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Complex Transcriptional Regulation of the *Saccharomyces cerevisiae CYB2* Gene Encoding Cytochrome b_2: CYP1(HAP1) Activator Binds to the *CYB2* Upstream Activation Site UAS1-B2

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Expression of the *Saccharomyces cerevisiae* gene encoding cytochrome b_2 (EC 1.2.2.3), *CYB2*, was investigated by direct analysis of mRNA transcripts and by measurement of the expression of *lacZ* fused to the *CYB2* control regions. These studies indicated that regulation of the *CYB2* gene is subject to several metabolic controls at the transcriptional level: inhibition due to glucose fermentation, induction by lactate, and inhibition in anaerobiosis or in absence of heme biosynthesis. Furthermore, we have shown that the *CYB2* promoter contains one *cis* negative regulatory region and two heme-dependent positive regions, one of which is controlled by the transcriptional regulator CYP1 (HAP1) which is involved in the modulation of the expression of several oxygen-regulated genes. The CYP1 (HAP1)-binding sequence was located by gel retardation and DNase I footprinting experiments and compared with the binding sequences previously characterized in detail ($UAS1_{CYC1}$, $UAS'_{CYP3\ (CYC7)}$, and UAS_{CTT1}).

Studies on the regulation of nuclear genes encoding mitochondrial proteins should provide a major contribution to our understanding of mitochondrial biogenesis in *Saccharomyces cerevisiae*. Several variables, including glucose fermentation, oxygen tension in the growth medium, and the availability of heme, are of particular interest because they all elicit significant changes in the levels of mitochondrial proteins of nuclear origin needed for the maintenance of a respiratory functional organelle. In this respect, the regulation of the expression of the respiratory components iso-1-cytochrome *c* (*CYC1*) and iso-2-cytochrome *c* (*CYP3* or *CYC7*) has been investigated (26, 32–34, 45). Detailed in vivo and in vitro studies of the upstream regions of both genes have demonstrated that their regulation is effected by positive elements which are known as upstream activation sites (UAS_S) (10, 21, 46). Within these elements, sequences have been identified which are the specific binding sites for characterized transcriptional activators (30). The transcriptional activator CYP1 (HAP1) (40) was shown to modulate the expression of *CYC1* and *CYP3* (*CYC7*) through an interaction with specific sequences: UAS1 for *CYC1* and UAS' for *CYP3* (*CYC7*) (30). The *CYP1* (*HAP1*) gene product could have a more general function as a coordinator of the activity of genes encoding proteins involved in various aspects of oxygen metabolism. Recently it has been shown that the expression of *CTT1* (encoding catalase T) and *HMG1* (encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase) is also mediated by the CYP1 (HAP1) transcriptional regulator (39, 44). In vivo *CYP1*-dependent UAS_S activity was found to be induced by heme (9). By using extracts, CYP1 (HAP1) binding to specific target sequences has been shown to be induced by hemin in vitro (12, 20, 30), suggesting that heme could serve directly as a ligand as steroids do for some hormone receptors in mammals (1a). The *CYP1* (*HAP1*) gene has been cloned, and its sequence has been determined (7, 29, 41). It encodes a protein of 1,483 amino acid residues which can be divided into three functional domains (7, 17, 18, 29, 42). At the N terminus there is a zinc finger domain which is involved in binding to specific DNA sequences (UAS_S). At the carboxyl end there is an acidic region necessary for transcriptional activation. Finally, in the middle of the protein, a distinct region which mediates heme induction has been characterized.

Yeast L-(+)-lactate cytochrome *c* oxidoreductase, or cytochrome b_2, is a soluble protein from the intermembrane space of mitochondria (13). It catalyzes the transfer of electrons from L-(+)-lactate to cytochrome *c*, which involves the formation of a specific complex between the two molecules (2, 19). At present, it is not clear whether cytochrome b_2 interacts preferentially with iso-1- or iso-2-cytochrome *c* during the electron transfer process. The level of cytochrome b_2 activity measured in crude extracts has been shown to be subject to respiratory adaptation (23, 34), and it was observed by Clavilier et al. (5) that the presence of the mutation *CYP1-18* considerably diminished cytochrome b_2 activity in the cell. Recently, the *CYB2* gene has been isolated and sequenced (12).

In this work we investigate the regulation of *CYB2* expression by direct analysis of mRNA transcripts and by the measurement of the expression of *lacZ* fused to the *CYB2* control regions. We have also analyzed how the *CYP1* (*HAP1*) product, identified as a positive transactive factor (8, 39, 41, 43, 44), exerted its control on the expression of the *CYB2* gene. In particular, we have identified the region upstream of the *CYB2* transcriptional start site that mediates *CYP1* (*HAP1*) activity. The implications of these findings for preferential transcriptional coordination between *CYB2* and *CYC1* are discussed.

MATERIALS AND METHODS

Bacterial and yeast strains. *Escherichia coli* TG1 [Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZM15*] and

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Dh5αF'[*endA1 hsdR17*(r_K m_K^+) *supE44 thi-1* λ⁻ *recA1 gyrA96 relA1* φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)*U169*] were used for plasmid propagation and maintenance. The *S. cerevisiae* strains used in this study were D261 (*MATa*/*MATα* prototroph) from L. Clavilier (Gif-sur-Yvette, France), FJ11-1B (*MATα his ade1 met cyp1-23*) and VP209-7B (*MATα ura3 leu2 cyc1-1*) from J. Verdière (Gif-sur-Yvette, France), Ole3-2b (*MATa hem1-12 gal*) from R. Labbe (Paris), and VP209-7B ΔCYP1 (*MATα ura3 cyc1-1 cyp1::LEU2*), VP209-7B ΔHEM1 (*MATα ura3 cyc1-1 hem1::LEU2*), and DBY-U4 (*MATα* Δ*his3 leu2 3-112 trp1-25 cyb2::URA3*) which were constructed in this laboratory.

Growth conditions. Yeast cells were grown on complete medium consisting of 2% yeast extract, 1% Bacto-Peptone (Difco Laboratories), and a carbon source: 10% glucose (repressed conditions or 2% galactose, 2% raffinose, 2% ethanol, or 2% lactate (unrepressed conditions). Minimal medium (0.67% yeast nitrogen base without amino acids), contained carbon sources as indicated above, the required amino acids at 40 μg/ml, and adenine or uracil at 20 μg/ml.

Transformation. *E. coli* was transformed by the method of Mandel and Higa (24). Yeast transformation was carried out by the LiCl procedure of Ito et al. (16).

DNA manipulation. Restriction endonucleases, T4 DNA ligase, alkaline phosphatase from calf intestine, DNA polymerase I, the large fragment from *E. coli*, DNase I, and T4 polynucleotide kinase were obtained from Biolabs and Boehringer and used in accordance with the supplier's recommendations. DNA sequencing was performed by the method described by Maxam and Gilbert (27). Restriction fragments were 5′ end labelled with T4 polynucleotide kinase (25).

Oligonucleotide synthesis. Oligonucleotides were synthesized with a model 7500 DNA synthesizer (Milligen) and purified by gel electrophoresis. The sequences of synthetic DNA fragments used as competitors in protein-DNA binding assays are shown in Fig. 8.

DNA preparation. Bacterial plasmids were prepared by an alkaline lysis protocol (25).

Electrophoresis of RNA, transfer to nitrocellulose sheets, and hybridization. Total RNA prepared by the method described by Maccechini et al. (23) was subjected to agarose gel electrophoresis in 50 mM boric acid–5 mM borate–10 mM sodium sulfate–5 mM methyl-mercuric hydroxide. After transfer to nitrocellulose filters, the RNA was hybridized with nick-translated DNA probes (38). In order to normalize for the amounts of mRNA, the same blots were rehybridized with an actin (*ACT1*) probe, which provides a reliable standard.

