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Organization of the Regulatory Region of the Yeast *CYC7* Gene: Multiple Factors Are Involved in Regulation

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Regulation of the *CYC7* gene of *Saccharomyces cerevisiae*, encoding iso-2-cytochrome *c*, was studied. Expression was induced about 20-fold by heme and derepressed 4- to 8-fold by a shift from glucose medium to one containing a nonfermentable carbon source. Deletion analysis showed that induction by heme depends upon sequences between −250 and −228 (from the coding sequence) and upon the *HAP1* activator gene, previously shown to be required for *CYC1* expression (L. Guarente et al., Cell 36:503–511, 1984). Thus, *HAP1* coordinates expression of *CYC7* and *CYC1*, the two genes encoding isologs of cytochrome *c* in *S. cerevisiae*. *HAP1-18*, a mutant allele of *HAP1*, which increased *CYC7* expression more than 10-fold, also acted through sequences between −250 and −228. In vitro binding studies showed that the *HAP1* product binds to these sequences (see also K. Pfeifer, T. Prezant, and L. Guarente, Cell 49:19–28, 1987) and an additional factor binds to distal sequences that lie between −201 and −165. This latter site augmented *CYC7* expression in vivo. Derepression of *CYC7* expression in a medium containing nonfermentable carbon sources depended upon sequences between −354 and −295. The interplay of these multiple sites and the factors that bind to them are discussed.

In *Saccharomyces cerevisiae*, cytochrome *c* exists as two isozymes, iso-1 and iso-2, that are encoded by distinct nuclear genes, *CYC1* and *CYC7*, respectively (6, 26). The primary sequences of these two cytochromes differ at approximately 16% of the amino acid residues (2). Both cytochrome products function comparably in the electron transport chain in the inner mitochondrial membrane. The reason for the existence of two isozymes of cytochrome *c* is not readily apparent. Perhaps, as for yeast enolase (4, 19) and alcohol dehydrogenases I and II (5), separate genes are differentially regulated to give separate isoforms under different physiological conditions. Consistent with this idea is the fact that the apo-iso-2-cytochrome *c* is synthesized, albeit at a low level, and is stable when cells are grown under heme-deficient conditions (anaerobic growth), whereas the apo-iso-1-cytochrome *c* is not (18). We hypothesize that the iso-2-cytochrome *c* is utilized when cells make a transition from an anaerobic to an aerobic environment. Other examples in *S. cerevisiae* in which multiple forms of a protein are encoded by separate genes are instances in which gene products are localized to different cellular compartments (30). For example, we have found that two nuclear genes encode citrate synthase. The product of *CIT1* targets to the mitochondria, whereas the product of *CIT2* is extramitochondrial (24).

Previous studies on regulation of cytochrome *c* expression in *S. cerevisiae* showed that the *CYC1* gene is regulated by levels of the cofactor heme and by the carbon source (12, 13). Expression is virtually absent under heme-deficient conditions, is induced by heme in medium containing glucose, and is derepressed further by shifting to medium containing a nonfermentable carbon source such as lactate. This regulation of *CYC1* is mediated by adjacent upstream activation sites UAS1 and UAS2 (12). UAS1 is responsible for basal levels of expression in glucose, while both sites contribute about equally to derepressed expression in lactate. If the UASs are deleted, no *CYC1* transcription occurs under any conditions. The activities of UAS1 and UAS2 depend upon distinct *trans*-acting activators. UAS1 is activated by the *HAP1* gene product, while UAS2 activity depends upon the combined action of the *HAP2* and *HAP3* gene products (12, 22, 23). We have recently shown that the *HAP1* product binds to a portion of UAS1 in vitro in a heme-dependent manner (20). This binding is evident in an acrylamide gel electrophoresis assay in which the mobility of a labeled UAS1 DNA fragment is retarded by the HAP1 protein in a yeast extract.

Studies of *CYC7* regulation indicate that this gene is also regulated by carbon catabolite control, but unlike *CYC1*, a basal level of expression is observed in the absence of heme (16). Studies of the *CYC7* regulatory region by Zitomer and colleagues have led to the proposal that the region contains separate sites that mediate positive and negative control (28, 29). Further evidence for positive control comes from the existence of a dominant mutation, *CYP1-18*, that is unlinked to the *CYC7* locus and substantially increases expression of that locus (3) acting via *CYC7* upstream regulatory sequences (14). Recent genetic analysis has shown that *CYP1-18* (here termed *HAP1-18*) is actually a mutation in the *HAP1* gene (21, 27). Biochemical analysis has shown that HAP1 binds to a site in *CYC7* upstream DNA (21).

In this report, we present an in vivo and in vitro analysis of the *CYC7* regulatory region, which had been altered by in vitro mutagenesis. The analysis was aimed at two fundamental questions. First, how is the regulation of the *CYC7* and *CYC1* genes coordinated? Second, how do regulatory sites and *trans*-acting factors interact to mediate multiple forms of physiological control?

