone also complemented the hem2-16 mutation of N28/U2, indicating that restoration of the respiratory-competent phenotype is not allele-dependent. The specific activity of ALA dehydratase activity increases in proportion to copy number of the cloned gene, the wild type allele HEM2 is likely to be contained within the 1.3-kb insert of pG32/ST7.

**Nucleotide Sequence of HEM2**—The nucleotide sequence of the 1.3-kb HindIII fragment in pG32/ST7 was determined by the method of Maxam and Gilbert (21) using the 5' end-labeled restriction fragments shown in Fig. 3. The sequence revealed a 1,029-nucleotide open reading frame beginning with an ATG initiation codon and extending through most of the fragment (Fig. 4). This open reading frame can code for a protein with a molecular weight of 37,837 whose sequence is

### Table II

| Strain          | Genotype                   | ALA dehydratase* |
|-----------------|----------------------------|------------------|
| W303-1A        | HEM2                      | 0.78             |
| W303VHEM2      | HEM2::LEU2                | 0.15             |
| C41/U1         | hem2-15                   | 0.08             |
| C41/U1/ST7     | hem2-15,HEM2 (on multicopy| 13.4             |
|                | plasmid)                  |                  |

* ALA dehydratase activity refers to nanomoles of porphobilinogen formed/mg protein/30 min. C41/U1/ST7 is a transformant of a wild type strain D272-10B/A1 were extracted with 1% deoxycholate and 1 M KCl at the indicated protein concentrations. One-half of the extract was oxidized with potassium ferricyanide and the other half reduced with sodium dithionite. Difference spectra were recorded at room temperature with a Cary Model 14 spectrophotometer.

![Fig. 2. Localization of HEM2 within the yeast genomic insert of pG32/T4. A partial restriction map of the nuclear DNA insert of pG32/T4 is shown on the upper left of the figure. The bars in the lower left of the figure depict the regions of the original insert subcloned in YEp352 to form the plasmids pG32/ST3-pG32/ST8. Dashed lines represent regions of the original cloning vector YEp24. The asterisk represents the junction of Sau3A and BamHI sites delimiting one end of the yeast DNA insert in pG32/T4. The ability of each plasmid to complement the respiratory deficiency of C41/U1 is denoted by the plus (complementation) and minus (no complementation) signs. The locations of restriction enzyme recognition sites are marked for HindIII (H), BamHI (B), EcoRI (E) and SstI (S). The location and orientation of HEM2 is indicated by the solid bar pointing in the direction of transcription.](image-url)

![Fig. 1. Difference spectra of mitochondrial cytochromes in wild type and in a HEM2 mutant. Mitochondria from N28 and from the parental wild type strain D272-10B/A1 were extracted with 1% deoxycholate and 1 M KCl at the indicated protein concentrations. One-half of the extract was oxidized with potassium ferricyanide and the other half reduced with sodium dithionite. Difference spectra were recorded at room temperature with a Cary Model 14 spectrophotometer.](image-url)
homologous to the recently reported sequence of human ALA dehydratase (30). The two proteins share 52% identical residues over the entire lengths of the polypeptide chains with only a few deletions/insertions required for the alignments (Fig. 5). The most highly conserved regions occur in the middle of the proteins where the homology is 62% based on identical residues and not taking into account conservative substitutions. This region includes a domain proposed to constitute the zinc-binding site of the enzyme (30). The sequences reported for four tryptic or chymotryptic peptides of bovine ALA dehydratase (30, 31) can also be aligned with the deduced sequence of the yeast protein (Fig. 5). These results confirm the identity of the cloned yeast gene as HEM2.

In Situ Disruption of HEM2—The disrupted allele HEM2::LEU2 was constructed using the method of one-step gene replacement (32). The 1.3-kb HindIII fragment containing the yeast HEM2 was ligated to the shuttle vector YEp352H,2 and the resultant plasmid was cleaved at the unique BamHI site within HEM2. A 3.0-kb BglII fragment containing the yeast LEU2 gene was isolated from plasmid YEp13 (33), ligated to the BamHI site of HEM2, and the 4.3-kb HindIII fragment bearing the disrupted HEM2 gene was isolated from the recombinant plasmid. This fragment was introduced into the diploid strain W303 homozygous for leu2, and leucine prototrophs were selected.

