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The CCAAT Box-Binding Factor Stimulates Ammonium Assimilation in *Saccharomyces cerevisiae*, Defining a New Cross-Pathway Regulation between Nitrogen and Carbon Metabolisms

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In *Saccharomyces cerevisiae*, carbon and nitrogen metabolisms are connected via the incorporation of ammonia into glutamate; this reaction is catalyzed by the NADP-dependent glutamate dehydrogenase (NADP-GDH) encoded by the *GDH1* gene. In this report, we show that the *GDH1* gene requires the CCAAT box-binding activator (HAP complex) for optimal expression. This conclusion is based on several lines of evidence: (i) overexpression of *GDH1* can correct the growth defect of *hap2* and *hap3* mutants on ammonium sulfate as a nitrogen source, (ii) Northern (RNA) blot analysis shows that the steady-state level of *GDH1* mRNA is strongly lowered in a *hap2* mutant, (iii) expression of a *GDH1-lacZ* fusion is drastically reduced in *hap* mutants, (iv) NADP-GDH activity is several times lower in the *hap* mutants compared with that in the isogenic wild-type strain, and finally, (v) site-directed mutagenesis of two consensual HAP binding sites in the *GDH1* promoter strongly reduces expression of *GDH1* and makes it HAP independent. Expression of *GDH1* is also regulated by the carbon source, i.e., expression is higher on lactate than on ethanol, glycerol, or galactose, with the lowest expression being found on glucose. Finally, we show that a *hap2* mutation does not affect expression of other genes involved in nitrogen metabolism (*GDH2*, *GLN1*, and *GLN3* encoding, respectively, the NAD-GDH, glutamine synthetase, and a general activator of several nitrogen catabolic genes). The HAP complex is known to regulate expression of several genes involved in carbon metabolism; its role in the control of *GDH1* gene expression, therefore, provides evidence for a cross-pathway regulation between carbon and nitrogen metabolisms.

Like many other microorganisms, *Saccharomyces cerevisiae* can utilize ammonium as a sole source of nitrogen. In baker's yeast, utilization of ammonia occurs exclusively via its incorporation into glutamate and glutamine (37). This process occurs in two ways. The first is a combination of two successive reactions catalyzed by the glutamine synthetase (16, 41) and the glutamate synthase (52), respectively. This pathway is of minor importance, as shown by the fact that mutants lacking glutamate synthase activity grow as well as wild-type cells in medium containing ammonium sulfate as the sole nitrogen source (39). The second pathway, which combines two reactions catalyzed, respectively, by NADP-linked glutamate dehydrogenase (NADP-GDH, product of the *GDH1* gene) (42, 44) and glutamine synthetase, constitutes the major pathway for the assimilation of ammonia. In ammonium sulfate medium, mutants lacking NADP-GDH activity grow at only about half the rate of wild-type cells (25).

The reductive amination of α-ketoglutarate by NADP-GDH is not only a key step for ammonia utilization but also an important connection point between carbon metabolism and nitrogen metabolism. For these reasons, regulation of *GDH1* gene expression is an important and probably complex issue. Activation of *GDH1* expression by the transcription factor Leu3p has been recently reported (30). This activator is involved in the control of branched-chain amino acid biosynthesis (22). Leu3p activates transcription only when α-isopropylmalate (α-IPM), the product of the first committed step in leucine biosynthesis, is present (59). Therefore, transcriptional activation by Leu3p is closely linked to the production of α-IPM by α-IPM synthase, which is feedback inhibited by leucine (6).

It has been previously reported (cited in reference 21) that mutations affecting another transcription factor, the HAP complex (stands for heme activator protein), lead to a defect in ammonia utilization. The HAP complex, also called yeast CCAAT box-binding factor, is a heteromeric complex composed of at least four subunits named Hap2p, Hap3p, Hap4p, and Hap5p (20, 27, 38, 47). Three of these subunits, Hap2p, Hap3p, and Hap5p, are required for DNA-binding activity, while Hap4p provides a transcriptional activation domain (38, 45). This complex has been shown to be involved in carbon-dependent regulation of the expression of several genes implicated in respiration (13). More recently, we have shown that this complex stimulates expression, at the transcriptional level, of several genes involved in mitochondrial biogenesis (11), and our recent data indicate that the yeast CCAAT box-binding factor plays a critical role in the regulation of energy metabolism (10). The fact that strains lacking *HAP2* or *HAP3* grow poorly on ammonium sulfate as the sole nitrogen source implicates the yeast CCAAT box-binding complex in the control of ammonia utilization. We have tested this hypothesis and show in the present report that the yeast CCAAT box-binding factor is required for optimal expression of *GDH1*.