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1. Strain 1-7a (*ura3-52 MAT*a *leu2-2 2-112 his4-519 ade6*) and its *hap1* and

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TABLE 1. Yeast strains

Strain	Relevant genotype	Source or reference
1-7a	*MAT***a** *ura3-52 leu2-3,-112 his4-519 ade1*	13
1-7a hap1	A *hap1* derivative of 1-7a	12
1-7a-hap1-D	1-7a derivative with a *LEU2* insertion in *HAP1*	20
1-7a *hem1::LEU2*	1-7a derivative with a *LEU2* insertion in *HEM1*	This study
DP31	**a**/α diploid homozygous for *HAP1-18*	P. Slonimski
TP25-4a	*MAT***a** *HAP1-18 hem1::LEU2 trp1 ura3-52*	This study
9a-1 hem1	*MAT*α *ura3-52 his4-519 ade6 hem1*	This study
LG2-1D	*MAT***a** *hap2-1 leu2-3, -12 his4-519 ade6*	12
BJWT-13a	*MAT*α *hap1 leu2-3, -12 ura3-52 trp1*	P. Drain

hap2 derivatives have been described (12; Table 1). 1-7a *hem1::LEU2* contains a *LEU2* insertion (on a *Sal*I-*Hpa*I fragment) in the *Sal*I site of *HEM1* (15). This insertion was introduced into 1-7a (25), selecting for Leu$^+$ and screening for acquisition of heme auxotrophy.

The *CYP1-18* mutation (here *HAP1-18*) has been shown to be a mutation in *HAP1* (27). The *HAP1-18* homozygous diploid strain DP31, generously provided by P. Slonimski, was allowed to sporulate, and segregants were isolated. A segregant was backcrossed to 1-7a, and the *HAP1-18* allele, segregating 2:2 in the cross, was identified as increasing expression of pTP101 2- to 3-fold in heterozygous diploids and 10- to 30-fold in haploids. One such *HAP1-18* segregant was crossed to 1-7a *hem1::LEU2*, and the *hem1* and *HAP1* markers were scored (Table 2). A segregant, TP25-4A (*MAT***a** *HAP1-18 hem1::LEU2 trp1 ura3-52*) was used in these studies.

Strain 9a-1 hem1 (*MAT*α *ura3-52 his4-519 ade6 hem1*) is a *hem1*$^-$ derivative of 9a-1 (12).

hap2 strains were obtained as segregants of an LG2-1D (*MAT***a** *hap2-1 leu2-3,-112 his4-519 ade6*)-BJWT-13A (*MAT*α *hap1 leu2-3,-112 ura3-52 trp1*) diploid. The *HAP1* and *HAP2* alleles were scored by effects on expression of *CYC1-lacZ* driven by UAS1 or UAS2 of the *CYC1* gene (see Table 5).

Media. Yeast cells were grown in a rich medium consisting of 2% yeast extract, 1% Bacto-Peptone (Difco Laboratories), and a 2% carbon source for inoculation into minimal medium for β-galactosidase assays. Minimal medium contained a 2% carbon source, required amino acids at 40 μg/ml, and adenine at 20 μg/ml. β-Galactosidase was assayed as described previously (9). The *hem1* mutants were grown in a medium supplemented with 500 ng of δ-aminolevulinate per ml for heme-deficient growth or 50 μg of δ-aminolevulinate per ml for heme-sufficient growth.

TABLE 2. Segregants of *HAP1-18* strain crossed with Hem1$^-$ strain[a]

Strain	Heme phenotype	β-Galactosidase level[b]
TP25-4A	−	216
TP25-4B	−	5
TP25-4C	+	15
TP25-4D	+	171

[a] 1-7a *hem1::LEU2* was crossed with a *HAP1-18* strain as described in Materials and Methods, and segregants of tetrads were tested for heme auxotrophy and *HAP1-18*. The *HAP1-18* allele was determined by the level of activation of a *CYC7-lacZ* gene on plasmid pTP101 in cells grown in heme-sufficient media. Thus, tetrad 4 shows 2:2 segregation of *hem1::LEU2* and the *HAP1-18* alleles.

[b] β-Galactosidase units are as previously defined (9).

TABLE 3. Plasmid characteristics

Plasmid	Upstream sequences	TATA box	Origin
pTP101	*CYC7*	*CYC7*	2μm
pTP106	*CYC7*	*CYC7*	ARS1, CEN4
pTP108	*CYC1*	*CYC7*	2μm
pTPLEU	*CYC7*	*LEU2*	2μm
pTP312G	*CYC1*, *Bgl*II linker	*CYC1*	2μm
pTP3′BZ	*CYC7*, *Bgl*II linker	*LEU2*	2μm

Plasmid constructions. The salient features of the plasmids described below are listed in Table 3.

(i) Construction of pTP101 and pTP106. To construct the *lacZ* fusion plasmid pTP101, we fused *CYC7* upstream and N-terminal coding sequences from pAB25 (a gift of B. Errede) to *lacZ* as follows (Fig. 1). First, we attached *Bam*HI linkers to an *Rsa* site at +15 in the *CYC7* coding sequence. Next, we inserted a *CYC7* fragment extending from a *Bam*HI site at −700 to the synthetic *Bam*HI site at +15 in the *CYC7* coding sequence into a backbone of pTP312G. pTP312G is identical to pLG312 (11, 13) except that it contains a *Bgl*II linker insertion at a *Sma*I site at −312 in *CYC1* upstream DNA. The backbone into which the *CYC7* fragment was inserted extended from a *Bam*HI site at the start of *lacZ* through vector sequences to the *Bgl*II site. Thus, pTP101 is analogous to pLG312 except that the DNA fused to *lacZ* consists of *CYC7* upstream sequences.