The site of integration of the LEU2 gene in one transformant W303VHEM2 was determined by Southern hybridization (Fig. 3). The open reading frame of the yeast gene is the same as that of the human gene and is indicated by arrows. The open bar shows the location of the open reading frame proposed to code for ALA dehydratase.

Fig. 3. Sequencing strategy. The locations of restriction enzyme recognition sites used for 5' end labeling are denoted by the symbols: BamHI (●), DdeI (○), HaeIII (○), HindIII (○), HinfI (△), MboI (●), PvuII (□), Rsal (▲), TaqI (○), and XbaI (○). The direction and approximate extents of the sequence obtained from each fragment are indicated by the arrows. The open bar shows the location of the open reading frame proposed to code for ALA dehydratase.

Fig. 4. Nucleotide sequence of HEM2 and flanking regions. The sequence shown includes the entire 1.3-kb HindIII fragment of pG32/ST7. The open reading initiated by the methionine codon at nucleotide +1 and ending at the termination codon at nucleotide +1028 has been translated into a protein sequence. The locations of the HindIII, BamHI, and XbaI sites are underlined for reference to Figs. 2, 5, and 6.
Staining of the gel (not shown) indicates that the entire DNA sample with EcoRI; clones were selected for leucine prototrophy. One such transformant, from W303VHEM2. W303 digested with HindIII. The migration of size standards are DNA from W303VHEM2 digested with HindIII; the blot was hybridized with the wild type HindIII fragment labeled by nick-translation. The results of the hybridization are shown in the part of the figure. The locations of the HindIII (H), BamHI (B), EcoRI (E), and XbaI (X) sites are indicated on the two maps. The direction of transcription of the HEM2 and LEU2 genes are denoted by the arrows. Following transformation of the isogenic diploid strain W303 with the linear 4.3-kb HindIII fragment, clones were selected for leucine prototrophy. One such transformant, W303VHEM2, was used for Southern hybridization analysis of its genomic DNA. Nuclear DNA was purified from W303 and from W303VHEM2 as described previously (22). The two genomic DNAs were digested with either EcoRI or HindIII and separated electrophoretically on a 1% agarose gel. Following transfer to nitrocellulose, the blot was hybridized with the wild type HindIII fragment labeled by nick-translation. The results of the hybridization are shown in the upper part of the figure. Lane 1, DNA from W303VHEM2 digested with EcoRI; lane 2, DNA from W303 digested with EcoRI; lane 3, DNA from W303VHEM2 digested with HindIII; lane 4, DNA from W303 digested with HindIII. The migration of size standards are marked in the margin. The 1.3-kb HindIII fragment containing the wild type HEM2 gene from W303 and W303VHEM2 appears to migrate in this gel at slightly different rates. Ethidium bromide staining of the gel (not shown) indicates that the entire DNA sample from W303 ran at a slightly retarded rate compared with the digest from W303VHEM2.

The diploid strain carrying one copy of the disrupted HEM2 allele was sporulated, and haploid colonies randomly picked from YPD medium supplemented with heme were tested for growth on minimal medium in the absence of leucine and on glycerol medium in the absence of heme. Out of 57 haploid progeny tested, 35 were leucine prototrophs (HEM2::LEU2) and required heme for growth on nonfermentable substrates (YEPG), while the remaining 22 were leucine auxotrophs (HEM2) and were respiratory-competent. Two leucine prototrophs, αW303VHEM2 and αW303VHEM2, were chosen as representative HEM2::LEU2 mutants. As mentioned above, these strains fail to form respiratory-competent diploid strains when mated to C41 or N28, thus confirming that the cloned HEM2 gene is the genetic element containing the hem2-15 and hem2-16 alleles.