Deletion experiments in the 5′-flanking region of *CYB2*. The 5-kb *Hin*dIII fragment from the recombinant plasmid pGB5C2R5 (12), which contains the entire *CYB2* gene plus 2 kb of its 5′-flanking region, was subcloned into the shuttle vector YEp351 (14) and pUC19 (47) to give the recombinant plasmids YEp2000 and pGB2000, respectively. Deletions in the 5′-flanking region of the *CYB2* gene were obtained by digestion of plasmid pGB2000 with *Hin*dIII and partial digestion with *Bgl*II, *Nhe*I, *Eco*RV, or *Sal*I. The obtained fragments (−446 to +2500, −250 to +2500, −230 to +2500, −137 to +2500, and −91 to +2500) were subcloned into restriction sites of the multiple cloning region of the shuttle vector YEp351 to give the recombinant plasmids YEp446, YEp250, YEp230, YEp137, and YEp91, respectively.

Western immunoblot analysis. Western blots were carried out as described by Guiard (12). Proteins were separated electrophoretically on 9% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and then analyzed by immunoblotting with polyclonal antibodies against the cytochrome b_2 protein.

Identification of *cis*-acting regulatory elements involved in the transcriptional control of *CYB2*. DNA subfragments of different lengths from the region −446 to −91 were obtained by using the restriction sites present (*Bgl*II, *Nhe*I, *Eco*RV and *Sal*I) and cloned into the multiple cloning site polylinker of the plasmid pUC19. They were recovered by cutting with *Sma*I and *Sal*I enzymes and were then ligated into the *Sma*I and *Xho*I sites of the *CYC1-lacZ* fusion vector pLG669Z (9) in place of the *CYC1* upstream activating sequences, as shown in Fig. 4. The DNA fragment which covers positions −250 to −200 of the *CYB2* promoter and which is flanked by *Sma*I and *Sal*I restriction sites was synthesized.

Enzyme assays. β-Galactosidase activity was assayed in yeast cells permeabilized with chloroform and sodium dodecyl sulfate as previously described by Guarente et al. (11). For each plasmid, a minimum of two transformants was assayed in triplicate, and values representing an average of three experiments (performed on separate days) were reported and expressed in Miller units (11).

Probes for electrophoresis DNA binding assays and footprinting. *CYB2* promoter fragments were derived from the plasmid pGB300, which contains the 309-bp *Bgl*II (−446 to −137) upstream region cloned into the *Bam*HI site of the polylinker of pUC19. Probes were prepared by digestion with *Eco*RI or *Hin*dIII and end labelling with polynucleotide kinase and [γ^{32}P]ATP by standard methods (25). Probes were then digested with *Hin*dIII or *Eco*RI to obtain the 309-bp fragment labelled at the opposite end; alternatively, DNA was digested with *Eco*RV to obtain the 216-bp (*Eco*RI-*Eco*RV) or the 93-bp (*Eco*RV-*Hin*dIII) fragment or with *Nhe*I to obtain the 114-bp (*Nhe*I-*Hin*dIII) fragment. Probes were then purified by polyacrylamide gel electrophoresis.

Preparation of yeast extracts for the binding assay. Cells were grown to an A_{600} of 2 and then harvested by centrifugation, and extracts were prepared as described by Pfeifer et al. (30).

Protein-DNA binding assay. The gel retardation assays were carried out as described in reference 30 with several modifications. γ-^{32}P-labelled fragments were incubated with crude cell extract (10 to 20 μg of protein) in 20 μl of 4 mM Tris HCl (pH 8)–15 mM NaCl–5% glycerol–0.5 mM mercaptoethanol–0.5 mM phenylmethylsulfonyl fluoride–0.05% bromophenol blue and with 1 to 10 μg of sonicated salmon sperm DNA as a nonspecific competitor. Reactions were carried out at 22°C for 10 min. Where it is indicated, hemin (final concentration, 40 μM), $MgCl_2$ (final concentration, 5 mM), or an oligonucleotide (100 ng) as specific competitor DNA was added. Protein-DNA complexes were loaded onto a 5% preelectrophoresed polyacrylamide gel (acrylamide-to-bisacrylamide weight ratio of 39:1). The gel was run in buffer containing 45 mM Tris HCl, 40 mM H_3BO_3, and 1.25 mM EDTA for 1 to 2 h at 20 mA at room temperature. Afterwards, gels were transferred to Whatman 3 MM paper, dried, and autoradiographed.

DNase I footprinting. DNase I protection experiments were performed by the method described by Pfeifer et al. (30) with several modifications. DNA-protein complexes, treated with 20 μg of DNase I per ml for 30 min were resolved on 5% polyacrylamide gels as described above and visualized by autoradiography of the wet gel at 4°C overnight. Bands corresponding to free and complexed DNA were excised, and DNA was eluted by shaking overnight at 60°C in 3 ml of buffer A (50 mM Tris HCl [pH 8], 10 mM

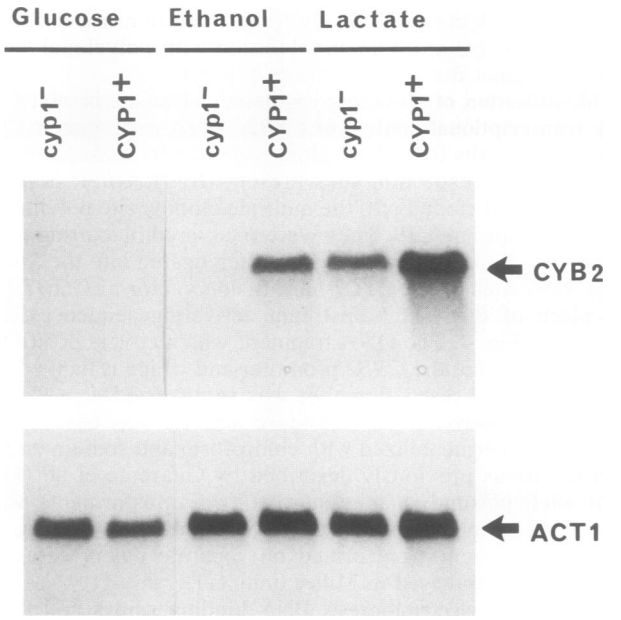


FIG. 1. Northern blot analysis of *CYB2* transcripts from *CYP1*$^+$ and *cyp1* mutant cells. Cells from strains D261 (*CYP1*$^+$) and FJ11-1B (*cyp1-23*) were grown in rich medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto-Peptone) plus 10% glucose, 2% ethanol, or 2% lactate to 1 or 2 generations before stationary phase. Total cellular mRNA was prepared by the method of Maccechini (23), and hybridization was carried out using the nick-translated 1.2-kb *Bam*HI-*Bgl*II fragment from plasmid pGB5C2H2 as a probe (12). Each lane contained 20 μg of total mRNA. The lower panel represents the same blot rehybridized with an actin (*ACT1*) probe.

