MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2302–2314
0270-7306/92/052302-13$02.00/0

Vol. 12, No. 5

Regulation of Yeast *COX6* by the General Transcription Factor ABF1 and Separate HAP2- and Heme-Responsive Elements

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Received 6 August 1991/Accepted 4 February 1992

Transcription of the *Saccharomyces cerevisiae COX6* gene is regulated by heme and carbon source. It is also affected by the HAP2/3/4 transcription factor complex and by *SNF1* and *SSN6*. Previously, we have shown that most of this regulation is mediated through UAS_6, an 84-bp upstream activation segment of the *COX6* promoter. In this study, by using linker scanning mutagenesis and protein binding assays, we have identified three elements within UAS_6 and one element downstream of it that are important. Two of these, HDS1 (heme-dependent site 1; between −269 and −251 bp) and HDS2 (between −228 and −220 bp), mediate regulation of *COX6* by heme. Both act negatively. The other two elements, domain 2 (between −279 and −269 bp) and domain 1 (between −302 and −281 bp), act positively. Domain 2 is required for optimal transcription in cells grown in repressing but not derepressing carbon sources. Domain 1 is essential for transcription per se in cells grown on repressing carbon sources, is required for optimal transcription in cells grown on a derepressing carbon source, is sufficient for glucose repression-derepression, and is the element of UAS_6 at which HAP2 affects *COX6* transcription. This element contains the major protein binding sites within UAS_6. It has consensus binding sequences for ABF1 and HAP2. Gel mobility shift experiments show that domain 1 binds ABF1 and forms different numbers of DNA-protein complexes in extracts from cells grown in repressing or derepressing carbon sources. In contrast, gel mobility shift experiments have failed to reveal that HAP2 or HAP3 binds to domain 1 or that *hap3* mutations affect the complexes bound to it. Together, these findings permit the following conclusions: *COX6* transcription is regulated both positively and negatively; heme and carbon source exert their effects through different sites; domain 1 is absolutely essential for transcription on repressing carbon sources; ABF1 is a major component in the regulation of *COX6* transcription; and the HAP2/3/4 complex most likely affects *COX6* transcription indirectly.

Cytochrome *c* oxidase, the terminal member of the mitochondrial respiratory chain in eukaryotes, is a key enzyme in the overall regulation of cellular energy metabolism (12, 55). This enzyme is a complex membrane protein composed of polypeptide subunits encoded by nuclear and mitochondrial genes (41). In the yeast *Saccharomyces cerevisiae*, cytochrome *c* oxidase consists of nine different polypeptide subunits (40). Three of the subunits (I, II, and III) are encoded by mitochondrial genes (*COX1*, *COX2*, and *COX3*, respectively). The six other subunits (IV, Va or Vb, VI, VII, VIIa, and VIII) are encoded by the unlinked nuclear genes *COX4*, *COX5a* or *COX5b*, *COX6*, *COX7*, *COX9*, and *COX8*, respectively (8, 31, 37, 40, 59, 60). *COX5a* and *COX5b* encode interchangeable isologs, Va and Vb, of subunit V (8). The other subunits are specified by unique genes present in single copy (63). Currently, it is thought that those subunits encoded by mitochondrial genes contain the catalytic redox centers of the enzyme and that those subunits encoded by nuclear genes are required for the regulation of catalysis or for assembly of the holoenzyme (42).

Given this division of labor between nuclear and mitochondrial genomes, it is likely that the nuclear genome-encoded subunits are instrumental in determining the overall level of functional cytochrome *c* oxidase molecules in a eukaryotic cell. With this in mind, an understanding of the regulation of these genes may provide important insight concerning the regulation of cytochrome *c* oxidase biogenesis and hence the regulation of cellular energy production. To approach this problem, we have been studying the regulation of the yeast *COX6* gene. The overall level of *COX6* transcription is activated by heme (51), repressed by glucose (60, 62), downregulated in the absence of a mitochondrial genome (13), and controlled by at least four *trans*-acting genes (*HAP2*, *HAP3*, *SNF1*, and *SSN6*) (51, 61). Previous studies have shown that transcription of *COX6* is glucose repressible in conjunction with the *HAP2* gene product (50) and that *SNF1*, which encodes a protein kinase (6, 7), and *SSN6* (5) are required to release *COX6* transcription from glucose repression (61). From these studies, it was not clear whether the HAP2/3/4 activator complex (16) affects *COX6* expression directly or indirectly. Also unclear was whether the HAP2/3/4 complex and the *SNF1-SSN6* pathway act through the same sites within the upstream activation sequence UAS_6.

To begin to understand how these environmental conditions and genetic pathways affect *COX6* transcription, we previously analyzed the effects of promoter deletion mutations on expression (50). These studies identified an upstream activation sequence, UAS_6, that is required for activation by heme and HAP2 and for release from glucose repression. This 84-bp segment of the *COX6* promoter contains two sequences that are similar to the sequence TNRTTGGT, which is a consensus recognition site for the HAP2/3/4 activator, and a sequence that matches the consensus site, $RTCR(Y)_3(N_3)ACG$, for ABF1 (also called SBF-B, TAF, SUF, GF1, and BAF1) (9, 10, 23, 24, 53), a multifunctional DNA-binding protein that can, depending on

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context, play a role in transcriptional activation, transcriptional repression, plasmid segregation, or DNA replication (3, 4, 47).

The goals of this study have been to (i) more precisely delineate the location of *cis* regulatory elements in UAS_6; (ii) identify which, if any, consensus HAP2 site is essential; and (iii) determine whether the ABF1 site functions in transcription. By using linker scanning mutagenesis and in vitro DNA binding assays, we have identified three elements within UAS_6 and one element downstream of it that are important. Two of these elements function in heme regulation, while the other two function in carbon source regulation. We have found that the ABF1 site within one of these elements, domain 1, binds ABF1 and is absolutely essential for expression on repressing carbon sources, and that the HAP2 consensus site which is adjacent to it confers HAP2/3/4-dependent control on *COX6* expression.

MATERIALS AND METHODS

Strains and growth conditions. The *S. cerevisiae* strains used in this study were CT155-7A (*MATα his4-580 trp1-289 ura3-52 hem1*) (52), JM43 (36), BWG1-7A (*matα leu2 his4 ade1 ura3*) (20), JP40 (BWG1-7A with *hap3-1*) (39), and SHY40 (BWG1-7A with *HAP3* disrupted by *HIS4*) (22). All strains were grown at 28 to 30°C. Strain CT155-7A was grown in SD synthetic minimal medium (46) containing yeast nitrogen base (Difco Laboratories) without amino acids, supplemented with appropriate amino acids (40 μg/ml), Tween 80 (0.1%), ergosterol (20 μg/ml), and methionine (40 μg/ml). To restore heme proficiency, the medium was supplemented with 100 μg of δ-aminolevulinic acid (δ-ALA) per ml. The carbon source was 8% dextrose, 2% raffinose, or 2% lactate, as indicated. Liquid cultures were grown with shaking (200 rpm), and cells were harvested in mid-logarithmic phase (1×10^7 to 2×10^7 cells per ml) by centrifugation and used immediately.

Plasmid DNA was propagated in *Escherichia coli* HB101 and DH5α grown in LB medium (32).

Plasmids. The following *COX6-lacZ* fusion plasmids that were used in this study were described previously: pMC61L (51); and pJDT64, pJDT73, and pJDT85 (50). Plasmid pJDT122 was constructed by linearizing pJDT73 with *Bam*HI at bp −590 and −135 followed by treatment with calf intestine alkaline phosphatase. Oligonucleotides (GATCCC CGTATCGCTCCATACGAGCCAATCAG; sense-strand sequence; GATCCTGATTGGCTCGTATGGAGCGATAC GGG; complementary-strand sequence) were annealed, gel purified, phosphorylated with T4 DNA kinase, and ligated with the pJDT73 DNA. The resulting recombinant plasmids were purified and sequenced (45). One having a single insertion of *COX6* sequences between −306 to −279 bp, and bordered by *Bam*HI restriction sites in the wild-type orientation with respect to the *COX6* gene, was obtained and designated pJDT122. Plasmids pJDT127 and pJDT128 have the same deletion of the *COX6* 5′ flanking region as does pJDT122 but were derived from pJDT36 (50) and have insertions of double-stranded oligonucleotides bordered by *Sst*I (at −590 bp) and *Bam*HI (at −135 bp) restriction sites. In pJDT127, the wild-type sequences between −292 to −289 bp have been replaced by the sequence GATC, which mutates the ABF1 recognition site. The inserted oligonucleotide has the sequence GAGCTCCGTATCGCTCCATGAT CGCCAATCAGGATC. In pJDT128, the wild-type sequences between −286 to −283 bp have been replaced by the sequence GATC, which mutates the HAP2 recognition site. The inserted oligonucleotide has the sequence GAGC TCCGTATCGCTCCATACGAGCGATCCAGGATC. Each of these oligonucleotides was produced from a longer single-stranded oligonucleotide that either could loop back on itself to self-anneal (pJDT127) or be annealed with a short 10-base priming oligonucleotide (pJDT128). Plasmids pJDT127 and pJDT128 were constructed from these oligonucleotides by synthesizing the second strand with DNA polymerase, digesting the plasmid with *Sst*I and *Bam*HI; gel purifying the double-stranded restriction fragment on a nondenaturing polyacrylamide gel, and ligating it into *Sst*I-*Bam*HI-digested pJDT36. Sequences were confirmed by DNA sequencing (45). Oligonucleotides used for all of these studies were synthesized by Operon Technologies (Alameda, Calif.). *CYC1* UAS DNA was obtained from plasmids pLG669Z (21) and pLGΔ-265 up1 (19). The *HAP2-lacZ* fusion plasmid, pJO61 (35), and the *HAP3-lacZ* fusion plasmid, pSH152 (22), were the generous gifts of L. Guarente (Massachusetts Institute of Technology).