To construct pTP106, we first deleted the 2μm origin of replication from pTP101 on an *Eco*RI fragment. Next, we inserted ARS1 and CEN4 on a *Hin*dIII fragment into a unique *Hin*dIII site just upstream of the *URA3* marker.

(ii) Construction of pTPLEU. pTPLEU replaces the *CYC7* TATA box-mRNA initiation region with that of the *LEU2* gene. To construct this plasmid, pTP101 was cleaved with *Xho*I and *Sac*I, liberating the *CYC7* initiation region and the first one-third of the *CYC7-lacZ* fusion. A *Xho*I-*Sac*I fragment from pLG3 (12) containing the *LEU2* initiation region and *LEU2-lacZ* sequences was inserted into the pTP101 backbone.

(iii) Construction of pTP108. In pTP108 the TATA box-mRNA initiation region of *CYC1* is replaced with that of *CYC7*. To construct this plasmid, the *Xho*I-to-*Sac*I fragment of pTP312G containing the *CYC1* TATA box-mRNA initiation region and first one-third of *CYC1-lacZ* was replaced with a *Xho*I-to-*Sac*I mRNA fragment of pTP101 containing the *CYC7* TATA box-mRNA initiation region and the first one-third of *CYC7-lacZ*. Like pTP312G, this plasmid contains a *Bgl*II site at −312 in *CYC1* upstream DNA.

(iv) 5′ deletions. 5′ deletions were made in two sets. Set 1 contains the *CYC7* TATA box-mRNA initiation region, and set 2 contains the *LEU2* mRNA initiation region. To construct set 1, *CYC7* upstream DNA was digested with *Bam*HI at −700, treated with *Bal31*, and, after *Bgl*II linkers were attached, digested with *Xho*I at −142. Deleted DNA was inserted into a backbone of pTP108 extending from the *Xho*I site through the *CYC7-lacZ* fusion to the *Bgl*II site.

To construct set 2, a plasmid analogous to pTPLEU was constructed by using, instead of pTP101, a deletion derivative of set 1 with *CYC7* sequences extending out to −354