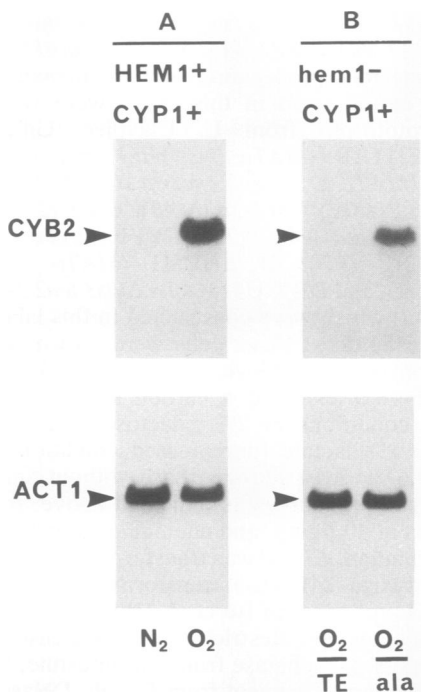


FIG. 2. *CYB2* transcription is activated by oxygen and heme. (A) Total cellular mRNA from D261 was prepared from aerobically (O_2) grown cells derepressed by growth in rich medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto-Peptone) plus 2% galactose to 1 or 2 generations before stationary phase. Anaerobic conditions (N_2) were achieved by bubbling argon through the cultures. Before harvesting, the anaerobic cultures were poisoned with 0.2 mg of cycloheximide per ml and chilled on ice for 30 min while still under argon. (B) Total cellular mRNA from the *hem1* strain Ole3-2b was prepared from cells derepressed by growth in rich medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto-Peptone) plus 2% raffinose in the presence of 0.2% Tween 80 and 30 μg of ergosterol per ml (TE) or in the presence of 30 μg of δ-aminolevulinic acid per ml (ala). Hybridization was carried out by using the nick-translated 1.2-kb *Bam*HI-*Bgl*II fragment from plasmid pGB5C2H2 as a probe (12). Each lane contained 20 μg of total mRNA. Lower panels represent the same blots reprobed with an actin (*ACT1*) probe.

EDTA, 100 mM NaCl) with 0.5 μg of sonicated salmon sperm DNA per ml. Extracted DNA was purified by absorption to Elutip-d columns (Schleicher and Schuell), eluted with 0.4 ml of buffer A containing 1 M NaCl, and ethanol precipitated. The DNA was then heated at 90°C for 5 min in denaturing buffer, chilled in ice, and loaded onto a 6% polyacrylamide denaturing gel (urea 6 M, TBE). DNA sequencing was performed by the method of Maxam and Gilbert (27).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to EMBL and GenBank and assigned accession number X03215.

RESULTS

Effect of carbon sources on *CYB2* mRNA synthesis. The level of cytochrome b_2 activity in *S. cerevisiae* is mainly related to growth conditions and to the effects of different carbon sources (35). To determine whether this regulation affects the *CYB2* mRNA level, we performed Northern (RNA) blot experiments with mRNA extracted from cells grown under different conditions. The wild-type strain D261 (*CYP1*$^+$) was used, and the *CYB2* mRNA was identified by using the nick-translated *Bam*HI-*Bgl*II (+490 to +1668) fragment containing a large internal *CYB2* coding sequence (12). The results presented in Fig. 1 show that the *CYB2* mRNA level is too low to be detectable when glucose is used as carbon source. *CYB2* mRNA synthesis is derepressed in aerobic cells grown on ethanol or lactate.

***CYB2* transcription is activated by oxygen and heme.** The amount of *CYB2* transcript was also investigated with respect to anaerobiosis and aerobiosis with cells grown on galactose. The results are illustrated in Fig. 2A and show that *CYB2* transcripts from the wild-type strain D261 cannot be detected under anaerobic conditions, whereas they accumulate in aerobic conditions. It has also been observed that heme exerts a positive control over the synthesis of many heme-containing proteins (15). To examine the effect of heme on expression of the *CYB2* gene we used the strain Ole3-2b that carries a mutation in *HEM1*, the structural gene for δ-aminolevulinic acid synthetase (the first enzyme in the heme biosynthesis pathway), and is consequently deficient in heme biosynthesis and respiration. Our analysis was done with cells grown on raffinose. Under these conditions (Fig. 2B), the *hem1* mutation blocks *CYB2* transcription, and the addition of δ-aminolevulinic acid (ala) to the growth medium overcomes this effect.

The *CYP1* (*HAP1*) locus controls *CYB2* transcription. Mutations at the *CYP1* (*HAP1*) locus have been observed to affect cytochrome b_2 activity (5). To determine whether this gene plays an important role in the regulation of the *CYB2* gene, a strain carrying the mutation *cyp1-23* at the *CYP1*

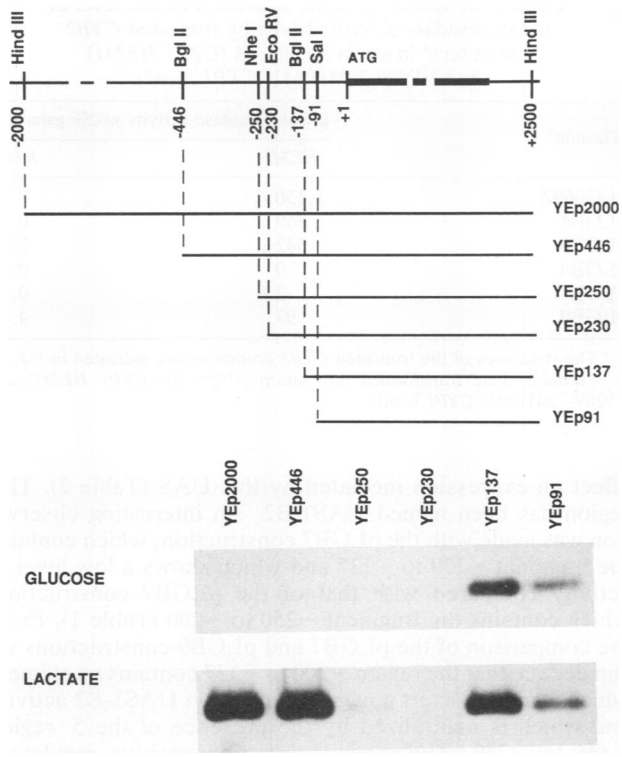
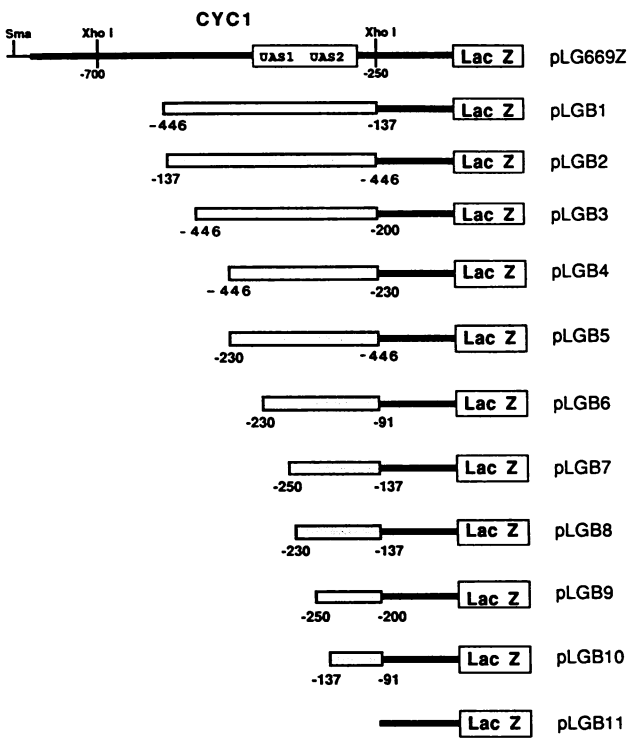


FIG. 3. Deletion analysis of *CYB2* promoter region. 5′ deletions in the upstream region of *CYB2* were constructed by using several restriction sites (*Hin*dIII, *Bgl*II, *Nhe*I, *Eco*RV, and *Sal*I) present in the 5′ end of the *CYB2* gene. Each construction was inserted into the shuttle vector Yep351 (14). Transformed cells (strain DBY-U4) carrying a chromosomal deletion of the *CYB2* gene (12) were grown in minimal medium plus 10% glucose or 2% lactate. Proteins were extracted and analyzed by immunoblotting with a polyclonal antibody preparation against the cytochrome b_2 protein (12).