Preparation of crude cell extracts from *S. cerevisiae*. Crude yeast cell extracts for both gel retardation and DNase I protection assays were made according to the method of Arcangioli and Lescure (1, 37). Generally, glucose-repressed extracts were made from CT155-7A cells grown to mid- to late log phase in YPD medium with 8% dextrose, while extracts from derepressed cells were made from cells grown to late log phase in YP-lactate (for strains CT155-7A and BWG1-7A) or in YPD with 2% dextrose to late log or early stationary phase (for strains JP40 and SHY40). Plasmid-containing cells were cultured in SD or SGAL (SD medium with 2% galactose instead of dextrose) under selection for the plasmid marker, to early log phase prior to preparation of crude cell extracts. Cells (25-ml cultures) were harvested by centrifugation and washed once in buffer A [200 mM Tris-HCl (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM Na_2EDTA, 7 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl chloride (PMSF), 10% (wt/vol) glycerol] by resuspension and centrifugation. The pelleted cells were resuspended in buffer A and chilled on ice. An equal volume of chilled glass beads was added, and the cells were disrupted by vigorous shaking for 5 min with a Vortex Genie mixer set on the highest setting. Cells were shaken for a total of 5 min, spread out over five 1-min intervals which were separated by 1-min intervals during which cells were kept on ice. The disrupted cell suspensions were incubated on ice for 30 min and then centrifuged at 9,000 × *g* for 60 min. The clear supernatant was removed carefully to a 30-ml Corex tube, and an equal volume of buffer B [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0), 5 mM EDTA, saturated $(NH_4)_2SO_4$] was added. The mixture was incubated on ice for 30 min and then centrifuged at 9,000 × *g* for 15 min. The pellet was resuspended in 0.2 ml of buffer C (20 mM HEPES [pH 8.0], 1 mM EDTA, 7 μM β-mercaptoethanol, 1 mM PMSF, 20% glycerol), aliquoted, frozen, and stored at −70°C. Prior to use, the protein concentration was determined by the method of Lowry et al. (30), using bovine serum albumin (BSA) as a standard.

Binding, gel retardation, and DNase I footprinting. The procedure for binding of proteins from crude extracts to end-labeled DNA was adapted from that of Dynan (11). Either 0.5 ng of a ^{32}P-end-labeled restriction fragment containing UAS_6 sequences (−185 to −340 bp) or a ^{32}P-labeled 32-bp double-stranded oligonucleotide corresponding to domain 1 DNA sequences was incubated in the presence of 80 μg of protein and 5 μg of the nonspecific competitor poly(dI-dC) · poly(dI-dC) (Sigma Chemical Co., St. Louis, Mo.) in

0.1 M TM buffer for final concentrations of 25 mM Tris-HCl (pH 7.9), 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, and 0.1 M KCl in a total volume of 50 μl for 20 min at room temperature. Gel retardation assays (17) were performed by adding 7 μl of loading mix (15% Ficoll 400, 0.1% bromophenol blue, 0.1% xylene cyanol) to the binding reaction and loading 10 μl of this mixture onto a 4% (40:1, acrylamide/bisacrylamide) polyacrylamide gel in 1× Tris-borate-EDTA. The gel was electrophoresed at 150 V (constant voltage) for about 2 h. After electrophoresis, gels were dried and exposed to Kodak SB or XAR autoradiography film.

End labeling of the restriction fragment containing UAS_6 or the double-stranded oligonucleotide corresponding to domain 1 sequences was performed in 15 μl of a mixture containing 50 mM Tris-Cl (pH 7.8), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μg of BSA per ml, 0.02 μM each dCTP, dGTP, and dTTP, 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol), and 0.5 to 1 U of Sequenase (U.S. Biochemical) for 15 min at 30°C. The reaction was stopped by the addition of 100 μl of Tris-EDTA buffer, and the entire mixture was passed through a G-50 Spun column. The void volume was collected, ethanol precipitated, and brought to 100 to 200 μl with distilled water for use in the gel retardation assays.

DNase I footprinting was done with crude extracts (11, 17), and reaction mixtures were loaded onto 6% sequencing gels. Size standards were DNA sequence ladders of known length.

Construction of LS mutations within the 5′ flanking region of the *COX6* gene. To obtain linker scanning (LS) mutations that span the 5′ flanking region of *COX6* between bp −341 and −180, we first made an extensive set of 5′ and 3′ deletions within this region. For this purpose, four plasmids derived from the 2μm-based high-copy-number plasmid pMC61L (51) were used. pMC61L carries 1.5 kbp of sequences upstream of the *COX6* initiation codon along with sequences specifying the first 21 codons of *COX6* fused in frame at the eighth codon of *E. coli lacZ*. These four pMC61L derivatives (pJDT22, pJDT30, pJDT37, and pJDT35), constructed by Trawick et al. (50), contain deletions at convenient locations within the 5′ flanking region of *COX6* and have *Bam*HI linkers inserted at the deletion sites.

Deletions were made bidirectionally from the *Bam*HI sites in the pMC61L derivatives by linearizing the plasmid DNA with *Bam*HI and resecting it with exonuclease BAL 31 as described previously (32). Procedures for stopping the digestion, filling in staggered ends, adding *Bam*HI linkers lacking 5′ PO_4 groups (dCCGGATCCGG; Bethesda Research Laboratories), and religating the ends were performed as described previously (50). Deletions were screened by restriction enzyme analysis followed by electrophoresis on high-resolution nondenaturing polyacrylamide gels (32). Deletion endpoints mapping within the desired region were determined by DNA sequence analysis (45), using Sequenase (U.S. Biochemical). Mutant plasmids, in which the wild-type *COX6* promoter sequence is substituted by the synthetic 10-bp *Bam*HI linker sequence at fixed positions spanning the 5′ flanking region of *COX6* between bp −341 and −180, were produced by using this set of 5′ and 3′ deletion mutants and the linker scanning strategy of McKnight and Kingsbury (33). To generate these LS mutants, the relevant 5′ and 3′ deletion mutants were recombined between the common *Bam*HI site at the deletion endpoints and the unique *Xho*I site within the vector (50). This was done by isolating the large backbone piece from the 3′ deletions and the smaller fragment from the 5′ deletions on low-melting-point agarose gels (54) and ligating the two pieces together. The predicted genotype of each LS mutation was confirmed by restriction analysis and by nucleotide sequencing. Overall, 21 linker substitution plasmids, as well as three linker insertion plasmids (whose endpoints mapped to the same or close to the same nucleotide), were constructed in this work (Fig. 1).

Plasmid DNA from *E. coli* was purified by the alkaline lysis method (2). Enzymes for restriction and ligation were used according to the recommendations of the suppliers (Bethesda Research Laboratories, Boehringer Mannheim, and New England Biolabs).

Analysis of LS mutant expression. Plasmids (10 μg of plasmid DNA in 10 μl) were introduced into *S. cerevisiae* cells by transformation, using the lithium acetate procedure (26). Transformants were selected for uracil phototrophy on SD medium supplemented with amino acids and δ-ALA, where appropriate. *URA*$^+$ transformant colonies were picked after 2 to 3 days and used for β-galactosidase assays as described previously (34, 50). Enzyme activity is expressed as nanomoles of *o*-nitrophenol produced per milligram of protein per minute, using a molar extinction coefficient for *o*-nitrophenol of 4.5×10^3 cm^{-1} M^{-1} at 420 nm (34). To determine the total cell protein per unit of optical density at 600 nm for each strain and growth condition used, the optical density at 600 nm of each sample was measured, the cell walls were disrupted by glass beads on a Vortex Genie mixer, and the protein concentration was determined in the presence of 2.5% sodium deoxycholate by the method of Lowry et al. (30).