Expression of COX5, COR1, ATP2, and CYC1 in a hem2 Mutant—The CYC1 gene of yeast coding for apo-iso-1-cytochrome c (34) has been reported to be transcriptionally regulated by glucose and heme (4, 5, 35). The regulation is mediated through several trans-acting factors that interact with sequence elements (UAS1 and UAS2) located in the 5'-flanking region of the CYC1 gene (4, 5). Glucose and heme are also known to affect the synthesis of other respiratory enzymes such as cytochrome oxidase and coenzyme QH2-cytochrome c reductase (3, 36, 37).

Two approaches were used to study transcription of genes coding for representative subunit polypeptides of respiratory complexes and of the mitochondrial ATPase in wild type or hem2 genetic backgrounds. The genes examined included COX5 coding for subunit 5 of cytochrome oxidase (10), COR1 coding for the 44-kDa subunit of coenzyme QH2-cytochrome c reductase (11), and ATP2 coding for the β subunit of F1 ATPase (12). The effect of heme on transcription of each gene was assessed by measuring the steady state levels of the mRNAs using Northern blot hybridization analysis and by expression of β-galactosidase in cells harboring chromosomally integrated fusions of 5'-flanking regions of the PET genes to the lacZ gene of E. coli.

The mRNAs for apo-iso-1-cytochrome c, subunit 5 of cytochrome oxidase, and the ATPase subunit were analyzed by Northern blot hybridizations of polyadenylated RNA from the hem2-16 mutant N28 and from the parental respiratory-competent haploid strain D273-10B/A1. Polyadenylated RNA from each strain was separated by agarose gel electrophoresis, transferred to diazobenzyloxymethyl paper, and probed with nick-translated fragments from the coding regions of each gene. The results of the hybridizations revealed a marked reduction of the cytochrome c mRNA in the mutant compared with the wild type (Fig. 7), as expected from the known heme dependency of transcription of the CYC1 gene. A similar decrease was seen in the mRNA coding for the cytochrome oxidase subunit but not for the β subunit of F1 (Fig. 7). The observed differences of the mRNA levels in the wild type and N28 were reflected in the amounts of detectable protein products assayed immunologically by reaction of Western blots of total mitochondrial proteins with antisera raised against cytochrome c, subunit 5 of cytochrome oxidase, and the β subunit of ATPase (data not shown).

To quantitate the loss of transcriptional efficiency in the heme-deficient genetic background, the 5'-flanking and the amino-terminal coding regions of CYC1, ATP2, COX5, and COR1 were fused in-frame to the seventh codon of lacZ. Specific restriction fragments of each gene were ligated into the multiple cloning site of the appropriate integrative vector to form the gene fusions (Table III and Fig. 8). The recom-
The promoters of the four different genes were selected which contained a wild type α-galactosidase activity after growth for 30–40 generations in nonselective YPD medium. Stable transformants containing each gene fusion were crossed to C41/U1 an active NcoI site within the URA3 gene and used to transform W303-1A or W303-1B to uracil prototrophy and bo genes, each gene fusion was crossed to C41/U1 and p54 meiotic segregants with the URA3 and hem2 markers were selected.

The α-galactosidase activity of the integrated lacZ fusions in the two genetic backgrounds was measured in log phase cells grown in either 8% glucose (repressed) or 2% galactose (derepressed). Expression of α-galactosidase directed by the promoters of the four different PET genes was assayed by measuring hydrolysis of o-nitrophenyl-β-d-galactoside in permeabilized cells (27). The results of such assays are summarized in Table IV. In the respiratory-competent background (HEM2), α-galactosidase activity was higher in cells grown on the nonrepressing sugar for each of the four gene fusions. The extent of glucose repression was approximately 10-fold for CYC1, COR1, and COX5 but was only 2.5-fold for ATP2. These results are consistent with earlier observations on the severity of glucose repression of the parent enzymes; while glucose represses cytochrome oxidase and coenzyme QH2-cytochrome c reductase activity by a factor of 10–20 (39), the mitochondrial ATPase is subject to only a 2–3-fold repression (40). In the hem2 mutants, the degree of glucose repression is

![Northern hybridization analysis of polyadenylated RNA from wild type and hem2 strains.](image)