MATERIALS AND METHODS

Plasmids, strains, and media. The yeast strains used in this study are BWG1-7a (*MAT***a** *ade1-100 his4-519 leu2-3,112 ura3-52*) (26), JO1-1a (*MAT***a** *ade1-100 his4-519 leu2-3,112 ura3-52 hap2*) (45), SHY40 (*MAT***a** *ade1-100 leu2-3,112 ura3-52 hap3*::*HIS4*) (27), Y404 (*MAT***a** *ade1-100 his4-519 ura3-52 hap4*::*LEU2*) (11), and Y406 (*MAT***a** *ade1-100 his4-519 leu2-3,112 hap4*::*URA3*) (11). Yeast rich medium is 1% (wt/vol) yeast extract–1% (wt/vol) Bacto Peptone. In this basic medium, either glucose, galactose, glycerol, or ethanol was added to the final concentration of 2% (wt/vol) as a carbon source. Yeast rich lactate medium was prepared as previously described (8). Minimal medium contains 2%

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(wt/vol) glucose or galactose, 0.17% (wt/vol) yeast nitrogen base without ammonium sulfate and amino acids (Difco), and 0.5% (wt/vol) ammonium sulfate. The growth medium was supplemented with appropriate requirements at the concentrations recommended by Sherman (57). Plates were made by adding agar to 2.5% (wt/vol). The NADP-GDH-overexpressing plasmid, named pUGDH1, was constructed by insertion of the 3-kb *Bam*HI fragment isolated from plasmid pCYG4 (44) carrying the complete *GDH1* gene at the unique *Bam*HI site of vector B2205 (2μm *URA3*), a derivative of pRS305 (58).

Plasmid manipulation in *S. cerevisiae*. Yeast transformation was performed by the lithium acetate procedure (31). Yeast plasmid DNA was extracted according to the method of Hoffman and Winston (29).

Construction of *lacZ* fusions and in vitro β-galactosidase (β-Gal) assays. Plasmid pCYG4, which contains the *GDH1* gene (44), was kindly provided by B. Hall, University of Washington. To construct *GDH1-lacZ* fusions, the 0.95-kb *Hin*dIII fragment isolated from plasmid pCYG4, carrying ca. 0.9 kb of the 5′-flanking region and 27 bp corresponding to the 9 N-terminal codons of *GDH1*, was fused in frame to the *E. coli lacZ* gene of either YEp353 (2μm *URA3*) or YEp363 (2μm *LEU2*) (43), generating two fusions, pGDH1-UZ and pGDH1-LZ, respectively.

To integrate the *GDH1-lacZ* fusion at the *GDH1* locus, the 0.95-kb *Hin*dIII fragment was isolated from plasmid pCYG4 and fused to the *lacZ* gene carried on the integrative vector YIp363 (*LEU2*) (43). The resulting construct, pIGDH1-LZ, was linearized at the unique *Nsi*I site located in the *GDH1* 5′-flanking region and then transformed into the strain BWG1-7a. Leu^+ transformants were selected and analyzed by Southern blotting to verify that a single copy of the construction was integrated at the *GDH1* locus (data not shown).

β-Gal assays were performed as described by Ruby et al. (54), with β-Gal units defined as $OD_{420} \times 1{,}000/OD_{600} \times t$ (minutes) × volume (milliliters) where OD_{420} is the optical density at 420 nm. In each experiment, at least three independent β-Gal assays were performed, and each assay was done on three independent transformants. The variation between assays was <20%.

Enzyme assays. Yeast total extracts for enzyme assays were prepared from 250-ml cultures. Cells were harvested at an OD_{600} of about 0.5 and then washed with 10 ml of extraction buffer and frozen at −80°C. The extracts were prepared by the method of Doherty (15), with some modifications. The cells were suspended in 0.5 ml of extraction buffer (0.1 M potassium phosphate [pH 7.4], 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, 20% glycerol) and vortexed four times for 20 s with glass beads (diameter, 450 to 500 μm). Disrupted cells were centrifuged for 2 min, and the resulting supernatant was used for enzyme assays. NAD-GDH and NADP-GDH activities were assayed by the method of Doherty (15). The specific activities are expressed in nanomoles of reduced coenzyme oxidized per minute per milligram of protein. In each experiment, at least two independent assays were performed. The variation between assays was <10%.