RESULTS

Construction and analysis of LS mutations. To identify important control elements within and downstream of UAS_6, we constructed a series of LS mutations that spanned the region from −341 to −186 bp as described in Materials and Methods. In most of the mutations, 10 ± 1 bp of the *COX6* promoter sequences was substituted by a 10-bp *Bam*HI linker, with the sequence 5′-CCGGATCCGG-3′ (Fig. 1). These LS mutations were constructed in plasmids with *COX6-lacZ* fusions containing 1.5 kbp of the 5′ flanking region of *COX6* (see Materials and Methods). The resulting plasmids are identical to the wild-type *COX6-lacZ* fusion, pMC61L, except for the inserted linker and the corresponding mutation. The effects of each mutation on expression were determined by assaying β-galactosidase levels in vivo and comparing these with those levels of β-galactosidase produced from pMC61L.

Each LS mutant plasmid was transformed into strain CT155-7A, a strain that carries a mutation in *HEM1*, the structural gene for δ-ALA synthetase (52). Previous studies have shown that *hem1* cells do not express heme-regulated genes, including *COX6* (51), *CYC1* (20), or *COX5a* (42, 52), presumably because of low intracellular levels of heme. When these cells are supplemented with δ-ALA, they are heme sufficient (18, 57) and essentially wild type in their expression of these genes. To monitor the effects of each LS mutation on regulation of *COX6* transcription by heme and carbon source, CT155-7A cells were grown in the presence or absence of δ-ALA or in repressing or derepressing carbon sources. Strain CT155-7A is carbon source repressed when grown in 8% glucose but derepressed when grown in 2% lactate. For these studies, cells carrying LS plasmids were grown in three different types of media: 8% glucose plus δ-ALA (repressing carbon source, heme-sufficient cells), 8%

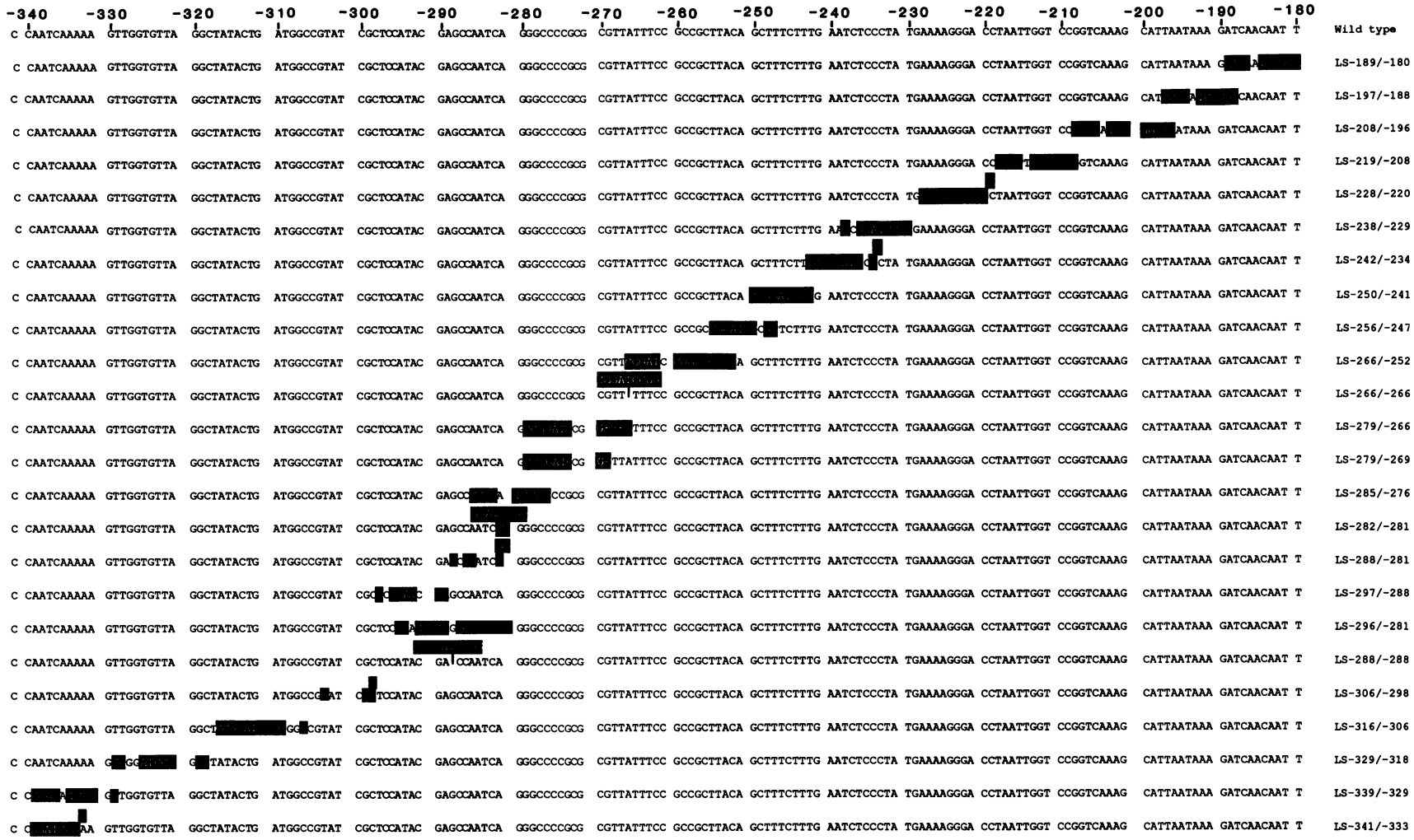

FIG. 1. Nucleotide sequences of LS mutations in the yeast *COX6* promoter. The nucleotide sequence of the wild-type promoter (sense strand), between bp −341 and −180 relative to the translation start site, is displayed on the top line. The nucleotide sequences of 24 LS mutants are shown below the wild-type sequence. The nomenclature of each LS mutant is derived from the parental 5′ and 3′ deletion mutants that were recombined for its construction. Mutated nucleotide residues are displayed in reverse contrast. In the cases where the two parental deletions do not match exactly, resulting nucleotide insertions or deletions are indicated on the 3′ border of the linker. In three instances, the *Bam*HI linker has undergone a change in sequence. As indicated, LS−250/−241 has a linker sequence of CGGGATCCGG, whereas both LS-266/-252 and LS-266/-266 show a 9-bp (CGGATCCGG) substitution.

TABLE 1. Expression from LS mutants

LS mutant	β-Galactosidase activity[a] for CT155-7A grown in[b]:		
	Repressing carbon source		Derepressing carbon source, Heme sufficient[e] (3)
	Heme sufficient[c] (1)	Heme deficient[d] (2)	
pMC61L (wild type)	31.2	1.3	128.7
LS-189/-180	27.0	1.2	119.9
LS-197/-188	36.2	1.8	127.4
LS-208/-196	36.1	1.4	126.5
LS-219/-208	17.8	0.8	115.6
LS-228/-220	54.9	5.7	112.6
LS-238/-229	32.1	1.4	133.2
LS-242/-234	53.7	2.7	120.7
LS-250/-241	30.1	1.3	138.0
LS-256/-247	43.1	3.3	137.7
LS-266/-252	28.7	7.3	134.2
LS-266/-266	30.7	9.4	122.4
LS-279/-266	13.5	1.5	140.5
LS-279/-269	17.8	0.8	125.8
LS-285/-276	1.3	0.1	24.7
LS-282/-281	7.7	0.8	117.0
LS-288/-281	1.9	0.7	56.5
LS-296/-281	0.3	0.0	28.4
LS-288/-288	15.5	0.8	124.4
LS-297/-288	5.3	0.3	101.2
LS-306/-298	10.4	0.5	124.7
LS-316/-306	41.0	1.7	117.5
LS-329/-318	45.3	2.2	124.5
LS-339/-329	43.0	2.1	137.9
LS-341/-333	29.2	1.4	117.7

[a] Units of β-galactosidase activity ([nanomoles of *o*-nitrophenol per milligram of protein per minute] × 100) are averages of six determinations; the range for these values was less than 20%.

[b] The activity of each plasmid was assayed in CT155-7A cells grown to mid-log phase in synthetic medium plus Tween, ergosterol, and methionine, with or without added δ-ALA, as described in Materials and Methods.

[c] Grown in the presence of δ-ALA. The carbon source was 8% dextrose.

[d] Grown in the absence of δ-ALA. The carbon source was 8% dextrose.