Fig. 7. Northern hybridization analysis of polyadenylated RNA from wild type and hem2 strains. Total yeast RNA isolated from the wild type (WT) parent D273-10B/A1 and from the hem2 mutant N28 was enriched for poly(A) RNA by passage through poly(U)-Sepharose 4B (Pharmacia LKB Biotechnology Inc.). Samples representing 10 µg of poly(A) RNA were separated on a 1% dodecylbenzylmethoxyethyl paper. The Northern blots were hybridized to nick-translated DNA probes containing portions of the coding regions from CYC1, COX5, and ATP2. The probe used for CYC1 was a 596-bp EcoRI-HindIII fragment containing 322 bp of coding sequence and 374 bp of 3′-flanking region (34). The COX5 probe was a PstI fragment of approximately 600 bp containing 302 bp of coding sequence and approximately 300 bp of 5′-flanking region (10). The ATP2 probe was a 400-bp BamHI-HindIII fragment containing 390 bp of coding sequence and 10 bp of 3′-flanking region (43). The COX5 probe detects two different transcripts, both of which are reduced in the hem2 mutant. We have no information concerning the difference in the two transcripts.

![Diagram](image)

FIG. 8. Fusion of lacZ to CYC1, COX5, ATP2 and COR1. The regions of CYC1, COX5, ATP2, and COR1 fused to the lacZ gene are shown by the solid lines (5′-flanking sequence) and the open bar (coding sequence). In each case the fusions were to the seventh codon of lacZ (dashed lines). Plasmid YlpC312 contains the 390-bp Smal(S)-EcoRI(E) fragment of CYC1 (34) in the integrative vector YIp356R (26). Plasmid YlpC2Z was constructed by ligating a 2.4-kb BamHI (B) fragment of ATP2 (12) to the BamHI site of Ylp353 (26). Plasmid YlpVZ has a 600-bp PstI(P) fragment with the COX5 (10) sequence cloned in Ylp356 (26). YlpC44Z was constructed by ligation of a 2.2-kb BamHI-KpnI(K) fragment with the COR1 sequence to the lacZ gene of Ylp356R (26). X, XhoI.

**Table IV**

| Strain Genotype          | Units of β-galactosidase |
|--------------------------|--------------------------|
|                          | Glucose | Galactose | Glucose | Galactose |
|                          | (+DP)   | (+DP)     | (+DP)   | (+DP)     |
| Wcz                     | HEM2, CYC1-lacZ           | 67     | 136     | 557     |
|                         | 65       | 111      | 36      | 371      |
| C41cz                   | hem2-15, CYC1-lacZ        | 2      | 10      | 15      |
|                         | 11       | 36       | 26      | 31       |
| Wbz                     | HEM2, ATP2-lacZ           | 212    | 590     | ND      | ND      |
|                         | 213      | 563     | ND      | ND      |
| C41Bz                   | hem2-15, ATP2-lacZ        | 193    | 523     | ND      | ND      |
|                         | 193      | 523     | ND      | ND      |
| Wvz                     | HEM2, COX5-lacZ           | 35     | 410     | 19      | 547     |
|                         | 35       | 410     | 19      | 547     |
| C41vz                   | hem2-15, COX5-lacZ        | 2      | 113     | 12      | 243     |
|                         | 2        | 113     | 12      | 243     |
| W44z                    | HEM2, COR1-lacZ           | 21     | 190     | 13      | 269     |
|                         | 21       | 190     | 13      | 269     |
| N2844Rz                 | hem2-10, COR1-lacZ        | 8      | 90      | 25      | 225     |
|                         | 8        | 90      | 25      | 225     |

**Table III**

| Gene   | Restriction fragment  | Upstream site | Downstream site | 5′-Flanking region (nucleotides) | Amino-terminal coding region (nucleotides) | Plasmid | Reference |
|--------|-----------------------|---------------|-----------------|---------------------------------|------------------------------------------|---------|-----------|
| CYC1   | SmaI                  | EcoRI         | 380             | 300                             | YEp356R                                 | 34      |           |
| COX5   | PstI                  | PstI          | 300             | 302                             | YEp356                                  | 10      |           |
| ATP2   | BamHI                 | BamHI         | 1260            | 1140                            | YEp353                                  | 12      |           |
| COR1   | BamHI                 | KpnI          | 1580            | 650                             | YEp356R                                 | 11      |           |