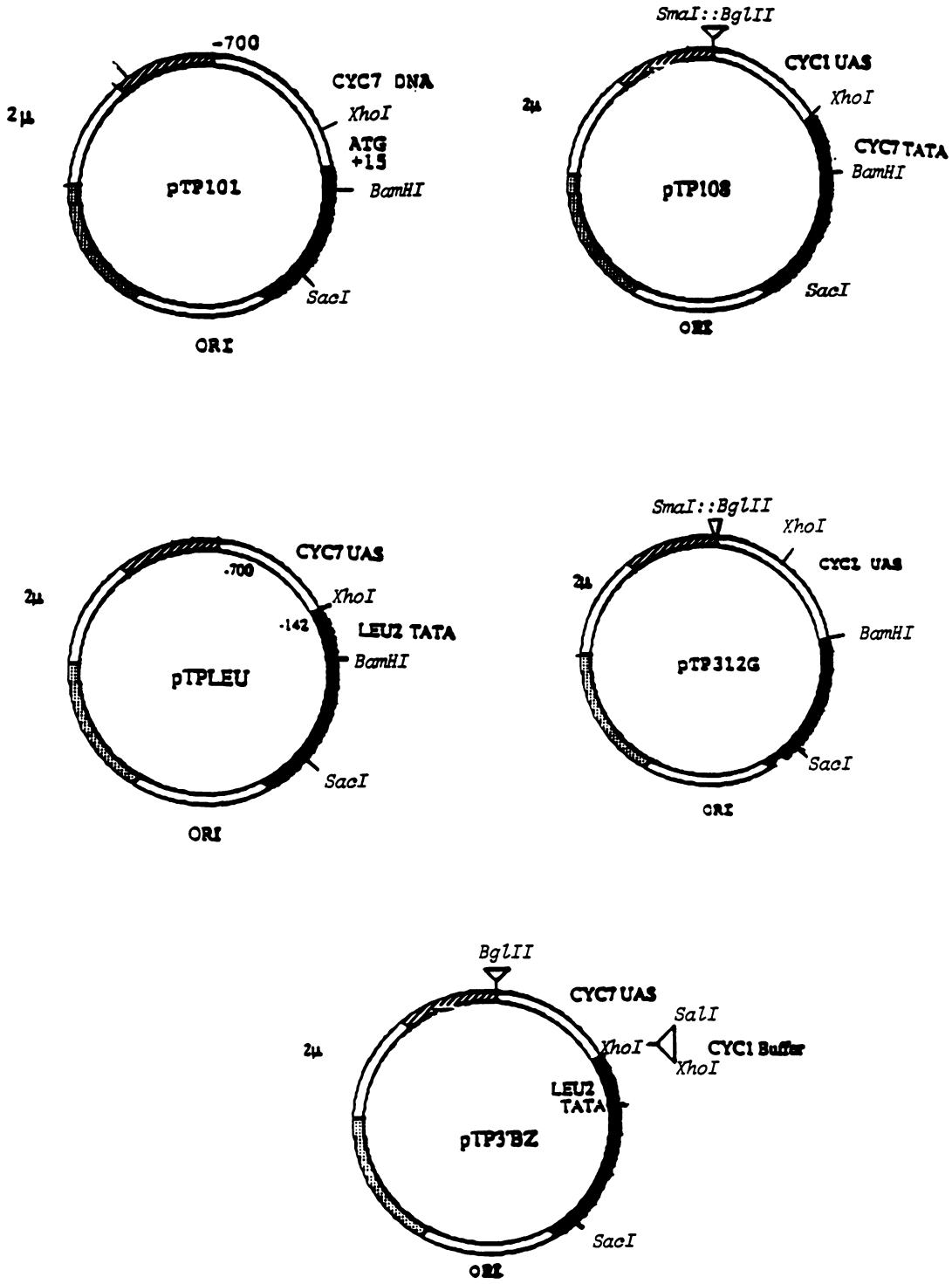

FIG. 1. Structure of *CYC7-lacZ* fusion-bearing plasmids. The plasmids were constructed as described in Materials and Methods. All carry the yeast 2μm origin (the 2.2-kilobase *Eco*RI fragment) and *URA3* marker (▨), the bacterial Amp^r gene (▩) and origin (ORI from pBR322), and various *lacZ* gene fusions (■). pTP101 carries a *CYC7-lacZ* fusion preceded by 700 base pairs of *CYC7* upstream DNA. pTP108 bears a *CYC7-lacZ* fusion with *CYC7* DNA extended out to −142. Upstream of this DNA is a *Sma-Xho* fragment bearing the *CYC1* UAS region. pTPLEU bears a *LEU2-lacZ* fusion with *LEU2* DNA extending out to −125. This segment is preceded by *CYC7* sequences extending from −142 to −700. pTP312G bears a *CYC1-lacZ* fusion preceded by *CYC1* upstream sequences. A *Bgl*II linker has been inserted into the *Sma*I site of this plasmid, as well as into pTP108. pTP3′BZ is analogous to pTPLEU except that *CYC7* upstream sequences extend only to −273. The plasmid has a *Bgl*II linker inserted at that site and a 300-base-pair buffer region for the construction of deletions.

TABLE 4. Regulation of *CYC7* expression[a]

Strain	Plasmid	β-Galactosidase level[b] on medium with:			
		Glucose (−heme)	Glucose	Glucose (+dp)	Lactate
1-7a	pTP101	0.3	5.5	25	40
	pLG312	0.04	140	270	460
1-7a hap1	pTP101	—[c]	0.8	0.8	4.2
	pTPLEU	—	0.6	—	—
9a-1 hem1	pTPLEU	0.2	5.0	—	—

[a] Strain 1-7a and its isogenic *hap1* derivative bearing plasmid pTP101, pLG312, or pTPLEU were grown in minimal medium containing 2% glucose or lactate. Heme-deficient cells were obtained by growing strain 9a-1 hem1 and an isogenic *hem1* derivative of 1-7a (1-7a *hem1*::*LEU2*) in medium containing 5 ng of δ-aminolevulinate per ml or supplemented with Tween 80, ergosterol, and methionine (see Materials and Methods). Deuteroporphyrin IX (dp), a heme analog previously shown to specifically derepress activity from UAS1 of *CYC1* (12), was added to 12 μg/ml. β-Galactosidase assays were performed as described in Materials and Methods.

[b] β-Galactosidase units are as previously defined.

[c] —, Activity was not determined.

only. This plasmid, pTP(−354L), was used as a starting point to construct additional 5′ deletions by the protocol described for set 1.

(v) 3′ deletions. To construct 3′ deletions which would fuse *CYC7* DNA to the *LEU2* TATA box-mRNA initiation region, we constructed plasmid pTP3′BZ (Fig. 1). This plasmid is analogous to pTPLEU except that it bears *CYC7* DNA extending out to −273 (a deletion endpoint isolated from set 2 described above) and contains a *Bgl*II linker at this site (at the *URA3-CYC7* boundary). To construct 3′ deletions, pTP3′BZ was digested with *Sal*I and *Bal31*, and *Xho*I linkers were attached. The deletion plasmids were then digested with *Xho*I and ligated to delete the buffer zone fragment. DNA was then digested with *Bgl*II, and the *CYC7* upstream DNA fragments were recloned into the pTP3′BZ *Xho*I-to-*Bgl*II backbone. DNA sequencing at the junctions of all the deletions described in this report was performed.

Probes for gel electrophoresis DNA-binding assay. *CYC7* probes were prepared by digesting appropriate deletion constructs with *Xho*I and *Bgl*II and end labeling with Klenow fragment and [α-^{32}P]TTP by standard methods (17). The probe was purified by polyacrylamide gel electrophoresis before use.

Gel electrophoresis DNA-binding assay. Protein-DNA complexes were resolved on high-ionic-strength polyacrylamide gels as previously described (20). Binding reactions were carried out in 20-μl volumes containing 4 mM Tris (pH 8.0), 40 mM NaCl, 4 mM $MgCl_2$, 5% glycerol, 0.5 ng of radiolabeled DNA probe, 1 μg of sonicated salmon sperm DNA, 40 μM hemin, and 10 μg of proteins. Reaction mixes were incubated for 25 min at 23°C and loaded immediately onto a 4% polyacrylamide gel in TBE buffer (90 mM Tris hydrochloride, 90 mM H_3BO_3, 2.5 mM EDTA). Electrophoresis was done at 25 mA until the bromophenol blue had run to the gel bottom. The gels were then transferred to Whatman 3MM filter paper, dried, and autoradiographed.