[e] Grown in the presence of δ-ALA. The carbon source was 2% lactate.

glucose minus δ-ALA (repressing carbon source, heme-deficient cells), and 2% lactate or raffinose plus δ-ALA (derepressing carbon source, heme-sufficient cells). Strain CT155-7A cannot grow on a derepressing carbon source minus δ-ALA; consequently, this condition could not be used.

Expression in heme-sufficient cells grown on a repressing carbon source. The β-galactosidase activities supported by each mutant construct are shown in Table 1, column 1. In general, mutations downstream of UAS_6, between −256 and −189, had little if any effect on expression. Two mutations produced a small effect. A small decrease in expression was seen with LS-219/-208, while a small increase was seen with LS-228/-220. LS-219/-208 altered the sequence TAATTGGT, one of three possible HAP2/3/4 binding sites in the *COX6* promoter. This sequence is the only one of these three sites in UAS_6 that is a perfect match to the HAP2/3/4 consensus binding site (13, 25, 30).

LS mutations that fall within UAS_6, between −340 and −256, localized three regions of interest. The first, domain 2, is defined by mutants LS-279/-266 and LS-279/-269. These mutants displayed a twofold reduction in activity. Previously, plasmid pJDT30, which carries a 4-bp deletion, from bp −279 to −276, had been shown to result in a two- to threefold decrease in activity (50), suggesting that these four base pairs are required for this site to function. Domain 2 appears to extend no further downstream than bp −269 and has a 5′ boundary that is most likely around bp −279. The second region of interest is defined by LS mutations that modify sequences between −276 and −306. Mutations in this region have a profound effect on expression. Mutant LS-285/-276 showed a strong decrease in activity (about 25-fold). This LS mutation changes domain 2, as well as a HAP2 consensus binding site located between bp −288 and −281 (5′-GCCAATCA-3′) on the DNA strand opposite that used for *COX6* transcription. This site is 87% similar to the HAP2/3/4 consensus sequence. Although this HAP2 site does not display a perfect match with the consensus, an alteration in it produced a much bigger effect than destroying the perfect HAP2 consensus site, between bp −218 to −211, in LS-219/-208. This is underscored by the finding that a substitution entirely within the HAP2 site in LS-288/-281 led to a 15-fold decrease in expression. This observation indicates that this site is a critical sequence for expression. Another linker insertion, LS-282/-281, at this site had a fourfold effect on activity; in this mutant, however, only a minor change is introduced into this HAP2 site (consensus changed to GCCAATCC; Fig. 1).

In mutant LS-296/-281, both the HAP2 site at bp −288 to −281 and the consensus recognition site for ABF1 (2, 40), between −302 and −290, have been altered. This mutation produced a very large (100-fold) reduction in activity. How much of this loss of activity can be assigned to the recognition site for ABF1? This question is addressed by use of two mutations that fall within the ABF1 consensus [RTCR $(N)_3(Y_3)$ACG) (9, 10)] but that leave the HAP2 site intact. LS-297/-288 exhibited a sixfold decrease in activity, whereas LS-306/-298 displayed a threefold reduction of *COX6-lacZ* expression. The higher expression of LS-306/-298 than of LS-297/-288 can be explained by the fact that in this LS mutant, the ABF1 site is only slightly changed [consensus changed to RTCYR$(N)_6$ACG; Fig. 1]. A linker insertion (LS-288/-288) that leaves both the ABF1 and HAP2 sites intact but that increases the distance between them by 10 nucleotides reduces activity by 50%. This finding, together with the fact that mutations in each site alone are not additive, raises the possibility that these two sites are not independent but act cooperatively.

LS mutations in sequences upstream of the ABF1 site (between bp −341 and −306) had little effect on expression. Thus, sequences within this region of UAS_6, including a third possible HAP2/3/4 binding site, between bp −332 and −325 (AAGTTGGT), are not essential for *COX6* expression.

Expression in heme-deficient cells grown on a repressing carbon source. There are at least two possibilities for how heme-responsive sites might act in regulating a heme-dependent gene such as *COX6*. A mutation in a heme-regulated site may lead to either a decrease in activity in heme-sufficient cells (as expected for a site that acts positively) or an increase in activity in heme-deficient cells (as expected for a negative site). To examine the effects of heme on the expression of *COX6*, cells transformed with mutant constructs were grown in the absence of δ-ALA under glucose-repressing conditions as described above.

Heme deficiency gave a 24-fold decrease in the expression of the *COX6-lacZ* fusion gene in transformants carrying the wild-type plasmid, pMC61L (Table 1; compare columns 1 and 2), similar to results published previously (51). Mutations introduced by the four linker scans that fall within the region bp −219 to −180 all produced wild-type heme-

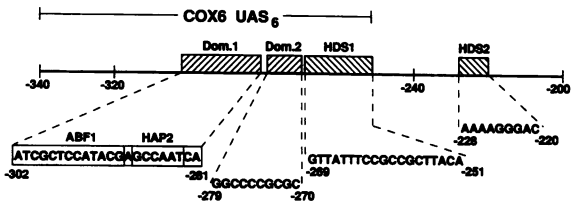

FIG. 2. Locations and sequences of *COX6* promoter elements. The location of UAS_6, defined by a previous deletion analysis (50), is shown at the top. Boxes indicate the limits of domain 1 (Dom.1), domain 2 (Dom.2), HDS1, and HDS2, as determined by linker scanning mutagenesis. Sense-strand sequences are shown for each element; the numbers below these sequences indicate distance from the translational initiation codon. The shaded regions within domain 1 indicate the ABF1 binding site and a HAP2/3 consensus sequence, as noted.

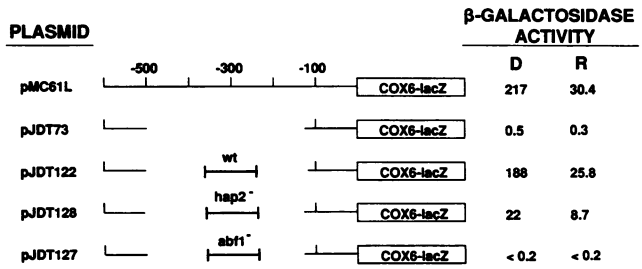

FIG. 3. Function of wild-type domain 1 and domain 1 with mutations at the HAP2 and ABF1 sites in glucose repression-derepression. Plasmids pJDT122, pJDT128, and pJDT127 were constructed as described in Materials and Methods. Each plasmid was transformed into strain JM43 and assayed for β-galactosidase activity as described in Materials and Methods. Cells were grown on either a repressing carbon source (R; 8% glucose) or a derepressing carbon source (D; 2% raffinose). β-Galactosidase units are expressed as (nanomoles of *o*-nitrophenol per milligram of protein per minute) × 100 and are averages of three determinations with a range of less than 10%. The sequences present in domain 1 in pJDT122 correspond to wild-type DNA sequences between −306 and −279 bp. The sequences present in domain 1 in pJDT128 are mutated between −286 and −283 bp in the HAP2 site; the sequences present in domain 1 in pJDT127 are mutated between −292 and −289 bp in the ABF1 site. These mutant plasmids carry domain 1 sequences that are otherwise identical to wild-type domain 1 in pJDT122.

dependent activation of *COX6-lacZ* expression (20- to 26-fold increase of expression in heme-proficient cells). This finding argues against participation of the HAP2 consensus site, between bp −218 and −211, in heme-dependent regulation of *COX6*. LS-228/-220 showed a significant increase in expression relative to the wild type, pMC61L, when grown without δ-ALA. Altering this sequence produced a 4.4-fold increase in *COX6-lacZ* activity in heme-deficient cells. Therefore, this sequence may contain a site that acts negatively in heme-deficient conditions. From the finding that LS-219/-208 and LS-238/-229, which alter sequences that surround this site, exhibited a wild-type level of activation by heme, it can be concluded that the 5′ boundary of this site is no further upstream than bp −228, whereas its 3′ boundary extends no further than bp −220. We have designated this site HDS2 (heme-dependent site 2).

LS-256/-247, LS-266/-252, and LS-266/-266, which alter sequences between bp −247 and −266, identify a second heme-responsive site. Like HDS2, this site acts negatively under heme-deficient conditions. LS-266/-252 and LS-266/-266, an insertion mutation, both produced large effects: 5.7- to 7.2-fold increases in expression relative to expression in pMC61L. Since LS-250/-241 and LS-279/-269 both produced wild-type heme-dependent activation, this site, designated HDS1, can be localized between bp −269 and −251. This region overlaps a site that was predicted previously (50) to be involved in heme regulation.