Protein determinations. Protein concentration was determined by using the Bio-Rad Protein Micro Assay System, with crystalline bovine serum albumin serving as the reference standard.

Site-directed mutagenesis. The three putative binding sites for the HAP complex present in the *GDH1* promoter at positions −438, −406, and −333 were specifically mutated by using the following synthetic oligonucleotides, respectively: GDA, 5′-ATCTGACCATCTGAATGCATGGCCTCGATA-3′; GDB, 5′-CTTATCGCAGAACCATGGCGCCGGAACCGG-3′; and GDC, 5′-CTTC TCTCTGCTGAGTCGACGTGGGGTCGT-3′.

The 0.95-kb *Hin*dIII fragment isolated from plasmid pCYG4 was cloned into plasmid Bluescript KS^+ (Stratagene) and mutagenized by the site-directed mutagenesis method described by Kammann et al. (32). The nucleotide sequence 5′-TGATTGGA-3′ corresponding to box A was converted into 5′-TGAATGCA-3′. Similarly, the nucleotide sequences 5′-ACCAATGC-3′ (box B) and 5′-TC CAATCA-3′ (box C) were converted into 5′-ACCATGGC-3′ and 5′-TC GACTCA-3′, respectively. The presence of the mutations results in the creation of the *Cla*I, *Nco*I, and *Sal*I restriction sites in the three boxes A, B, and C, respectively. The mutated 0.95-kb *Hin*dIII fragments from these constructs were then fused to the *lacZ* gene carried on the expression vector YEp363 (2μm *LEU2*) (43). The fusions obtained were named pGDH1-ΔA, pGDH1-ΔB, and pGDH1-ΔC, respectively (see Fig. 3). The 0.5-kb *Hin*dIII-*Nsi*I fragment isolated from the fusion pGDH1-ΔA, carrying the ΔA mutation, was used to replace the 0.5-kb *Hin*dIII-*Nsi*I fragment of the fusion pGDH1-ΔC. The resulting fusion, named pGDH1-ΔAC, carries both the ΔA and ΔC point mutations (see Fig. 3). Similarly, a fusion named pGDH1-ΔBC, carrying both the ΔB and ΔC point mutations, was constructed with the 0.5-kb *Hin*dIII-*Nsi*I fragment isolated from the fusion pGDH1-ΔB (see Fig. 3). The presence of the mutation(s) in each construct was verified by sequencing.

RNA extraction and Northern (RNA) blot analysis. RNA extraction (56) and Northern blot analysis (55) were carried out as described previously. The Northern blot was probed both with a 0.6-kb *Cla*I fragment of the *ACT1* gene from the plasmid pYA208 (23) and a DNA fragment purified from the studied genes. The following fragments were used as probes for genes *GDH1*, *GLN3*, and *GLN1*, respectively: (i) a 3-kb *Bam*HI fragment of the plasmid pCYG4, (ii) the 2.3-kb *Sac*I-*Xho*I fragment of the plasmid pPM4 (40), and (iii) the 1.1-kb *Sal*I-*Xho*I fragment containing the *GLN1* gene (purified from the plasmid pPM5 kindly provided by B. Magasanik, Massachusetts Institute of Technology). [α-^{32}P] dCTP-labelling of the probe was done by the random-priming procedure (Ready to Go Kit; Pharmacia).