Extract preparation. Extracts were prepared as described previously (20). Cells grown to an A_{600} of 1.0 were harvested by centrifugation, suspended in extraction buffer [200 mM Tris hydrochloride (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 7 mM 2-mercaptoethanol], and disrupted by agitation with glass beads. Extracts were centrifuged for 1 h at 10,000 × *g*. The supernatant was collected and precipitated by the addition of 100% $(NH_4)_2SO_4$ in protein buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 5 mM EDTA) to a final $(NH_4)_2SO_4$ concentration of 40%. The protein pellet was suspended in protein buffer containing 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 7 mM 2-mercaptoethanol.

RESULTS

***CYC7* is regulated by catabolite repression and heme.** To quantitate the degree of regulation of *CYC7* by catabolite repression and heme, we constructed a gene fusion of the first five codons of *CYC7* to *lacZ*. This fusion, preceded by about 700 nucleotides of *CYC7* upstream sequences, was placed on a multicopy plasmid (pTP101) (see Materials and Methods; Fig. 1) and introduced into strain 1-7a *hem1*::*LEU2*. β-galactosidase levels for pTP101 and pLG312, a multicopy yeast plasmid bearing a *CYC1-lacZ* fusion (13), were determined under a variety of growth conditions. *CYC7* was regulated both by catabolite repression and by the availability of heme (Table 4). In the absence of heme, low basal levels of transcription were observed that were induced 17-fold by heme addition (Table 4). A shift from glucose- to lactate-containing medium resulted in an additional four- to sixfold derepression (Table 4). Derepression of a similar magnitude could be elicited by addition of the heme analog, deuteroporphyrin IX, previously shown to derepress UAS1 of *CYC1*. The same regulation was observed for a single-copy plasmid, pTP106, bearing a *CYC7-lacZ* fusion and for fusions integrated into the chromosome at *CYC7* (data not shown). Thus, although expressed at only 5% of the levels of *CYC1*, *CYC7* exhibited a pattern of regulation much like that of *CYC1*. One distinguishing feature of *CYC7* control was that pTP101 expression in the absence of heme was not completely abolished, providing a low basal level of the iso-2-cytochrome *c*.

***CYC7* expression requires the *HAP1* product.** Next we examined whether *CYC7* expression from pTP101 depends upon the *CYC1* activator locus *HAP1*, required for UAS1 activation, or *HAP2*, required for UAS2 activation. While the *hap2* mutation did not affect levels of expression of *CYC7* (Table 5), mutations in *HAP1* reduced expression about 10-fold, to levels observed in a *HAP1* strain deprived of heme (Tables 4 and 5). Thus, the *HAP1* regulatory gene links the expression of the two genes encoding cytochrome *c* in *S. cerevisiae*.

We wished to determine whether *HAP1* or other loci mediated regulation of *CYC7* by heme and carbon source. Thus, we compared the ability of the gene fusion to derepress in *HAP1* and *hap1* strains in lactate or glucose medium

TABLE 5. Expression in *HAP1* and *HAP2* mutants[a]

Strain	*HAP1*	*HAP2*	β-Galactosidase level[b]		
			pTP101	UAS1	UAS2-UP1
TP10-6A	+	+	9.2	189	23
TP10-8A	−	+	.49	2.4	11
TP10-8B	−	+	.51	4.0	12
TP10-8C	+	−	20	40	1.4
TP10-8D	+	−	14	39	.54

[a] LG2-1D (*hap2*) and BJWT-13A (*hap1*) were crossed as described in Materials and Methods. Segregants were transformed with plasmids containing UAS1 or UAS2-UP1 (12) driving expression of *CYC1-lacZ* to score for *HAP1* and *HAP2*, respectively. Both *hap1-1* and *hap2-1* segregated 2:2 in tetrad 8 and other tetrads not shown. TP10-6A is a *HAP1 HAP2* segregant from tetrad 6.

[b] β-Galactosidase units are as previously defined (9).

TABLE 6. 5′ deletions in the *CYC7* promoter[a]

Deletion	β-Galactosidase level[b]			
	HAP1		*HAP1-18*	
	Glucose	Lactate	Glucose (heme sufficient)	Glucose (heme deficient)
−354	2.7	11	125	
−352			165	
−347			53	
−340			65	
−334	0.5	3.3	37	
−329			65	
−328			45	
−312	1.0	2.5	58	
−311			107	
−295	1.4	1	57	
−282	1.1	0.4	45	
−273	0.7	0.2	36	0.1
−262	0.6	0.3	10	
−250	0.3	0.3	20	0.2
−246			1.2	
−225			0.2	
−201	0.1		0.1	

[a] Deletions with indicated endpoints were constructed as described in Materials and Methods. Plasmids bearing deletions were introduced into BWG1-7a (*HAP1*) or TP25-4A (*HAP1-18*).
[b] β-Galactosidase units are as previously defined (9).

supplemented with the heme analog deuteroporphyrin IX (Table 4). No induction by deuteroporphyrin IX was observed in a *hap1* background. Rather, levels like those seen in a heme-deprived strain were observed. However, induction of comparable magnitude in lactate was observed in *hap1* and *HAP1* strains, although the overall levels remained low in *hap1* cells. These data suggest that heme induction is mediated by the *HAP1* locus but other factors may play a role in lactate derepression of *CYC7*.