Construction of lacZ gene fusions
approximately the same as in wild type, although the specific activity of \( \beta \)-galactosidase is lower for all except the ATP2 fusion. When grown on galactose, the most significant reduction of \( \beta \)-galactosidase expression in the hem2 background is seen for the CYC1 fusion (greater than 60-fold), followed by the COR1 (20-fold) and COX5 (4-fold) fusions. The specific activity of \( \beta \)-galactosidase driven by the ATP2 promoter was nearly identical in the wild type and hem2 backgrounds, indicating that this gene is not regulated by heme.

That the effects seen were directly related to the absence of heme was confirmed by the \( \beta \)-galactosidase activity measured in hem2 mutants grown in the presence of the heme analogue deuteroporphyrin IX. This compound has been shown to stimulate transcription of CYC1, although it is not an intermediate in heme biosynthesis (5). The results shown in Table IV indicate that the lowered expression of \( \beta \)-galactosidase in the hem2 mutants can be overcome by the inclusion of deuteroporphyrin IX in the growth media. In the case of the CYC1 and the COX5 fusions, the \( \beta \)-galactosidase activity of hem2 mutants grown in galactose supplemented with the heme analogue is increased to approximately one-half the levels measured in wild type cells. Under the same conditions, the COR1 fusion expressed nearly identical levels of \( \beta \)-galactosidase in both the wild type and hem2 genetic backgrounds.

**DISCUSSION**

The respiratory defect of two independently isolated pet strains assigned to complementation group G32 has been attributed to mutations in HEM2, the structural gene of yeast ALA dehydratase. The mutants have reduced levels of cytochromes, are not complemented by a known hem2 mutant, and are deficient in ALA dehydratase. The ability of pet mutants to utilize glucose or galactose as a carbon source in the absence of added unsaturated fatty acids and ergosterol is surprising in view of previous studies (7) indicating that mutations in enzymes of the heme biosynthetic pathway prevent growth of such strains on both fermentable and nonfermentable substrates. The growth phenotype of the hem2 mutants reported here can be explained if one assumes that the lower sufficient heme to be made for sterol and fatty acid synthesis but not for respiration. This interpretation is consistent with the observation that a stringent mutation in HEM2 constructed by in situ disruption of the gene confers a requirement of ergosterol and unsaturated fatty acid (Tween 80) or of heme for growth on glucose.

To clone the HEM2 gene, C41/U1, a mutant carrying the hem2-15 allele, was transformed with a yeast genomic library. The recombinant plasmid pG32/T4, isolated for its ability to complement the hem2 mutation, was used to subclone the gene on a 1.3-kb HindIII fragment. The HindIII fragment was found to contain an open reading frame capable of coding for a protein of 38 kDa. The following evidence indicates that the reading frame codes for yeast ALA dehydratase. 1) The 1.3-kb HindIII fragment can complement two independent hem2 mutations. 2) Mutants with a disrupted copy of the gene lack ALA dehydratase and do not complement hem2 mutants. 3) Transformants harboring the gene on an autonomously replicating plasmid have 17 times higher ALA dehydratase activity than wild type yeast. 4) The primary sequence of the protein predicted from the nucleotide sequence of the gene is homologous to the recently reported sequence of human ALA dehydratase (30).

The CYC1 gene of \( S \). \textit{cerevisiae} is known to be transcriptionally regulated by glucose and by intracellular heme (4, 5, 35). The requirement of heme for transcription has been demonstrated with hem1 mutants blocked in heme biosynthesis due to lesions in the \( \delta \)-aminolevulinic synthetase, the first enzyme of heme biosynthesis (1). Such mutants show only low levels of transcription of CYC1 (5). Transcription of the gene can be restored to normal levels by supplementing the medium with \( \delta \)-aminolevulinic acid or with the heme analogue deuteroporphyrin IX (5). It was of interest to examine whether a similar requirement of heme exists for transcription of other nuclearly encoded components of the respiratory chain and affiliated phosphorylation system. Both subunits 5 of cytochrome oxidase and the 44-kDa core 1 subunit of coenzyme \( QH_2 \)-cytochrome c reductase are essential for the synthesis of the functional enzymes, although they have no electron carrier functions. The \( \beta \) subunit of \( F_1 \) is also an essential component of the mitochondrial ATP synthetase (41).