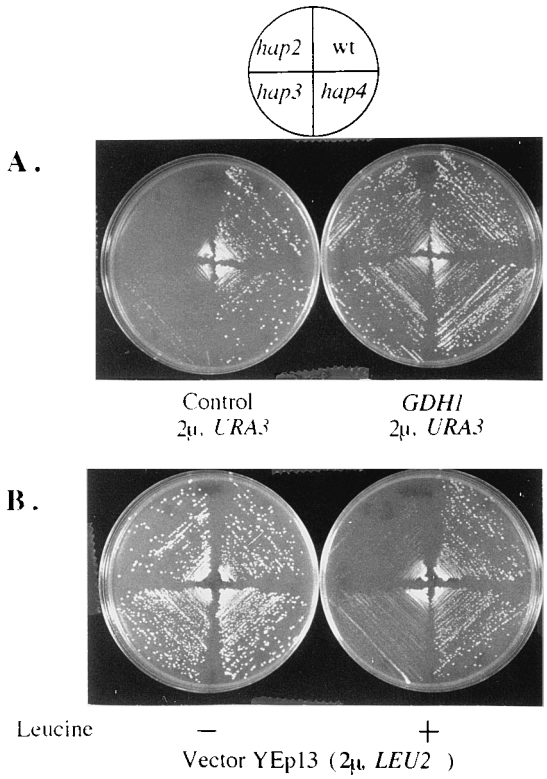

FIG. 1. Phenotypic analysis of the *hap* mutants on ammonium sulfate medium. (A) Suppression of the slow-growth phenotype of the *hap2* and *hap3* mutants by overexpression of the *GDH1* gene. Transformants were streaked out on minimal medium supplemented with adenine, histidine, and leucine. wt, *hap2*, *hap3*, and *hap4* stand for the wild-type and *hap2*, *hap3*, and *hap4* mutant isogenic strains, respectively. The growth phenotype was scored after 3 days at 28°C. (B) Restoration of the *hap* mutant phenotype by leucine excess. Yeast strains were transformed with the high-copy-number YEp13 vector (4) carrying the *LEU2* marker. Transformants were streaked out on minimal medium supplemented with adenine, histidine, and uracil. Leucine was added at a final concentration of 60 μg/ml. 2μ, 2μm (plasmid).

RESULTS

The slow-growth phenotype of the *hap* mutants on ammonium sulfate can be suppressed by overexpression of *GDH1*. It has been reported (cited in reference 21) that *hap* mutants present a growth defect on ammonium sulfate as a nitrogen source. Such a phenotype has been previously described for *gdh1* mutants (25), and since the HAP complex is known to activate transcription of several genes, we have hypothesized that low levels of expression of the *GDH1* gene in the *hap* mutants could result in a *gdh1* phenocopy. We have therefore tested the ability of the *GDH1*-overexpressing plasmid to restore growth of the *hap* mutants. As shown in Fig. 1A, a wild-type growth on ammonium sulfate medium can be restored by transforming the *hap* mutants with a *GDH1*-overexpressing plasmid. The cloning vector was used as a control in this experiment, and no effect on growth was observed. This result strongly suggests that the HAP complex plays some stimulatory role in expression of the *GDH1* gene. Since *GDH1* activation by Leu3p has been previously shown to be reduced in response to leucine (30), we have tested whether the presence of leucine in the medium affects the *hap* mutant phenotype. Because the available *hap* strains carry the *leu2* marker,

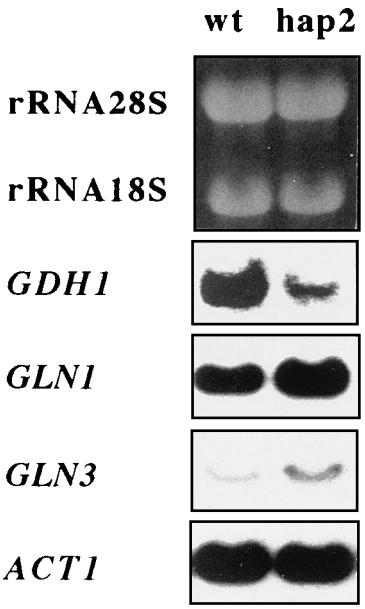

FIG. 2. Northern blot analysis of the *GDH1*, *GLN1*, and *GLN3* gene transcripts. Total RNA of two isogenic wild-type BWG1-7a (wt) and mutant JO1-1a (*hap2*) strains was extracted from cells grown on rich galactose medium. Hybridizations were done with α-^{32}P-radiolabelled probes made from restriction fragments specific for each gene (see Materials and Methods). The rRNAs (stained with ethidium bromide) and an actine probe (*ACT1*) were used to monitor equal loading of the lanes. Each hybridization was done independently, and the blots were assembled for the figure.

we first transformed them to prototrophy with the YEp13 plasmid (4) carrying the *LEU2* gene. The transformed strains were then tested for growth on ammonium sulfate in the presence or in the absence of leucine. The results presented in Fig. 1B clearly show that the *hap2* and *hap3* strains can utilize ammonium as a nitrogen source when no leucine is added to the medium. Conversely, the addition of leucine to the medium restablishes the growth defect (Fig. 1B). Our results show that the *hap* mutants grow normally on ammonium sulfate in the absence of external leucine or when *GDH1* is overexpressed, suggesting that the growth defect is observed only when both leucine and *hap2* or *hap3* mutations are present.