***CYC7* regulation occurs via upstream sequences.** Regulation of many yeast genes is known to occur via upstream activation sites (10). To determine whether this is also the case for *CYC7*, a hybrid promoter was constructed consisting of the TATA box-mRNA initiation region of the *LEU2* gene, preceded by *CYC7* sequences that reside upstream of a *Xho*I site at −142. This *LEU2* segment does not function unless an upstream activation site is provided (12). The levels of expression, and the requirements of this hybrid promoter for heme and *HAP1*, on plasmid pTPLEU were identical to those on pTP101 (Table 4). Thus, sequences upstream of the *Xho*I site at −142 determine the levels of expression of *CYC7* and regulation of the gene by heme levels and *HAP1*.

Mapping the HAP1-responsive site in the *CYC7* regulatory region. To map more precisely the upstream sequences that

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-354          -340          -320          -300
AGCAGCCGGGTTATAGCGCCCCTTATTGAATTATTTTCCTTCGTGCCTTCTCTGA
              -280          -260          -240
GAAGGGTCTGCAGTCCCCCGCCGAGGGGTCTTTTCCCACCTTCTCAAAGCTAATAGCGAT
              -220          -200          -180
AATAGCGAGGGCATTTATTCAAGTTCCAACTACTATAAGTGGCCGCAAGGGGCAAAGACA
              -160          -140
AAGGCACACAACATATATATATATCGTGTTGTGAAGCTCGAG
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FIG. 2. *CYC7* upstream DNA sequence. Sequences from −354 to the *Xho*I site at −142 are shown. Underlined sequences indicate the HAP1-binding site previously identified (22).

TABLE 7. 3′ deletions of the *CYC7* promoter[a]

Deletion	β-Galactosidase level[b] with *HAP1-18* on glucose medium:	
	Heme sufficient	Heme deficient
−143	70	<0.1
−147	57	<0.1
−160	51	
−164	57	
−165	37	<0.1
−168	62	
−171	67	
−172	45	
−173	34	
−182	29	
−186	13	<0.1
−187	11	
−188	12	<0.1
−193	7	<0.1
−194	6	<0.1
−195	6	<0.1
−196	9	
−212	13	<0.1
−213	16	<0.1
−214	13	<0.1
−228	2	<0.1
−249	0.2	
−259	0.2	
−263	0.2	

[a] 3′ deletions were constructed as described in Materials and Methods. Plasmids bearing deletions were introduced into TP25-4A, and β-galactosidase levels were assayed.
[b] β-Galactosidase levels are as previously defined.

mediate regulation of *CYC7*, we constructed an extensive series of 5′ and 3′ deletions as described in Materials and Methods. The 5′ deletions begin at a site 354 nucleotides upstream of the AUG codon (Fig. 2). *CYC7* sequences between the deletion endpoint and the *Xho*I site at −142 were fused to the TATA box-mRNA initiation region of the *LEU2* gene, in turn, fused to *lacZ*. 3′ deletions extend upstream from the *Xho*I site. DNA between −273 and the 3′ deletion endpoints was fused to *LEU2* sequences as described above.

These deleted constructs were introduced into strains bearing the *HAP1* or *HAP1-18* allele. The *HAP1-18* mutation is a dominant mutation of *HAP1* that results in higher expression of *CYC7* (Table 2; 3, 21, 27). The higher levels of expression resulting from the *HAP1-18* mutation increased the sensitivity of the assay with mutant constructs. As shown below, *HAP1-18* does not alter regulation of *CYC7*. We believe that it simply reflects a stronger interaction between *HAP1* and its target sequence. An analysis of 5′ deletions indicated that multiple regions were involved in the expression of *CYC7* (Table 6). Deletions of sequences between −352 and −347 gave rise to about a threefold decrease in expression. Thus, a site designated A1, with a 5′ border between −352 and −347, augments *CYC7* expression. Interestingly, −350 to −345 contains the sequence GCCGGG (Fig. 2), a portion of the HAP1-binding sequence in UAS1 (20). (The removal of sequences between −700 and −352 as a prelude to the construction of our deletion sets resulted in a two- to threefold reduction in expression. It is likely, therefore, that sequences that are necessary for optimal levels of expression of *CYC7* extend even further upstream than −352.) Several deletions ending between −340 and −273 did not give any consistent further decrease in expression. A deletion ending at −262 resulted in a fourfold further