Estimations of the steady state concentrations of the mRNAs for the cytochrome oxidase subunit 5 in wild type and in a hem2 mutant have confirmed earlier evidence, based on measurements of translatable RNA, that transcription of the COX5 gene is regulated by heme (42). Similar analyses of mRNAs for the \( \beta \) subunit of \( F_1 \) showed no appreciable differences in wild type and the mutant strain. The requirement of heme for transcription of the COX5 and of the COR1 gene was substantiated by measurements of \( \beta \)-galactosidase activity in strains carrying chromosomally integrated fusions of the promoter regions of each gene to lacZ. The expression of lacZ driven by the COX5 promoter was approximately 4 times lower in the hem2 mutant than in wild type yeast when the two strains were grown on galactose. A similar effect of heme was seen when lacZ was fused to the upstream region of COR1; the absence of heme in this case caused a 20-fold lowering of \( \beta \)-galactosidase activity. These data suggest that transcriptional regulation by heme is not confined to genes whose products have a direct function in electron transport (e.g., hemoproteins) but may be a more general mechanism for modulating the synthesis of a broader spectrum of mitochondrial proteins of nuclear origin needed for the maintenance of a respiratory functional organelle. The effect of hem2 mutations on transcription of COX5 and CYC1 appears to be more severe based on the levels of the mRNAs detected by Northern hybridization than by measurements of the \( \beta \)-galactosidase activity expressed in the lacZ fusion assays. This apparent discrepancy is most likely due to the use of intensifying screens in the exposure of the Northern blots, a technique known to accentuate the signals of more radioactive bands due to a nonlinear response of the x-ray film.

The lacZ fusion experiments demonstrate that both COX5 and COR1 are additionally regulated by glucose. The expression of \( \beta \)-galactosidase in fusions to the upstream regions of the two genes was approximately 10 times greater when cells were grown in galactose than in glucose. An effect of comparable magnitude was seen for CYC1 whose product apo-\( \text{iso-1-cytochrome c} \) is known to be glucose-repressed (4, 5, 35). In each case, glucose inhibited \( \beta \)-galactosidase expression whether the fusions were integrated in the wild type or in the hem2 mutant, suggesting that glucose and heme exert their effects separately. An examination of the upstream regions of COX5 and COR1 failed to reveal sequences homologous to the UAS1 or UAS2 regulatory elements of CYC1 (4, 5). The identification of the regulatory elements responsible for controlling transcription of COX5 and COR1 will require deletion analysis of the 5'-flanking regions of these genes.

Somewhat different results were obtained when lacZ was fused to the upstream region of the ATP2 gene. First, the level of \( \beta \)-galactosidase of cells grown in galactose was 2-3 times higher than in cells grown in glucose. This is consistent with earlier observations that the mitochondrial ATPase activity of yeast grown under glucose-repressed or -derepressed
for expert technical assistance, to Dr. Edith Gollub for her generosity. Tially identical β-galactosidase activities were detected in the wild type and the hem2 mutant indicating that transcription of the ATP2 gene is probably not influenced by heme. Transcriptional regulation by heme therefore may be restricted to nuclear genes whose products are subunits of respiratory complexes.

Acknowledgments—We wish to express our gratitude to Anna Akai for expert technical assistance, to Dr. Edith Gollub for her generosity in providing us her collection of hem mutants of yeast, and to Dr. L. Guarante for helpful suggestions.