The LS mutation within the HAP2 consensus binding site between bp -288 and -281 (LS-288/-281) produced very low expression in both heme-deficient and heme-sufficient conditions. Thus, it is not possible to determine whether this site participates in heme-dependent activation or whether it is merely required for expression per se. The LS mutations (LS-297/-288 and LS-306/-298) that modify the ABF1 site did not affect heme regulation. Because LS mutations altering sequences upstream of bp −306 had little or no effect on activity under both heme-deficient and heme-sufficient conditions, it is unlikely that sequences between bp −340 and −306 play a role in heme regulation of *COX6*.

Expression in heme-sufficient cells grown on a derepressing carbon source. LS mutations that altered sequences between −256 and −180, downstream of UAS_6, had little effect on expression in cells grown on a derepressing carbon source. Similarly, mutations that modified sequences between −341 and −296 and −276 and −252 within UAS_6 had little or no effect on expression. In contrast, mutations between −276 and −296 reduced expression by as much as fivefold. These mutations, LS-285/-276, LS-288/-281, and LS-296/-281, modified sequences within the HAP2 consensus site and to a lesser extent within the ABF1 binding site. The effects of these mutations on expression in cells grown on a derepressing carbon source are not as great as in cells grown on a repressing carbon source. In repressing media these mutations reduce expression 16- to 100-fold, but in derepressing media they reduce expression only 2- to 5-fold. The differential effects of the mutants in repressed and derepressed cells suggest that this region of UAS_6 is functional in carbon source regulation of *COX6*.

***COX6* promoter elements.** The LS mutations described above have revealed four elements that are important for *COX6* transcription (Fig. 2). Two of these, HDS1 (between −269 and −251 bp) and HDS2 (between −228 and −220 bp), act negatively in heme regulation. Expression from mutants at these sites is increased in heme-deficient cells. A third element, domain 2 (between −279 and −270 bp), acts positively on heme-sufficient cells grown on a repressing carbon source. Mutations at this site have no effect on transcription in cells grown on a derepressing carbon source. The fourth element, domain 1 (between −302 and −281 bp),

TABLE 2. Evidence that domain 1 is not involved in heme regulation

Plasmid	*COX6* upstream DNA (kb)	β-Galactosidase activity[a] in CT155-7A cells that are:	
		Heme sufficient[b]	Heme deficient[c]
pMC61L	1.5	38.8	0.8
pJDT73	1.5–0.59/0.135–0	0.23	0.3
pJDT122	1.5–0.59/0.31–0.28/0.135–0	26.7	16.2

[a] Units of β-galactosidase activity ([nanomoles of *o*-nitrophenol per milligram of protein per minute × 100) are averages of three determinations; the range for these values was less than 10%.
[b] Cultured in the presence of added δ-ALA.
[c] Cultured without added δ-ALA.

TABLE 3. Expression of *COX6-lacZ* fusions in *hap3* mutant cells

Plasmid[a]	Plasmid genotype[b]	β-Galactosidase activity[c] in strains:	
		BWG1-7a (*HAP*$^+$)	JP40 (*hap3-1*)
pJDT122	*ABF1*$^+$/(*HAP2/3/4*)$^+$	304	103
pJDT127	*abf1*/(*HAP2/3/4*)$^+$	0.2	<0.1
pJDT128	*ABF1*$^+$/*hap2/3/4*	100	97.4

[a] Construction of plasmids is described in Materials and Methods.
[b] Plasmid genotype refers to consensus binding sites in domain 1 that have been altered.
[c] Cells from each strain were cultured in SD medium, harvested, and assayed as described in Materials and Methods. β-Galactosidase activity is given as (nanomoles of *o*-nitrophenol per milligram per minute) protein × 100.

acts positively in heme-sufficient cells grown on either a repressing or derepressing carbon source. This element contains an ABF1 consensus site (between −302 and −290 bp) and a possible HAP2 consensus site (between −288 and −281 bp). It is essential for transcription in cells grown on a repressing carbon source.

Domain 1 is sufficient for glucose repression-derepression but does not function in heme regulation. As mentioned above, mutations within domain 1 lead to differential effects on expression in cells grown on repressing or derepressing carbon sources. However, because some mutations within this region supported very little expression in cells grown on a repressing carbon source, we were unable to determine conclusively whether this site functions in carbon source control or heme regulation of *COX6* transcription. To address this question, we constructed recombinant plasmids in which the entire *COX6* UAS_6 element was replaced by wild-type domain 1 or a mutant domain 1 in which either the HAP2 or ABF1 consensus sites was mutated (Fig. 3). One plasmid, pJDT122, carried domain 1 but no other *COX6* promoter elements. Two other plasmids, pJDT127 and pJDT128, have sites that are similar to domain 1 but carry mutations at either the ABF1 or HAP2 site, respectively. Expression driven by these plasmids was compared with that supported by plasmid pMC61L, which carries all of the promoter sequences required for *COX6* expression, and plasmid pJDT73, which is deleted for that region of the *COX6* promoter (between −540 and −135 bp) that is required for expression. From the results presented in Fig. 3, it is clear that the wild-type domain 1 is sufficient for nearly wild-type levels of expression in either repressing or derepressing medium. Moreover, it is clear that expression from pJDT122 exhibits the same level of glucose repression-derepression as does pMC61L, which carries the entire *COX6* promoter. In both cases, the level of expression in raffinose (a derepressing carbon source that supports levels of *COX6* expression that are similar to those in lactate) is sevenfold higher than in glucose. Plasmid pJDT128, which has a mutant HAP2 site but a wild-type ABF1 site, also supports carbon source-dependent regulation, although to a lesser extent than does either pJDT122 or pMC61L. This finding suggests that the ABF1 site contributes in part to carbon source control but implies that the HAP2 site is required as well. The HAP2 site does not function as an activator when the ABF1 site is mutated in plasmid pJDT127; expression was completely abolished. From the results in Table 2, it is also clear that domain 1 contributes little, if at all, to heme regulation. The level of expression supported by pJDT122 was nearly the same in heme-deficient and heme-sufficient cells.

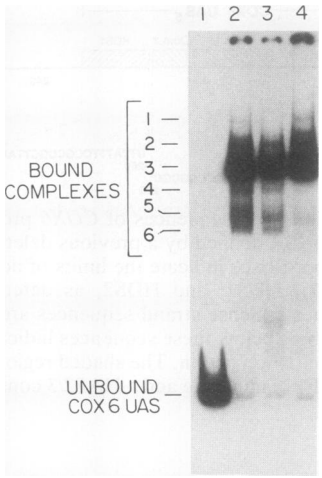

FIG. 4. Gel mobility shift electrophoresis assays with DNA containing *COX6* UAS. A *Bam*HI restriction fragment containing bp −185 to −340 of *COX6* UAS (i.e., the *COX6* promoter) was obtained from pJDT85 (50), 5′ end labeled with [α-^{32}P]dATP and Sequenase enzyme (U.S. Biochemical), incubated with 80 μg of protein from crude cell extracts, and electrophoresed on a 4% polyacrylamide gel. The positions of the unbound *COX6* UAS DNA and the major protein-DNA complexes are indicated. Lanes: 1, unbound *COX6* UAS DNA; 2, extract derived from CT155-7A cells grown in 8% dextrose plus δ-ALA (repressing carbon source, heme sufficient); 3, extract from CT155-7A cell grown as in 8% dextrose minus δ-ALA (repressing carbon source, heme deficient); 4, extract derived from CT155-7A cells grown in 2% lactate plus δ-ALA (derepressing carbon source, heme sufficient).

Domain 1 functions as a HAP2/3-dependent UAS element. Although the promoter of *COX6* has three possible HAP2 binding sites (between bp −332 and −325, −288 and −281, and −218 and −211), it is clear from results for the LS mutants described above that only sequences at the site between bp −288 and −281 were required for expression. This site falls within domain 1. To determine whether this site functions in HAP2/3/4 regulation of *COX6* transcription, we transformed a *hap3* mutant, JP40, and an isogenic *HAP3*$^+$ strain, BWG1-7A, with pJDT122, pJDT127, and pJDT128. Transformants carrying each plasmid were grown in SD medium and assayed for β-galactosidase activity (Table 3). Each of these transforming plasmids contains a *COX6-lacZ* fusion gene whose expression is controlled by a UAS_6 element containing only domain 1 that is wild type in pJDT122, mutated to abolish the ABF1 recognition site in pJDT127, and mutated to abolish the HAP2 consensus site in pJDT128. Expression from pJDT122 was threefold lower in the *hap3-1* mutant than in the isogenic *HAP3*$^+$ strain. Expression from pJDT128 was threefold lower in the *HAP3*$^+$ strain but was not reduced in the *hap3-1* mutant. pJDT127 supported barely detectable expression in either strain. These results indicate that the HAP2 consensus site between −288 and −281 bp is a HAP3-responsive element and that this site is inoperative in the absence of the ABF1 site. Thus, this HAP2 consensus site does not function as an independent UAS-like element. However, it is required for full domain 1-mediated expression (compare pJDT122 with pJDT128).