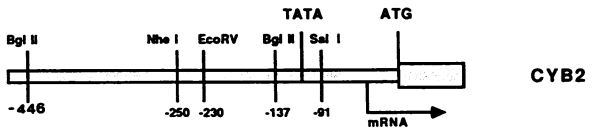


FIG. 4. Identification of *cis*-acting regulatory elements involved in the transcriptional control of *CYB2*. The DNA fragments obtained from the 5′ end region of *CYB2* (between −460 and −90) were tested for their ability to mediate gene expression by using them to replace the *CYC1* UASs in the plasmid pLG669Z (9). The resulting plasmid constructions were used to transform yeast strains. As shown in Table 1, the transformed yeast strains VP209-7B (*CYP1*$^+$ *HEM1*$^+$) and VP209-7B ΔCYP1 (*cyp1* mutant *HEM1*$^+$) were grown in minimal media supplemented with 10% glucose or 2% ethanol or 2% ethanol plus 2% lactate. The yeast strains VP209-7B (*CYP1*$^+$ *HEM1*$^+$) and VP209-7B ΔHEM1 (*CYP1*$^+$ *hem1* mutant), described in Table 2, were grown in minimum media supplemented with 2% galactose or with 2% galactose plus 30 μg of ergosterol per ml and 0.2% Tween 80, respectively. β-Galactosidase activity was assayed as described in Materials and Methods.

(*HAP1*) locus (43) was used, and the level of *CYB2* transcripts has been investigated by Northern blotting. The results of these experiments are presented in Fig. 1 and show that the level of *CYB2* mRNA in this genetic context and especially under ethanol growth conditions is significantly reduced. Nevertheless, with the *cyp1-23* mutant, the transcriptional induction by lactate is conserved but the wild-type level of mRNA is not attained.

Deletion analysis of the upstream region of the *CYB2* gene. The deletions in the upstream region of *CYB2* are illustrated in Fig. 3 and were produced as described in Materials and Methods. Cells carrying a deletion of the chromosomal *CYB2* gene were transformed with plasmids which carried various deletions and were then grown in the presence of glucose or lactate. The level of expression of the cytochrome b_2 protein was determined by Western blotting. The results show that the deletion of DNA sequences upstream of −446 does not affect *CYB2* expression. The catabolic repression by glucose and the derepression by lactate (or ethanol [data not shown]) is retained. Deletions between −446 and −91 seem to eliminate all control. In the constructions Yep137 and Yep91, *CYB2* expression becomes constitutive, but with a higher level in Yep137 where the putative TATA box element is conserved (12). As a result of the data obtained in this experiment, further studies will be focused on the upstream region between −460 and −91.

Identification of *cis* regulatory regions in the *CYB2* promoter. Important sequences for the control of gene expression are often dispersed in the region upstream from the transcription initiation site. The transcriptional start site for the *CYB2* gene has been located between positions −34 and −44 upstream of the ATG site (12). On the basis of the experiment described above (Fig. 3), we constructed a set of plasmids containing DNA subfragments of different lengths from the region −446 to −90 which had been cloned into a *CYC1-lacZ* fusion vector in place of the *CYC1* upstream activating sequences (UASs) in order to define regions upstream of *CYB2* required for its expression (Fig. 4). The resulting plasmids were transformed into the wild-type strain VP209-7B (*CYP1*$^+$), and transformants were assayed for β-galactosidase activity under different metabolic conditions (Table 1). Mutant strains were constructed in which the

TABLE 1. Effects of carbon sources and the *CYP1* locus on the level of β-galactosidase driven by truncated *CYB2* promoters[a] in strain VP209-7B (*CYP1 HEM1*)

Plasmid[b]	β-Galactosidase activity on the following carbon source:		
	Glucose (10%)	Ethanol (2%)	Ethanol (2%) and lactate (2%)
pLG669Z	284	840	1,280
pLGB1	12	460	1,400
pLGB2	18	446	1,428
pLGB3	127	465	1,200
pLGB4	4	46	170
pLGB5	2	4	8
pLGB6	0.2	0.2	0.3
pLGB7	2.7	4	15
pLGB8	0.2	0.3	0.3
pLGB9	37	147	380
pLGB10	0.3	0.3	0.3
pLGB11	0.1	0.2	0.2

[a] The structures of the truncated *CYB2* promoters are indicated in Fig. 4.
[b] Plasmids were transformed into strain VP209-7B (*CYP1 HEM1*).

CYP1 (*HAP1*) gene and the *HEM1* gene (encoding δ-aminolevulinic acid synthetase) were partly deleted and disrupted with DNA fragments containing the *URA3* or a *LEU* gene, respectively. The hybrid promoters, which displayed a substantial level of expression in the wild-type strain, were analyzed in these two new genetic contexts. The effects of the *cyp1* and *hem1* mutations are presented in Table 2 and 3, respectively. Data obtained from these experiments demonstrate that the fragment −446 to −137 inserted into the pLGB1 construction permits all the regulatory effects already revealed by the mRNA analysis. This hybrid promoter presents high sensitivity to the growth conditions and is strongly affected by the *cyp1* and the *hem1* mutations. In unrepressed galactose conditions the effect of the *hem1* mutation is stronger than that of the *cyp1* mutation. In addition, this sequence can be inverted without an impairment of its activity (pLGB1 and pLGB2 constructions). The removal of the region −200 to −137 (pLGB3 construction) decreases only the glucose repression effect in the wild-type strain, compared with the pLGB1 or pLGB2 construction. The same experiments performed with the constructions pLGB9 and pLGB4 allow the characterization of two *cis*-acting positive regulatory elements. The first is in a DNA fragment of 50 bp from the pLGB9 construction; it requires heme biosynthesis to maintain its activating property (Table 3), and disruption of the *CYP1* (*HAP1*) gene exerts a major effect on expression mediated by this UAS (Table 2). This region has been named UAS1-B2. An interesting observation was made with the pLGB7 construction, which contains the fragment −250 to −137 and which shows a low level of activity compared with that of the pLGB9 construction, which contains the fragment −250 to −200 (Table 1). From the comparison of the pLGB7 and pLGB9 constructions we can deduce that the region −200 to −137 contains an element which is able to exert a negative effect on UAS1-B2 activity and which is neutralized by the presence of the 5′ region −446 to −250. The second *cis*-acting positive regulatory element, UAS2-B2, is located in a wide region between −230 and −446 (pLGB4 construction). Its activating properties are related to the growth conditions (repression with glucose utilization, derepression with ethanol, and induction with lactate compared with ethanol) and are affected by the absence of heme biosynthesis but are independent of the product of *CYP1* (*HAP1*) gene (Tables 1, 2, and 3).

TABLE 2. Effects of carbon sources and the *CYP1* locus on the level of β-galactosidase driven by truncated *CYB2* promoters[a] in strain VP209-7B ΔCYP1 (*cyp1 HEM1*)

Plasmid[b]	β-Galactosidase activity on the following carbon source:		
	Glucose (10%)	Ethanol (2%)	Ethanol (2%) and lactate (2%)
pLG669Z	7.8	381	387
pLGB1	0.9	49	200
pLGB3	2.4	61	214
pLGB4	2.2	48	220
pLGB7	0.6	0.7	1.5
pLGB9	2.2	5	7

[a] The structures of the truncated *CYB2* promoters are indicated in Fig. 4.
[b] Plasmids were transformed into strain VP209-7B ΔCYP1 (*cyp1 HEM1*).