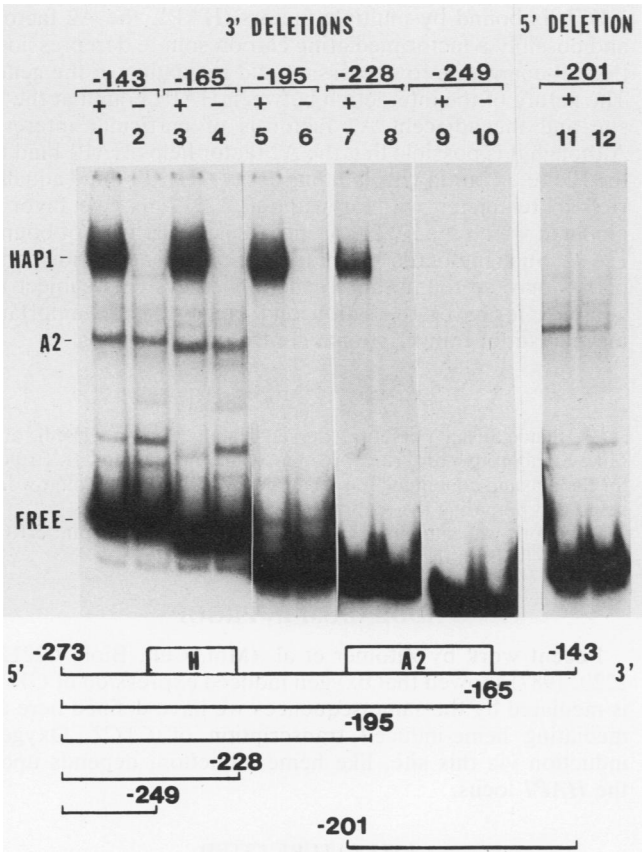

FIG. 3. Detection of protein-DNA complexes by using crudely fractionated yeast extracts. Extracts prepared from *hap1* cells (lanes 2, 4, 6, 8, 10, and 12) and *HAP1* cells carrying pHAP1 (lanes 1, 3, 5, 7, 9, and 11), a high-copy-number plasmid bearing the *HAP1* gene, were assayed for binding to sets of radiolabeled deletion fragments. Binding reactions were carried out in the presence of 40 μM hemin and 10 μg of salmon sperm DNA. The diagram at the bottom shows the parent plasmid (top line) and the four 3′ and one 5′ deletion fragments assayed. Each line represents the region of *CYC7* UAS DNA present in the deletion fragment. The H site defines the *HAP1*-responsive site in vivo and HAP1-binding site in vitro (21). This site also mediates heme induction in vivo. The A2 box is defined by in vivo augmentation of *HAP1*-dependent expression and by in vitro binding of a *HAP1*-independent binding factor.

reduction in activity, while a deletion ending at −246 was extremely defective in activity. We conclude that a region critical to *CYC7* activity has a 5′ border between −262 and −246 (the H site). Because constructs retaining the H site still responded to *HAP1-18*, we infer that HAP1 acts via this site.

3′ deletions mapped sequences required for expression to the H site. Deletions with endpoints between −143 and −182 did not cause any consistent reduction in expression (Table 7). Deletions ending between −186 and −214 were variable in their effects, giving rise, on average, to a fivefold decrease in activity. Deletion to −228 caused another large reduction in expression, while deletions to −249 or further abrogated expression. These experiments suggest that sequences important in *CYC7* expression extend as far downstream as −182. However, constructs retaining sequences downstream of −228 still responded to *HAP1-18* (data not shown). Thus, our interpretation of the 3′ deletion data, borne out by in vitro experiments described below, is that the 3′ border of the H site lies between −214 and −228 while an additional site that augments expression (A2) lies between −182 and −214.

Mapping sequences responsive to heme induction and catabolite control. As noted above, induction by heme but not by a shift to growth in a nonfermentable carbon source was blocked in a *hap1* strain. This finding suggested that additional factors beyond HAP1 are involved in catabolite regulation, whereas HAP1 might mediate heme induction. We therefore wished to determine the location of *CYC7* sequences mediating these forms of derepression relative to the *HAP1*-responsive site (H site).

The 3′ deletion analysis located sequences responsible for heme induction upstream of −228 (Table 7), while 5′ deletion analysis showed that sequences downstream of −250 suffice for heme induction (Table 6). Thus, the H site and the heme induction site map to the same region.

The 5′ deletion analysis (Table 6), however, showed that sequences involved in lactate derepression were distinct from the H site. Derepression was about 4-fold in strains bearing constructs deleted to −354, was reduced to about 2.5-fold in a strain with a construct deleted to −312, and was abolished in a strain with a construct deleted to −295. Thus, we conclude that sequences involved in catabolite derepression lie between −354 and −295. Present in this interval is the repeated sequence CCTTC at −317 to −313 and at −309 to −305 (Fig. 2).

Binding of factors to regions H and A2. We wished to determine whether HAP1 bound to regions A1, H, or A2 identified above. We therefore tested DNA fragments in the gel retardation assay (7, 8). This assay allows the detection of any factors in yeast extracts that bind to these regions. The results of this assay performed on a series of deletions across the H and A2 sites are shown in Fig. 3. Crudely fractionated yeast extracts from strains containing *HAP1* on a high-copy-number plasmid (pHAP1) or mutant in *HAP1* were mixed with these DNAs, and binding was assayed (1).

Two primary complexes were observed. One complex was formed regardless of whether a *HAP1* or *hap1* strain was used to prepare the extracts. Formation of this complex was due to the binding of a factor to sequences in the A2 region. A 3′ deletion ending at −165 left binding intact, whereas a deletion extending to −195 abolished binding. 5′ deletions mapped the site bound by this factor to sequences downstream of −201. We presume that this factor is responsible for the activity of the A2 site in vivo. High-mobility complexes seen in this assay were not competed with unlabeled *CYC7* DNA and were not studied further.