Major DNA-protein complexes that form with UAS_6 map to domain 1. To further understand UAS_6, we have performed in vitro DNA binding studies. A *Bam*HI restriction fragment from pJDT85 (50) containing the entire UAS_6 sequence,

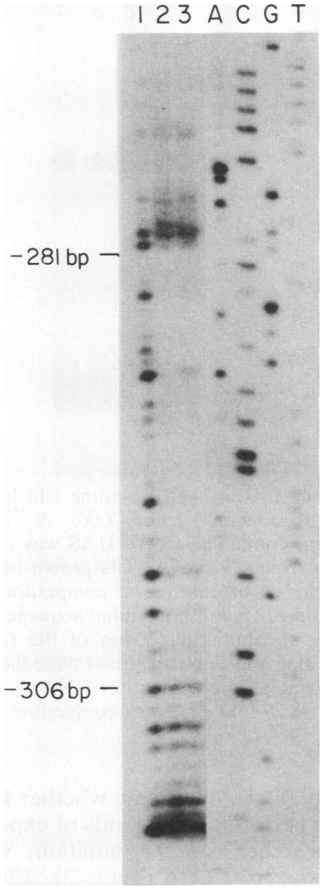

FIG. 5. DNase I protection assay of UAS_6. Crude cell extracts (80 μg of protein) were incubated with *COX6* UAS DNA derived from pJDT35 (50), treated with DNase I according to Dynan (11), and then run on a 6% polyacrylamide–urea sequencing gel. Lanes: 1, no extract; 2, extract from CT155-7A grown with δ-ALA (heme sufficient); 3, extract from CT155-7A grown without δ-ALA (heme deficient). Size markers were DNA nucleotide sequencing lanes A, C, G, and T. The coordinates given are in base pairs 5′ of the *COX6* initiation codon.

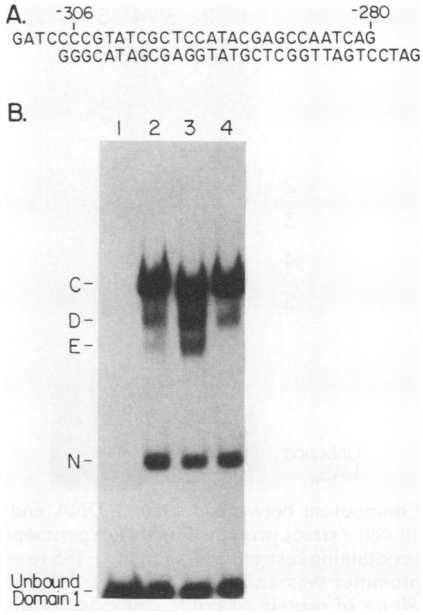

FIG. 6. Gel mobility shift electrophoresis assays with DNA containing domain 1. A double-stranded 32-bp oligonucleotide (A), containing the footprinted region (−306 to −280 bp) shown in Fig. 5 and bordered by *Bam*HI sites (to facilitate cloning and end labeling), was incubated with extracts from CT155-7A grown in 8% dextrose minus δ-ALA (lane 2), 8% dextrose plus δ-ALA (lane 3), and 2% lactate plus δ-ALA (lane 4). Lane 1 received no cell extract. Incubation and electrophoresis were performed as described in to the legend to Fig. 4. C, D, E, and N refer to the retarded complexes.

between −185 and −340 bp, was isolated, end labeled with ^{32}P, incubated with extracts from yeast cells grown in various conditions, and assayed by gel electrophoresis mobility shift assays. The results from a typical experiment are shown in Fig. 4. UAS_6 DNA forms different numbers of DNA-protein complexes when incubated with extracts from cells grown on a repressing carbon source plus δ-ALA (heme sufficient), a repressing carbon source minus δ-ALA (heme deficient), or a derepressing carbon source plus δ-ALA. In all three extracts, a major complex (band 3) and a minor complex (band 2) are observed. In addition, minor complexes are seen in some extracts but not others. Band 1 is found in heme-sufficient cells grown on either a repressing or derepressing carbon source but is absent in heme-deficient cell extracts. Bands 4 to 6 are present in cells grown on a repressing carbon source but are reduced or missing in extracts from cells grown on a derepressing carbon source.

Typically, more than 90% of the labeled *COX6* UAS probe used in our gel shift studies is bound in band 3. Consequently, it is possible to footprint the region of UAS_6 bound to this DNA-protein complex, using crude cell extracts and DNase I treatment. UAS_6 DNA that was end labeled at either −340 or −256 bp was mixed with 80 μg of extract protein from heme-sufficient or heme-deficient cells grown on glucose, under the same conditions used for the gel retardation assays discussed above. They were treated with DNase I, and the products were analyzed on a 6% polyacrylamide–6 M urea sequencing gel. The footprint of one strand of UAS_6 is shown in Fig. 5. A footprint of the opposite strand (not shown) was identical. The region of UAS_6 that was protected from DNase I was between −306 and −280 bp from the *COX6* initiation codon. This region corresponds exactly to domain 1.

These findings imply that domain 1 is the major protein-binding element within UAS_6. To confirm this conclusion, we performed two types of experiments. First, we examined whether a double-stranded synthetic oligonucleotide corresponding to the sequence between −306 to −280 bp bound proteins in crude cells extracts; second, we examined whether this synthetic oligonucleotide would compete with UAS_6 in gel shift assays. From Fig. 6, it is obvious that domain 1 by itself has protein binding activity and that the pattern of proteins bound are different in extracts from glucose-repressed and -derepressed cells. They are only slightly affected by heme deficiency. In each case, there is a major band (band C), just as with UAS_6. In the experiment shown in Fig. 7, the 32-bp synthetic double-stranded oligonucleotide was used as an unlabeled competitor against ^{32}P-labeled UAS_6 DNA in cell extracts from heme-sufficient glucose-repressed CT155-7A cells. Increasing amounts of competitor DNA removed binding of both the major and minor bands. At high concentrations of competitor DNA, the intensity of band 5 increases. This band probably represents a protein-DNA complex that forms with UAS_6 sequences that are outside of domain 1.

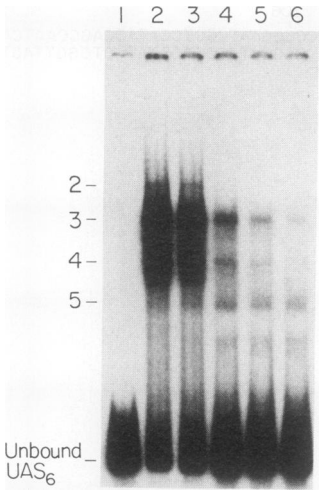

FIG. 7. Competition between domain 1 DNA and UAS_6 DNA for binding of cell extract protein. For this experiment, the *Bam*HI *COX6* UAS-containing restriction fragment, −185 to −340 bp, from the *COX6* promoter was end labeled with [α-^{32}P]dATP and incubated with 80 μg of protein of crude cell extracts from CT155-7A cells grown in 8% dextrose (glucose-repressing conditions) plus δ-ALA and increasing amounts of unlabeled double-stranded domain 1 oligonucleotide, described in the legend to Fig. 6. Lanes: 1, unbound UAS_6 DNA incubated without cell extract; 2, UAS_6 DNA incubated with cell extract but no unlabeled domain 1 DNA; 3 to 6, UAS_6 DNA incubated with cell extract and a 10-fold excess (lane 3), 50-fold excess (lane 4), 100-fold excess (lane 5), or 200-fold excess (lane 6) of unlabeled domain 1 DNA. The positions of complexes 2 through 5 are indicated.

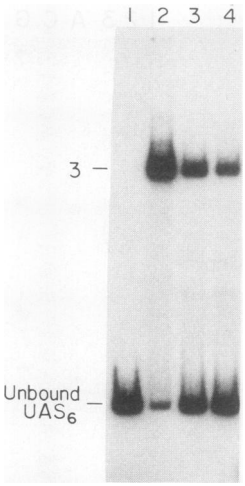

FIG. 8. Evidence that an ABFI binding site from an unrelated gene competes with domain 1 from *COX6*. A ^{32}P-labeled *Bam*HI restriction fragment containing *COX6* UAS was incubated with 80 μg of cell extract from CT155-7A cells grown in 2% lactate plus δ-ALA in the presence or absence of competitor DNA (a 288-bp fragment of plasmid pLG669Z containing sequences −382 to −620 bp upstream from the initiation codon of the *CYC1* gene [21]). Conditions for binding and electrophoresis were the same as for Fig. 4. Lanes: 1, no extract; 2, no competitor DNA; 3, 10-fold excess of competitor DNA; 4, 25-fold excess of competitor DNA.