TABLE 3. Effects of heme starvation on the level of β-galactosidase activity driven by truncated *CYB2* promoters[a] in strain VP209-7B (*CYP1 HEM1*) and VP209-7 ΔHEM1 (*CYP1 hem1*)

Plasmid[b]	β-Galactosidase activity at 2% galactose	
	HEM1	*hem1*
pLG669Z	1,150	8
pLGB1	569	0.5
pLGB3	542	2.9
pLGB4	9	0.8
pLGB7	2.6	0.9
pLGB9	97	4.6

[a] The structures of the truncated *CYB2* promoters are indicated in Fig. 4.
[b] Plasmids were transformed into strain VP209-7B (*CYP1 HEM1*) and VP209-7 ΔHEM1 (*CYP1 hem1*).

Localization of the binding site of the *CYP1* (*HAP1*)-dependent complex. The binding of proteins to the labelled 323-bp *Bgl*II fragment of the *CYB2* promoter was analyzed in a *CYP1*$^+$ and *cyp1* mutant context by using a DNA gel retardation assay. When a crude cell extract of the wild-type strain (grown on 2% galactose) was tested, several complexes were observed (Fig. 5A and B). We attribute the retardation band with the higher molecular weight to a specific *CYP1* (*HAP1*)-dependent complex by virtue of the following arguments.

(i) This retardation band is absent when a *cyp1*-negative cell extract is tested (Fig. 5).

(ii) Specific competition is observed when an unlabelled DNA fragment containing the UAS1 region of *CYC1* (Fig. 5) [whose sequence has been identified by Pfeifer et al. (30) as a target of the *CYP1* (*HAP1*) product] is added.

(iii) The binding is strongly increased when heme is added to the incubation mixture. This stimulation by heme in vitro is a property of the formation of *CYP1* (*HAP1*)-dependent complexes and has been demonstrated for the interactions of *CYP1* (*HAP1*) with the UAS1 of *CYC1* (20), with the UAS′ of *CYP3* (*CYC7*) (30), and with the UAS_{CTT1} of *CTT1* (44). The amount of *CYP1* (*HAP1*)-dependent complex is increased by the addition of $MgCl_2$ and heme to the incubation mixture; however, $MgCl_2$ alone appears to have no effect. The Mg^{2+} ions could contribute to the interaction or could stabilize the complex in the presence of heme by an independent or synergistic effect. In subsequent experiments, the incubation mixture always contained $MgCl_2$ (5 mM) in addition to heme.

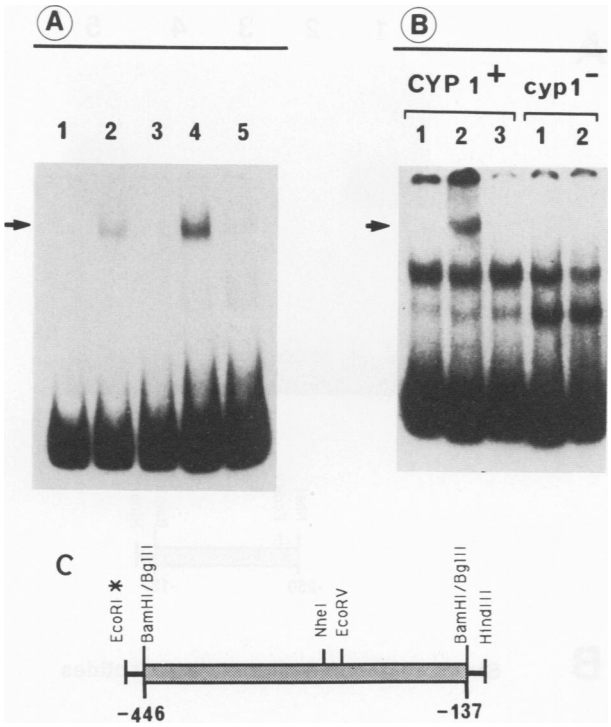


FIG. 5. CYP1 protein binding to the 323-bp *Bgl*II fragment of the CYB2 promoter. (A) The labelled 323-bp fragment was incubated with crude yeast extract prepared from VP209-7B (*CYP1*$^+$) cells grown in rich medium (1% [wt/vol] yeast extract, 1% [wt/vol] Bacto-Peptone) plus 2% galactose. Binding reactions were carried out in the absence (lane 1) or in the presence (lane 2) of heme (40 μM). The effect of the addition of $MgCl_2$ (5 mM) (lane 3) was also tested. Lanes 4 and 5 show the result of a binding reaction when both heme (40 μM) and $MgCl_2$ (5 mM) were added in the absence or in the presence of 100 ng of unlabelled oligonucleotide containing the $UAS1_{CYC1}$ sequence, respectively. The arrows indicate the retardation band corresponding to the *CYP1* (*HAP1*)-dependent complex. (B) DNA retardation experiments with crude yeast extracts from *CYP1*$^+$ and *cyp1* mutant cells were carried out in the absence (lane 1) or in the presence (lane 2) of heme (40 μM) and $MgCl_2$ (5 mM). Lane 3 shows the competition by the unlabelled oligonucleotide containing the $UAS1_{CYC1}$ sequence in the presence of heme (40 μM) and $MgCl_2$ (5 mM). (C) Restriction map of the 323-bp fragment of the *CYB2* upstream sequence is shown. The probe was prepared by digesting with *Eco*RI, labelling with polynucleotide kinase and [γ-^{32}P]ATP, and cutting with *Hin*dIII. The oligonucleotide sequence containing $UAS1_{CYC1}$ is described in Fig. 8.