A second complex, labeled HAP1, has been analyzed in detail and was recently shown to be due to the binding of the *HAP1* gene product to sequences between −251 and −229 (21). Binding of HAP1 to these sequences was highly stimulated by heme. Thus, the H site responds to *HAP1* and to heme levels in vivo and binds the *HAP1* product in vitro in a heme-dependent manner. Attempts to demonstrate the binding of HAP1 to the A1 site have yielded inconsistent results, which are not presented.

DISCUSSION

In this study, we examined control of the *CYC7* gene of *S. cerevisiae*. We showed that, like *CYC1*, *CYC7* is regulated by two signals, heme and carbon catabolite control. Induction by heme was abolished by a loss-of-function mutation in the *HAP1* gene, which encodes the activator for the upstream activation site UAS1 of the *CYC1* gene. Thus, the *HAP1* product coordinates the induction by heme of the two

yeast genes encoding cytochrome *c*. This result clarifies previous findings that a dominant *HAP1* mutation, *1-18*, results in a large increase in *CYC7* expression (3).

In the case of UAS1 of *CYC1*, *hap1* mutants were totally defective in transcription under all physiological conditions. For *CYC7*, *hap1* mutants or wild-type cells grown under heme-deficient conditions retained a low residual activity. This activity was subject to derepression when *hap1* cells were shifted from medium containing glucose to one with a nonfermentable carbon source such as lactate. Thus, control of *CYC7* by heme via *HAP1* and control by carbon source appear to be distinct. Our 5′ deletion data suggest that carbon source control is mediated by a site with a 5′ border between −354 and −295 (from the coding sequence). Deletion of this site prevented derepression of *CYC7* in lactate. We suggest that a factor separate from HAP1 interacts with this region to augment expression of *CYC7* when cells grow in nonfermentable carbon sources. We have not, as yet, identified this factor or any gene that might encode it. It is conceivable that the sequence CCTTC, appearing twice in this region, at −317 and −309, is a functional component at this site (Fig. 2). Indeed, a 5′ deletion ending at −312 removes one of these pentamers and was intermediate in its ability to be derepressed. It is possible that HAP1 itself also plays a role in catabolite control. The *hap1* strain did not derepress in lactate medium to levels observed in the wild type. Furthermore, extracts prepared from lactate-grown cells contain higher levels of *HAP1*-binding activity as measured by gel electrophoresis DNA-binding assays (K. Pfeifer and L. Guarente, unpublished data).

5′ and 3′ deletions were used to map the *HAP1*-responsive site, termed H, to sequences between −250 and −214. Deletion of the H site reduced *CYC7* expression to undetectable levels in glucose media. Constructs which deleted all DNA upstream of the H site, but left the H site intact, displayed a reduced level of expression but still responded to the *HAP1-18* mutation, indicating that the *HAP1* product interacts with the H site. Such constructs all responded to heme induction. Moreover, in vitro binding experiments using yeast extracts and labeled H-site DNA clearly showed that a protein bound to the H site in extracts prepared from *HAP1* but not *hap1* cells. We have recently shown that this protein is encoded by *HAP1* and depends upon heme in vitro for its binding (21). By DNAase I and methylation interference footprinting, we localized the HAP1-binding site to the 23-base-pair region from −229 to −251. The binding site is centered on the 9-base-pair direct repeat TAATAGCGA (Fig. 2).

The activity of the H site was augmented about fivefold by sequences between −214 and −182, the A2 site. In vitro binding experiments identified a factor that bound to A2. This factor is not HAP1, since it could be found in extracts prepared from *hap1* mutant cells.

The H site delineated here is part of a region previously identified by Wright and Zitomer (29) as crucial to positive control of *CYC7* expression. Wright also identified by 5′ deletion analysis and by construction of internal deletions a negative site located at −300, the removal of which elevated expression. Our data appears to be at odds with these findings (Table 6) and instead suggest that these sequences actually play a role in positive regulation of expression in cells growing on a nonfermentable carbon source. The reason for this discrepancy is unclear but may relate to differences in the yeast strains used in the two studies.

The *CYC7* UAS, like UAS1 (21) and UAS2 (S. Hahn, J. Olesen, S. Forsburg, and L. Guarente, unpublished data) of *CYC1*, is bound by multiple factors (HAP1, the A2 factor, and possibly a factor mediating carbon source derepression) that all contribute to expression and regulation of the gene. The nature of the interaction between HAP1 bound at the H site and the adjacent A2 factor is of particular interest. Although it is possible that the A2 factor helps HAP1 bind to the H site, in our in vitro binding assay, HAP1 bound equally well to templates with or without A2. Thus, we favor a model in which the A2 factor increases the activity of bound HAP1. Such multicomponent transcriptional activation complexes may be found more generally in systems subject to complex forms of regulation and constitute an important mechanism of transcriptional control in eucaryotes.

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ADDENDUM IN PROOF

Recent work by Zitomer et al. (Mol. Cell. Biol. **7**:2212–2220, 1987) showed that oxygen induced expression of *CYC7* is mediated by the same sequences we have defined here as mediating heme-induced transcription of *CYC7*. Oxygen induction via this site, like heme induction, depends upon the *HAP1* locus.

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