REFERENCES

1. Granick, S., and Beale, S. E. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 46, 33–203
2. Bard, M., Woods, R. A., and Haslam, J. M. (1974) Biochem. Biophys. Res. Commun. 56, 324–330
3. Saltzgaber-Muller, J., and Schatz, G. (1978) J. Biol. Chem. 253, 304–310
4. Guarante, L., and Mason, T. L. (1983) Cell 32, 1279–1296
5. Guarante, L., Lalonde, J. E., and Alani, E. (1984) Cell 36, 503–511
6. Miyake, S., and Sugimura, T. (1968) J. Bacteriol. 96, 1977–2003
7. Gollub, E. G., Liu, K., Dayan, J., Adlersberg, M., and Sprinson, D. B. (1977) J. Biol. Chem. 252, 2946–2954
8. Urban-Grimal, D., and Labbe-Bois, R. (1981) Mol. Gen. Genet. 183, 55–92
9. Cumesky, M. G., McEwen, J. E., Ko, C., and Poyton, R. O. (1983) J. Biol. Chem. 258, 13418–13421
10. Koerner, T. J., Hill, J., and Tzagoloff, A. (1985) J. Biol. Chem. 260, 9513–9515
11. Tzagoloff, A., Wu, M., and Crivellone, M. (1986) J. Biol. Chem. 261, 17163–17169
12. Saltzgaber-Muller, J., Kunapuli, S. P., and Douglas, M. G. (1983) J. Biol. Chem. 258, 11465–11470
13. Tzagoloff, A., Akai, A., and Fourny, F. (1976) FEBS Lett. 65, 391–396
14. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) J. Biol. Chem. 250, 8219–8235
15. Botstein, D., and Davis, R. W. (1982) in Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression (Strathern, J., N., Jones, E. W., and Broach, J. R., eds) pp. 607–636, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Dieckmann, C. L., and Tzagoloff, A. (1983) Methods Enzymol. 97, 355–360
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18. De Barreiro, O. L. C. (1967) Biochim. Biophys. Acta 139, 479–486
19. Urata, G., and Granick, S. (1963) J. Biol. Chem. 238, 811–820
20. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Maxam, A. H., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560–564
22. Myers, A. M., and Tzagoloff, A. (1985) J. Biol. Chem. 260, 15371–15377
23. Laughon, A., and Gesteland, R. F. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6827–6831
24. Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5350–5354
25. Schmidt, R. S., Richardson, C. B., Gillham, N. W., and Boynton, J. E. (1983) J. Cell Biol. 96, 1451–1463
26. Myers, A. M., Tzagoloff, A., Kinney, D. M., and Lusty, C. L. (1986) Gene (Amst.) 45, 299–310
27. Guarante, L. (1983) Methods Enzymol. 101, 181–191
28. Murphy, M. J., Siegel, L. M., Kamin, H., and Rosenthal, D. (1973) J. Biol. Chem. 248, 2891–2894
29. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) Yeast 2, 163–167
30. Wetmur, J. G., Bishop, D. F., Cantelmo, C., and Desnick, R. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7703–7707
31. Lingner, B., and Kleinschmidt, T. (1983) Z. Naturforsch. Teil C Biochem. Biophys. Biol. Virol. 38, 1099–1061
32. Rothstein, R. J. (1983) Methods Enzymol. 101, 202–212
33. Broach, J. R., Strathern, J. N., and Hicks, J. B. (1979) Gene (Amst.) 8, 121–133
34. Smith, M., Leung, D. W., Gillam, S., Astell, C. R., Montgomery, D. L., and Hall, B. D. (1977) Cell 16, 753–761
35. Zitomer, R. S., Montgomery, D. L., Nichols, D. L., and Hall, B. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3627–3631
36. Schatz, G., and Mason, T. L. (1974) Annu. Rev. Biochem. 43, 51–87
37. Van Loon, A. P. G. M., de Groot, R. J., van Eyk, E., van der Horst, G. T. J., and Grivell, L. A. (1982) Gene (Amst.) 20, 323–337
38. Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Tzagoloff, A. (1984) Mitochondria, Plenum Publishing Corp., NY
40. Tzagoloff, A. (1989) J. Biol. Chem. 244, 5027–5033
41. Senior, A. L., and Wine, G. (1983) J. Membr. Biol. 73, 105–124
42. Gollub, E. G., and Dayan, J. (1985) Biochem. Biophys. Res. Commun. 128, 1447–1454
43. Takeda, M., Vassarotti, A., and Douglas, M. G. (1985) J. Biol. Chem. 260, 15458–15465