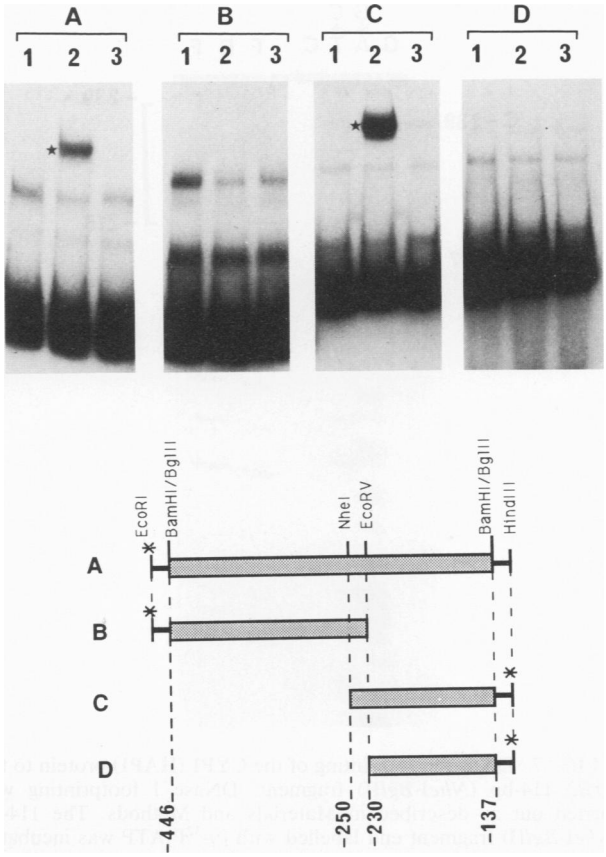


FIG. 6. Localization of the *CYP1* (*HAP1*)-dependent complex binding to *CYB2* promoter. DNA binding was carried out with crude yeast extract from VP209-7B (*CYP1*$^+$) cells grown in rich medium plus 2% galactose. Asterisks indicate the retardation band corresponding to the *CYP1* (*HAP1*)-dependent complex. The labelled DNA fragments used are the 323-bp *Bgl*II fragment (A), the 230-bp *Bgl*II-*Eco*RV fragment (B), the 114-bp *Nhe*I-*Bgl*II fragment (C), the 94-bp *Eco*RV-*Bgl*II fragment (D). Lanes 1, without heme and $MgCl_2$; lanes 2, with heme (40 μM) and $MgCl_2$ (5 mM) added; lanes 3, competition with unlabelled oligonucleotide containing the $UAS1_{CYC1}$ sequence in the presence of heme (40 μM) and $MgCl_2$ (5 mM). On the bottom of the figure a restriction map of fragments and the labelling sites is shown. Probes were derived from pUC19 containing the 323-bp *Bgl*II region of the *CYB2* promoter. DNA was digested by *Eco*RI or *Hin*dIII and end labelled with polynucleotide kinase and [γ-^{32}P]ATP. Different fragments were obtained by cutting the DNA with *Hin*dIII, *Eco*RV, or *Nhe*I. Each experiment with the different fragments was independently performed. The distance migrated by the probes does not appear to be in correlation with their own molecular weight. The oligonucleotide sequence containing $UAS1_{CYC1}$ is described in Fig. 8.

To localize the *CYP1* (*HAP1*)-dependent protein-binding site, crude extracts of strain VP209-7B (*CYP1*$^+$) were prepared and tested for their ability to form complexes with the labelled *CYB2* promoter fragments. As shown in Fig. 6 with the two fragments *Bgl*II (323 bp) and *Nhe*I-*Bgl*II (114 bp), a prominent retarded band is observed in the presence of heme which is unable to compete with an excess of unlabelled synthetic oligonucleotide which covers the UAS1 sequence of the *CYC1* gene. When the 230-bp (*Bgl*II-*Eco*RV) or the 94-bp (*Eco*RV-*Bgl*II) labelled fragment was used, we could not observe the *CYP1* (*HAP1*)-dependent protein binding. These results suggest that the site of binding is localized around the *Eco*RV restriction site; cutting by *Eco*RV disrupts the binding sequence and so prevents complex formation. This analysis is in agreement with the results obtained previously, which show that the fragment −250 to −200 is able to activate the transcription in *cis* in a *CYP1* (*HAP1*)-dependent manner.

The protected DNA region in the *CYP1* (*HAP1*)-dependent complex was determined by DNase I footprinting experiments. As shown in Fig. 7, a 22-bp protected region (−239 to −218) is observed. This sequence covers the *Eco*RV site, as was predicted by DNA retardation experiments. To corroborate this result, oligonucleotides which contained strictly the *CYP1* (*HAP1*)-binding site from the *CYC1*, *CYP3* (*CYC7*), and *CYB2* promoters were synthesized and used as specific competitors in the interaction between the *CYP1* product and the labelled DNA fragment

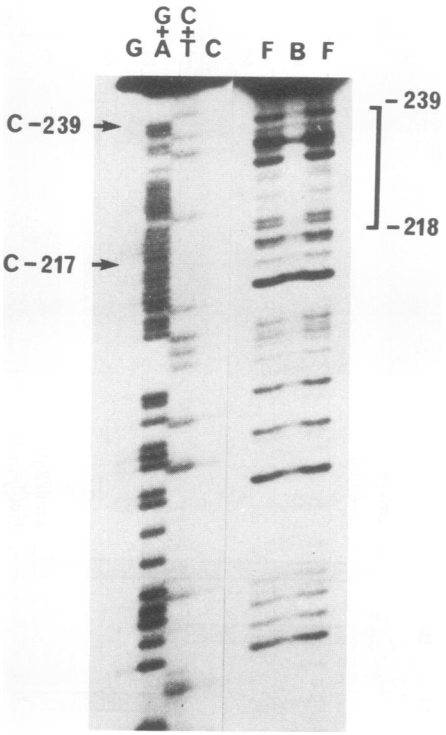


FIG. 7. DNase I footprinting of the CYP1 (HAP1) protein to the *CYB2* 114-bp (*Nhe*I-*Bgl*II) fragment. DNase I footprinting was carried out as described in Materials and Methods. The 114-bp (*Nhe*I-*Bgl*II) fragment end labelled with [γ-^{32}P]ATP was incubated with a crude yeast extract of *CYP1*$^+$ cells in the presence of heme (40 μM) and $MgCl_2$ (5 mM) and treated with DNase I. Free (F) and bound (B) DNA were separated on a 5% polyacrylamide gel, eluted, and then analyzed on a standard 6% sequencing gel by the method of Maxam and Gilbert (27). The protected sequence (−239 to −218) is bracketed.

*Nhe*I-*Bgl*II (−250 to −137). These three oligonucleotides are effective competitors of UAS1-B2 (Fig. 8, lines 2, 3, and 4). In this experiment, an oligonucleotide with a sequence unrelated to that of the CYP1 (HAP1)-binding site (Fig. 8, line 5) was used as a negative control and shows that the competition observed with the three binding sites is specific.

DISCUSSION

In this paper we have shown that the carbon and oxygen sources are the major regulatory factors which determine the steady-state level of *CYB2* mRNA. Growth of cells in high concentrations of glucose or in the absence of oxygen, deficiency in heme biosynthesis, and mutations affecting the *CYP1* (*HAP1*) locus have similar effects on the synthesis of cytochrome b_2: the steady-state level of its mRNA is strongly reduced. What is the precise relationship between these factors? The mechanism(s) by which growth on glucose causes repression of many genes is unknown, but genetic analyses of glucose repression done in recent years have identified several genes involved in this general regulatory system which affect a large number of genes besides those encoding apoheme proteins (3). Mutations in these genes should be tested for their effect on *CYB2* regulation. Anaerobic regulation could be reduced to the single parameter of intracellular heme concentration, as has been proposed by Guarente et al. (9). In that case the heme concentration could reflect the relative level of oxygen available to cells. It has been demonstrated that the transcription of *CYC1* is activated by the *CYP1* (*HAP1*) product in response to this inducer. In this study, we found that the *CYP1*