ABF1 binds to domain 1. To determine whether ABF1 binds to domain 1, we did two experiments. First, we tested an ABF1 site from another gene for its ability to compete with domain 1 sequences in UAS_6. For this experiment, we used the ABF1 site from the *ANB1* gene as unlabeled competitor DNA. The *ANB1*-ABF1 sequences used in this study have been shown previously to compete with other genes for the binding of ABF1 (9). From Fig. 8, it is apparent that increasing amounts of *ANB1*-ABF1 competitor DNA reduced the amount of the major protein-DNA complex (band C) formed with UAS_6 sequences and shifted labeled UAS DNA sequences to the bottom of the gel, where unbound UAS_6 migrates. The second experiment was designed to determine whether ABF1 is present in the retarded complexes observed in gel mobility experiments with domain 1 DNA. For this assay, we used a polyclonal antiserum to purified ABF1 protein (obtained from P. Rhode and J. Campbell, California Institute of Technology). Previously, this serum has been shown to bind ABF1-containing DNA-protein complexes and shift them to the top of the gel in gel shift assays (44). From Fig. 9, it is clear that this antiserum also shifts the position of each protein-DNA complex formed between domain 1 and proteins in extracts from glucose-repressed cells to the top of the gel. In contrast, preimmune serum had no effect. These results indicate that the anti-ABF1 serum recognizes bands C, D, and E and therefore suggest that each of these bands contains ABF1. At the moment, the identity of band N (Fig. 6) is unknown.

Domain 1 does not bind HAP2/3/4. The results presented above indicate that domain 1 can function as a HAP2/3/4-dependent UAS element and suggest that this is the only region of the *COX6* promoter that responds to this transcription factor complex. To determine whether HAP2/3/4 binds to domain 1, we performed two kinds of experiments. First, we examined whether a *hap3* mutation, which leads to reduced expression of *COX6* (Table 3), affects the DNA-protein complexes that are observed in gel retardation assays with UAS_6 or domain 1. Second, we tested for binding between domain 1 and *HAP2-lacZ* or *HAP3-lacZ* fusion proteins in crude cell extracts. From Fig. 10, it is clear

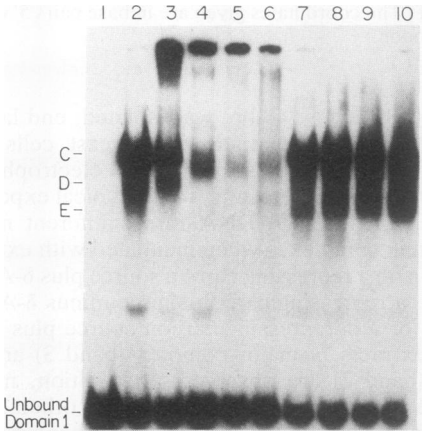

FIG. 9. Protein-DNA complexes formed with domain 1 DNA are recognized by anti-ABF1 serum. Eighty micrograms of extract protein, from CT155-7A cells grown in 8% dexrose plus δ-ALA, was incubated with a ^{32}P-labeled 32-bp double-stranded oligonucleotide corresponding to domain 1 DNA sequence and either antiserum to ABF1 (lanes 3 to 6) or preimmune serum (lanes 7 to 10). Lanes 3 to 6 received 1, 5, 10, and 20 μl of anti-ABF1 serum, respectively; lanes 7 to 10 received 1, 5, 10, and 20 μl of preimmune serum, respectively. Lane 1 received no cell extract, and lane 2 received extract and ^{32}P-labeled probe but no sera.

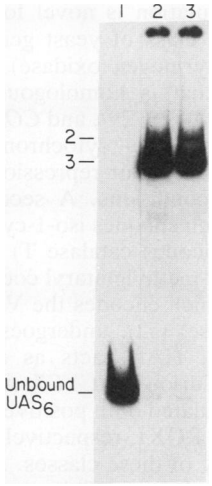

FIG. 10. Evidence that protein-DNA complexes that form with UAS_6 DNA are unaffected by a *hap3* mutation. A *Bam*HI restriction fragment containing *COX6* UAS_6 sequences was labeled with ^{32}P and incubated with 80 μg of protein from crude cell extracts of strain BWG1-7A (lane 2) or SHY40 (lane 3). Both strains were grown to late log phase in 2% dextrose and were hence derepressed. Lane 1 contained labeled probe but no cell extract.

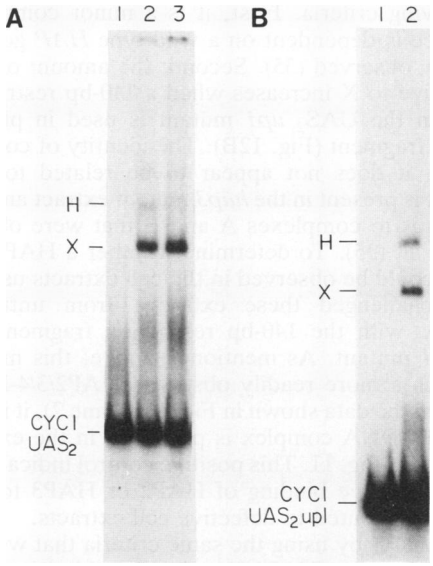

FIG. 12. Evidence that cell extracts that do not form complexes between HAP2/3/4 and domain 1 DNA are competent to form complexes between HAP2/3/4 and *CYC1* UAS_2 DNA. A 140-bp *Xho*I-*Sma*I restriction fragment containing wild-type *CYC1* UAS_2 or *CYC1* UAS_2 *up1* sequences was isolated from pLG669Z or pLGΔ265 up1 (19), respectively, and end labeled with [α-^{32}P]dATP and Sequenase. An aliquot (0.5 ng) of this restriction fragment was incubated with 80 μg of protein and 5 μg of the nonspecific competitor poly(dI-dC) · poly(dI-dC) and subjected to gel shift electrophoresis as described in Materials and Methods. (A) Cell extracts were prepared from *HAP*$^+$ cells (strain BWG1-7A; lane 2) or *hap3* cells (strain JP40; lane 3) grown in YP-lactate (derepressing medium). Lane 1 received no cell extract. (B) The cell extract from CT155-7A (lane 2), a *HAP*$^+$ *hem1* strain, that was used for the experiment shown in Fig. 11 was used. Lane 1 received no cell extract.

that the pattern of DNA-protein complexes that are observed with UAS_6 are the same in wild-type and *hap3* mutant cell extracts. This finding suggests that the major DNA-protein complexes that we observed in our gel shift assays do not contain the HAP2/3/4 complex. For the second experiment, we made use of fusion proteins containing the coding regions of *HAP2* and *HAP3* fused in frame to the coding region from the *E. coli lacZ* gene. These fusion proteins are under the control of the *GAL4* promoter and

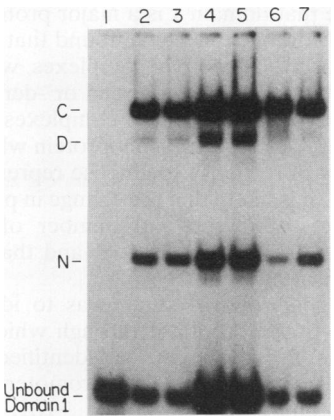

FIG. 11. Evidence that *HAP2-lacZ* and *HAP3-lacZ* fusion proteins do not bind domain 1 DNA. A ^{32}P-labeled 32-bp double-stranded oligonucleotide corresponding to domain 1 DNA sequence was incubated with 80 μg of cell extract protein from CT155-7A cells grown in a derepressing carbon source (galactose) plus δ-ALA and carrying a *HAP2-lacZ* fusion plasmid (pJO61; lane 2) or a *HAP3-lacZ* fusion plasmid (pSH152; lane 3). Lane 1 contained labeled oligonucleotide but no cell extract; 4 and 5 are identical to lanes 2 and 3 except they contained five times the amount of labeled probe; lanes 6 and 7 used extracts made from untransformed CT155-7A cells grown on YP–8% dextrose and YP–2% lactate, respectively. These control cells did not carry fusion plasmids.

have been shown previously to bind to UAS_2 of the *CYC1* gene (22, 35) and produce distinct and slower-migrating bands in gel retardation assays. For our experiments, the *HAP2-lacZ* fusion plasmid pJO61 and the *HAP3-lacZ* fusion plasmid pSH152 were transformed into strain CT155-7A. Cells were grown in derepressing medium containing galactose (to induce expression) and δ-ALA. As seen in Fig. 11, no new bands were seen with extracts from cells carrying either plasmid when compared with untransformed CT155-7A.