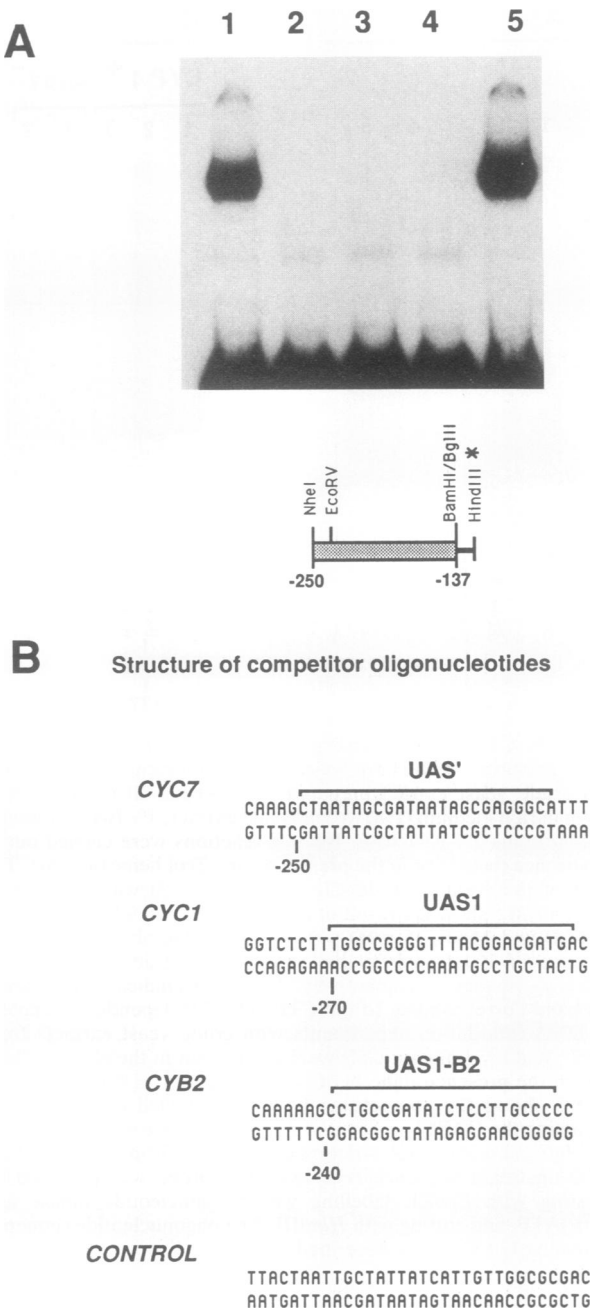


FIG. 8. Competition analysis using the labelled DNA fragment −250 to −137 containing the CYP1 (HAP1) binding site. (A) Gel retardation experiments were done in the presence of heme (40 μM) and $MgCl_2$ (5 mM). Lane 1, retardation band corresponding to the *CYP1* (*HAP1*)-dependent complex in the absence of competitor; lanes 2, 3, and 4, competition with 100 ng of cold oligonucleotide covering UAS1-B2$_{CYB2}$, UAS1$_{CYC1}$, and UAS′$_{CP3\ (CYC7)}$, respectively; lane 5, competition with 100 ng of competitor oligonucleotide unrelated to a CYP1 (HAP1) binding site (control). (B) Structure of the synthetic DNA fragments used as competitors in protein DNA binding assays.

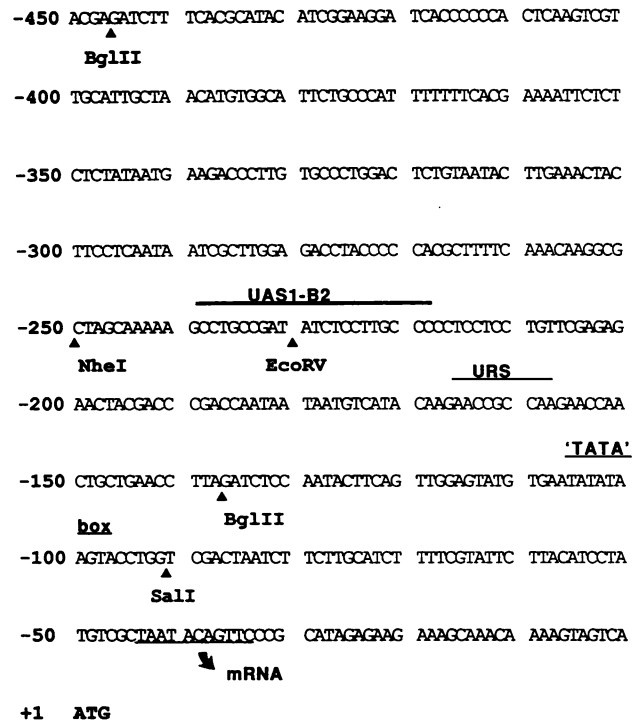


FIG. 9. Sequence of the *CYB2* regulatory region. Specific domains and restriction sites are indicated.

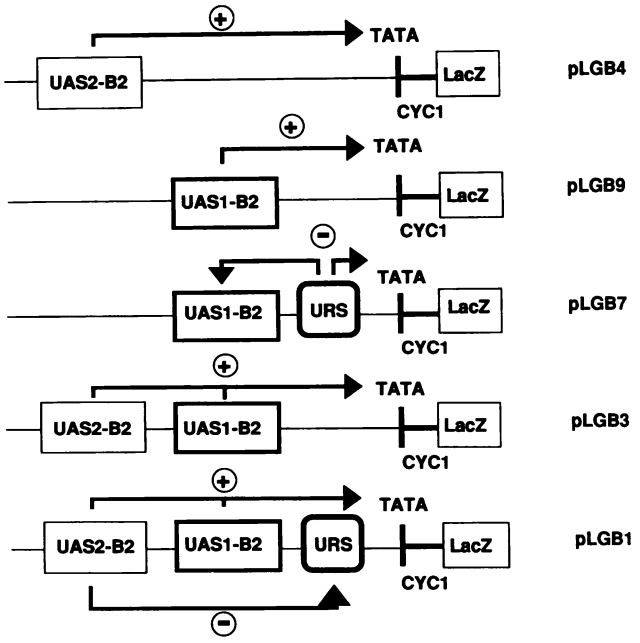


FIG. 10. Schematic representation of the regulatory elements in different constructions used to analyze the 5'-flanking region of the *CYB2* gene. DNA fragments isolated from the 5'-flanking region of the *CYB2* gene were substituted for the *CYC1* UASs and tested for their ability to regulate gene expression (Fig. 4 and Tables 1 and 2). With the pLGB1 construction, a working model for the *CYB2* regulation is proposed. +, transcription activation; −, inhibition.

(*HAP1*) gene plays an interesting role in the regulation of *CYB2*. Analysis of *CYB2* mRNA from a *cyp1* mutant demonstrates that the *CYP1* (*HAP1*) gene product also exerts a positive control on the expression of *CYB2*, as it does for *CYC1*, *CYP3* (*CYC7*), *CTT1*, and *HMG1* expression (8, 39, 40, 44). In this genetic context the induction by lactate remains possible, even if the mRNA level of *CYB2* does not reach the level of the wild-type strain. From these results we can propose that a specific induction of *CYB2* transcription by lactate exists and can act either independently of the *CYP1* (*HAP1*) locus or in a synergistic manner.

A detailed study of the 5'-flanking sequence of the *CYB2* gene (Fig. 9) reveals that most of the *cis* regulatory sites are located between nucleotides −446 and −90. A dissection of this region gives rise to a working model and a possible explanation of how transcription of the *CYB2* gene is regulated (Fig. 10). According to our results, *CYB2* is mainly regulated via three functionally distinct *cis*-acting elements (two positive and one negative) present in the DNA fragment spanning nucleotides −446 to −137. The two positive regions have the general characteristics of the upstream activation sequences described above in the transcriptional control of a large number of genes in *S. cerevisiae*. The activity of the first one (from −250 to −200), UAS1-B2, is abolished in the heme-depleted *hem1* mutant and in a strain carrying a *cyp1*::*LEU2* disruption. These data demonstrate that heme-stimulated UAS1-B2 activity is mediated by the action of the CYP1 (HAP1) transcriptional activator, as has been shown by Guarente et al. (8) and Pfeifer et al. (29) for UAS1 of the CYC1 gene. The second upstream activation site, UAS2-B2, is contained in a large fragment of DNA (−446 to −250) and has not been precisely localized. Its activity is repressed by glucose, derepressed in response to the shift to nonfermentable carbon sources such ethanol, and induced by lactate, compared with ethanol. It is not under the control of *CYP1* (*HAP1*) gene, but it is affected by the absence of heme biosynthesis. This activation region presents some close similarities with *UAS2* of *CYC1*, whose activity is controlled by the *HAP2*, *HAP3*, and *HAP4* products (8). These two *CYB2* UAS_S elements show strong synergistic effects in their activity, as has been demonstrated between UAS1 and UAS2 of *CYC1*. Preliminary results show that *CYB2* transcription is affected in the *hap2-1* mutant strain from the Guarente laboratory (1). Identification of the *cis*-acting region involved in this process is in progress. The third region, between −200 and −140 and downstream from UAS1-B2, can directly or indirectly repress the activation effect of the *CYP1* (*HAP1*) gene and could be the target site for a negatively acting factor. This effect is suppressed when the region −446 to −250 is present. At present we cannot decide whether this last effect is due to UAS2-B2 itself or to another *cis*-acting element present in the fragment −446 to −250. In addition, the presence of this third region increases the glucose repression 10-fold and could be involved in the catabolic repression process. In a significant number of cases, a DNA region associated with the negative control of yeast gene regulation has been described (36, 46). In the regulation of *CAR1* transcription, a 9-bp palindromic sequence, AGCCGCCGA, which possesses characteristics of a repressor-binding site has been shown by Sumrada and Cooper to play a central role in the induction process (37). The DNA fragment containing this upstream repression sequence has been called URS and can be specifically neutralized by a *cis*-acting element, the upstream induction sequence, associated with *CAR1* induction (37). Its insertion 3' to the *CYC1* UAS1 inhibits the transcriptional activity of this UAS. Related