As a positive control for this experiment, we examined whether a HAP2/3/4-DNA complex could be detected in our extracts from CT155-7A cells with DNA sequences that are known to bind HAP2/3/4. For this control, we used a 140-bp restriction fragment that contains UAS_2 from the yeast *CYC1* promoter. Previously, it has been observed that the UAS_2 sequences in this fragment bind HAP2/3/4. This binding is abolished in cells that carry mutations in the *HAP2* or *HAP3* gene and is enhanced when the UAS_2 sequences carry the *up1* mutation (35). To identify a HAP2/3/4-DNA retarded complex, we first used the 140-bp fragment from a wild-type *CYC1* gene and extracts from isogenic *HAP*$^+$ and *hap3* strains. From the gel shift experiment shown in Fig. 12A, it is clear that two retarded complexes are observed. The minor complex, H, is present in the *HAP*$^+$ extract but not in the *hap3* extract, whereas the major complex, X, is present in both extracts. Complex H is a HAP2/3/4-DNA complex by

the following criteria. First, it is a minor complex whose appearance is dependent on a wild-type *HAP* genotype, as previously observed (35). Second, the amount of this complex relative to X increases when a 140-bp restriction fragment from the UAS_2 *up1* mutant is used in place of the wild-type fragment (Fig. 12B). The identity of complex X is unknown; it does not appear to be related to HAP2/3/4 because it is present in the *hap3* mutant extract and probably corresponds to complexes A and B that were observed by Olesen et al. (35). To determine whether a HAP2/3/4-DNA complex could be observed in the cell extracts used for Fig. 11, we challenged these extracts, from untransformed CT155-7A, with the 140-bp restriction fragment from the UAS_2 *up1* mutant. As mentioned above, this mutant fragment gives a more readily observed HAP2/3/4-DNA complex. From the data shown in Fig. 12B (lane 2), it is clear that a HAP2/3/4-DNA complex is produced in the extracts that were used for Fig. 11. This positive control indicates that our failure to observe binding of HAP2 or HAP3 to domain 1 cannot be attributed to defective cell extracts.

In summary, by using the same criteria that were used to establish that the HAP2/3/4 complex binds to UAS_2 of *CYC1*, we found that it does not bind to domain 1 of *COX6*. Because this site contains the HAP2/3-responsive element of the *COX6* promoter, it is likely that the HAP2/3/4 complex affects *COX6* transcription indirectly, via another transcription factor whose expression or function it modulates.

DISCUSSION

In this study, we have analyzed *cis* elements that are involved in the regulation of *COX6* transcription. By using linker scanning mutagenesis, we have identified four elements that are important. Two of these, HDS1 and HDS2, mediate regulation of *COX6* by heme. The third element, domain 2, is required for maximal transcription in cells grown on a repressing carbon source but appears to be nonfunctional in cells grown on a derepressing carbon source. The fourth element, domain 1, is essential for transcription in cells grown on a repressing carbon source and is required for optimal transcription in cells grown on a derepressing carbon source. The major protein binding sites of UAS_6 lie within this element.

Previously, we have found that *COX6* expression is regulated positively by heme and that the sequences that mediate part of this regulation are within UAS_6 (50, 51). In these previous studies, we used both high- and low-copy-number plasmids and found similar results. Here, using high-copy-number plasmids carrying LS mutations, we have localized the *cis* elements that are involved in this regulation more precisely. One of these sites, HDS1, lies within UAS_6, between −269 and −251 bp. Mutations in this site produced a substantial increase in expression in heme-deficient but not in heme-sufficient cells. The other site, HDS2, is located downstream of UAS_6, between −228 and −220 bp. Mutations at this site produced an increase in expression in heme-deficient cells and a smaller increase in expression in heme-sufficient cells. No sequence similarity between these two heme-dependent sites and other characterized heme elements, such as those in front of *CYC1* (38), *CYC7* (38, 65), *ANB1* (27), or *COX5b* (25), is apparent. Neither sequence acts positively in the presence of heme. Instead, both appear to be sites through which a negative factor(s) acts in the absence of heme. The presence of heme apparently abolishes negative action through these sites and enables heme-dependent activation of *COX6* expression. This type of heme-dependent regulation is novel for yeast genes regulated by heme. One class of yeast genes, *HEM13* (which encodes coproporphyrinogen oxidase) (64), *ANB1* (which encodes a protein that is homologous to a translational intiation factor, eIF4d) (28, 29), and *COX5b* (which encodes the Vb subunit isoform in cytochrome *c* oxidase) (52), undergoes a heme-dependent repression, i.e., an inactivation under aerobic conditions. A second class of genes, including *CYC1* (which encodes iso-1-cytochrome *c*) (19, 37, 38), *CTT1* (which encodes catalase T) (56), *HMG1* (which encodes 3-hydroxy-3-methylglutaryl coenzyme A reductase) (49), and *COX5a* (which encodes the Va subunit isoform in cytochrome *c* oxidase) (52), undergoes a heme-dependent activation. In these, HAP1 acts as an activator in the presence of heme. Curiously, *CYC7*, which encodes iso-2-cytochrome *c*, is regulated both positively and negatively by heme, via HAP1 and ROX1, respectively (43, 58, 65). *COX6* does not fall into any of these classes. Although its expression is increased in heme-sufficient conditions, it is not activated by HAP1 in a *CYC1*-like manner (51).

In an earlier study (50), we reported that UAS_6 sequences between −341 and −250 bp are sufficient for glucose repression-derepression of *COX6*. In this study, we have localized this regulation to domain 1, located between −302 and −281. In addition, we have shown that domain 2, located between −279 and −270 bp, is required for maximal expression in cells grown on a derepressing carbon source. The results for the LS mutants presented here allow us to conclude that glucose expression of *COX6* is not mediated solely by a transcriptional repressor because no mutation led to expression levels in glucose that were equal to the level of expression observed in cells grown on a derepressing carbon source. Therefore, derepression of *COX6* expression is probably due to a positive effector of transcription. The localization of carbon source control of *COX6* expression to domain 1 is interesting because this element binds ABF1 and contains a site, adjacent to the ABF1 binding site, through which the HAP2/3/4 complex works. By means of gel shift electrophoresis and DNase I footprinting, we have been able to demonstrate that domain 1 is a major protein binding site within UAS_6. Moreover, we have found that it forms different numbers of DNA-protein complexes when incubated with extracts from glucose-repressed or -derepressed cells. ABF1 is present in each of these complexes. Recently, we have found that ABF1 is a phosphoprotein whose phosphorylation changes in response to glucose repression-derepression (48). Thus, it is likely that the change in phosphorylation of ABF1 affects the type and number of protein-DNA complexes that form with domain 1 and that this, in turn, affects the transcription of *COX6*.

One of the goals of this study was to identify the element(s) of the *COX6* promoter through which HAP2 regulates expression. Previously, we had identified three possible HAP binding sites within the *COX6* promoter (50). These are between −332 and −325, −288 and −281, and −218 and −211 bp. From the results of our linker scanning mutagenesis study, it is clear that only the site between −288 and −281 bp is important for *COX6* expression. This site lies within domain 1 and, as shown here, functions as a HAP2/3-dependent element. This element is necessary but not sufficient for domain 1 function. In fact, it requires a functional ABF1 site, suggesting that the factor(s) that works through this site acts cooperatively with ABF1. A paradoxical aspect of our studies is that although this site mediates the activation of *COX6* by HAP2 and HAP3 and is similar in sequence to a HAP2 consensus site, it does not bind the

HAP2/3/4 complex, at least in vitro. The simplest explanation for this finding is that HAP2/3/4 does not bind sequences between −288 and −281 bp but exerts its effect indirectly by regulating either the expression or function of a factor that does bind to this site. An alternative explanation is that because the sequences within this site are not a perfect HAP2 consensus, it is a very weak binding site for HAP2/3/4, and in vitro binding is unstable and therefore not observed. In this light, it is interesting that HAP2/3/4 binding to *CYC1* UAS_2 is difficult to observe in vitro unless one uses an *up1* mutation of UAS_2 (Fig. 12) (14). Although it is possible that ABF1 helps stabilize binding of HAP2/3/4 to domain 1, this explanation is unlikely for our results because ABF1 is clearly present in our extracts. Is it possible that ABF1 competes with the HAP2/3/4 complex in vitro, under our binding conditions, but facilitates binding in vivo? Binding studies with a purified active HAP2/3/4 complex and a determination of whether and how it interacts with ABF1 will be required to resolve this question.

ACKNOWLEDGMENTS

We thank S. Silve for critically reading the manuscript. We further acknowledge M. Droste, D. Duhl, and L. Dircks for helpful discussions.

This work was supported by a gift from Leslie and Justine Bailey and by grants GM 34827, GM30228, and GM 39324 from the National Institute of Health to R.O.P.

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