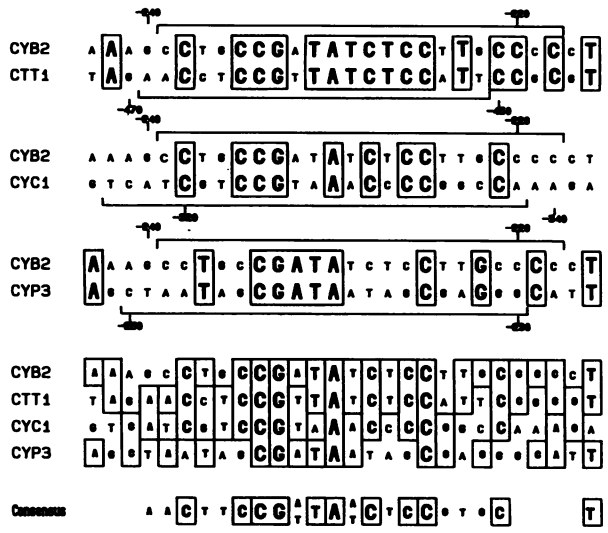


FIG. 11. Footprinting comparison of CYP1 (HAP1) DNA complexes at UAS1-B2 of *CYB2*, UAS of *CTT1*, UAS1 of *CYC1*, and UAS′ of *CYP3* (*CYC7*). Brackets indicate sequences protected from DNase I by the CYP1 (HAP1) protein. The orientation of $UAS1_{CYC1}$ has been inverted to obtain an optimal alignment with the other sequences.

sequences have been found in 14 other genes. A similar sequence, AACCGCCAA (−166 to −158), is also present in the DNA fragment −200 to −140 of the *CYB2* promoter (Fig. 9). Luche et al. (22) recently demonstrated by gel retardation assay that the URS element from *CAR1* could bind protein(s) and that the DNA fragment −169 to −153 of the *CYB2* gene containing this homologous sequence was an effective competitor of the *CAR1* URS and vice versa. On the basis of these observations, the authors raised the possibility that all of these homologous sequences found upstream of various yeast genes could be the target for the same protein or family of proteins. In the case of *CYB2*, such homology seems to be physiologically significant. A working model to account for the behavior of the negative element present in the *CYB2* promoter similar to that of Cooper is proposed (Fig. 10) and is currently being investigated.

By gel retardation assays, we have demonstrated a *CYP1* (*HAP1*)-dependent fixation on the upstream region of *CYB2* which is mediated by hemin. This result supports the idea already developed that hemin could function as the metabolic signal to activate transcription through a direct interaction with the CYP1 (HAP1) protein (9, 30, 44). It would be very interesting to know how the product of the *CYP1* (*HAP1*) gene interacts with this inducer and how it becomes competent to activate transcription and whether this process occurs in the cytoplasm or in the nucleus.

DNase I footprinting experiments have shown that the sequence recognized by the *CYP1* (*HAP1*) product is a 22-bp element (−239 to −218). The size of this site is quite similar to the described protected region on $UAS1_{CYC1}$, $UAS'_{CYP3\ (CYC7)}$, and UAS_{CTT1} (30, 44). In Fig. 11 the CYP1 (HAP1) protein-binding sequence of the *CYB2* upstream region is aligned with the corresponding regions of the *CYC1*, *CYC7* (*CYP3*), and *CTT1* genes. As for *CTT1* and *CYC1* (44), a high similarity between $UAS1\text{-}B2_{(CYB2)}$ and $UAS_{(CTT1)}$ can be observed: 15 of 22 bp are common and 10 of 11 bp in the core of the protected region are identical. When the three binding sites are aligned, the overall sequence similarity decreases significantly and only 8 bp are identical. The CYP1 (HAP1) protein-binding sequence of the *CYP3* (*CYC7*) upstream region shows no obvious similarity with those of the other three. Two hypotheses can be proposed to explain this observation. (i) CYP1 DNA binding occurs even though there is no large region of sequence similarity and even though the different target sequences are variant forms of a single consensus sequence, as has been proposed by Cerdan and Zitomer (4). It would be interesting to know whether an apparent absence of similarity among sequences of nucleotides could still yield similar three-dimensional structures. Methylation interference assays show that UAS_{CTT1}, $UAS1_{CYC1}$, and $UAS'_{CYP3\ (CYC7)}$ have similar contacts with the CYP1 (HAP1) protein. From the alignment of the different DNase I footprint sites (Fig. 11), a short consensus sequence with four nucleotides which are 100% conserved and seven others which are 75% conserved can be deduced. (ii) Alternatively, the similarity among $UAS1\text{-}B2_{CYB2}$, UAS_{CTT1}, and $UAS1_{CYC1}$ and their lack of similarity with $UAS'_{CYP3\ (CYC7)}$ would suggest that CYP1 (HAP1) target sequences may belong to different classes of sites. Although it is not possible to eliminate either of these hypotheses at the moment, most evidence tends to support the second. It has been proved that several mutations affecting residues in the CYP1 (HAP1) protein at the base of the zinc finger DNA binding domain, a structure similar to a set of fungal activators including GAL4 (7, 29), abolish or decrease differentially the specific binding to $UAS1_{CYC1}$ and $UAS'_{CYP3\ (CYC7)}$ (17, 29). The fact that UAS′ of *CYP3* (*CYC7*) also competes for binding to CYP1 (HAP1) with UAS1-B2 of *CYB2* and UAS1 of *CYC1* suggests, as has been proposed by Pfeifer et al. (29), that the DNA-binding domain of CYP1 (HAP1) could contain several overlapping DNA-recognition sites. In addition, another example has been reported in the case of a liver DNA-binding protein (6). This molecule is able to specifically recognize multiple nucleotide sites in regulatory regions of transthyretin, α_1-antitrypsin, albumin, and simian virus 40 genes, which contain only minimal sequence similarities.

With this study of *CYB2* transcription, we have added a new example to the growing list of genes whose expression is regulated by oxygen tension in the growth medium and by the intracellular heme level and which are activated by *CYP1*. Our investigation provides new data which allow a better understanding of the coregulation of the genes encoding proteins involved in various aspects of oxidative metabolism in different subcellular compartments and which also emphasize the role of the *CYP1* gene product as a regulator in this process. With the involvement of the *HAP2* gene in the regulation of *CYB2* (1) and *CYC1*, we indicate a particular and subtle coregulation mechanism of both genes. Finally, we have shown that the transcriptional machinery which controls *CYB2* expression involves complex molecular interactions; experiments aimed at a more detailed characterization of the *CYB2 cis*-acting regions and the identification of new *trans*-acting factors are in progress.

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