***GDH1* expression is activated in the presence of the HAP complex.** The results presented above strongly suggested that synthesis of NADP-GDH in the presence of leucine was not sufficient to sustain normal growth of the *hap* mutants on ammonium sulfate. To confirm this hypothesis, we tested expression of the *GDH1* gene in wild-type and *hap* mutant strains.

The regulation of *GDH1* was first tested at the transcriptional level by Northern blot analysis. A *GDH1*-specific probe was hybridized to total RNA isolated from isogenic wild-type and *hap2* mutant strains. Results presented in Fig. 2 clearly show that expression of *GDH1* is strongly diminished in the absence of Hap2p.

Regulation of *GDH1* was also assayed with *lacZ* fusions carried on two different vectors with either *LEU2* or *URA3* as a selective marker (see Materials and Methods for details of the construction). Given that Hap2p is part of the HAP complex, we have tested the effect on expression of the *GDH1* gene of mutations in either *HAP2*, *HAP3*, or *HAP4* encoding three subunits of this complex. As shown in Table 1, *GDH1-lacZ* expression is drastically decreased in *hap2* and *hap3* mutants. Interestingly, the *hap4* mutation has a much weaker effect on expression of the fusion, suggesting that this subunit is less required than Hap2p and Hap3p for activation of the *GDH1* gene. This result supports the observation that growth of the *hap2* and *hap3* mutants on ammonium sulfate as a sole nitrogen source is much slower than that of the *hap4* mutant (Fig. 1). Since no difference in *GDH1* expression between *hap2* and *hap3* strains was found, either of these strains was used for further experiments. The levels of expression of the *GDH1-lacZ* fusion were found to be significantly different, depending on the vector carrying the fusion. The level of expression of the fusion is lower when it is carried on the *URA3* vector compared with that of the *LEU2* construct (Table 1). This effect could either be the result of a copy number difference between the two constructs or it could be due to the addition of leucine in the medium when the *URA3* vector is used. Indeed, a two- to fourfold inhibition of *GDH1* expression in the presence of leucine has been previously reported (30).

Finally, NADP-GDH activity was measured in vitro and was found to be four to six times lower in the *hap* strains than in the wild-type strain (Table 1). In this assay, the effect of the *hap4* mutation appears greater than the effect of the same mutation on the expression of the *GDH1-lacZ* fusion; the reason for this discrepancy could be that the enzymatic assay measures a second NADP-GDH activity independent of Gdh1p. Indeed, a gene named *GDH3*, encoding a potential second NADP-GDH, was recently found during systematic sequencing of the yeast genome (5). In this experiment, the NAD-GDH activity was used as a control, since it is known not to be affected by the HAP complex (7) and, indeed, the same activity was found in all four strains (Table 1).

Two CCAAT box sequences are required for optimal expression of *GDH1*. The activation of *GDH1* expression in the presence of HAP was further supported by the finding of three potential HAP binding sites (TNATTGGT) (19) upstream of the *GDH1* gene at positions −333, −406, and −438 relative to the ATG.

Each of these sequences was independently mutated. The mutated promoter sequences were then reintroduced upstream of the *GDH1-lacZ* fusion as described in Materials and Methods. As shown in Fig. 3, mutations in box A or box C result in a 30% decrease in *GDH1-lacZ* expression, while the mutation in box B has no effect on expression of the fusion. The role of box A and box C was further confirmed by studying

TABLE 1. Effect of mutations in the *HAP2*, *HAP3*, and *HAP4* genes on expression of *GDH1-lacZ* fusions and GDH specific activities

Strain[a]	Activity			
	β-Gal assay with fusion[b]:		GDH assay[c]	
	pGDH1-UZ	pGDH1-LZ	NADP-GDH	NAD-GDH
wt	176	541	583	101
hap2	32	67	102	102
hap3	24	55	100	80
hap4	93[d]	377	154	100

[a] wt, *hap2*, *hap3*, and *hap4* stand, respectively, for the BWG1-7a (wild type), JO1-1a, SHY40, and Y406 isogenic strains.

[b] β-Gal activity was measured as described in Materials and Methods. Cells were grown in minimal galactose medium. Assays were performed in the presence or in the absence of leucine (60 μg/ml) with respectively, the pGDH1-UZ (*URA3* 2μm) and pGDH1-LZ (*LEU2* 2μm) fusions.

[c] Cells were grown in minimal galactose medium supplemented with adenine, histidine, leucine, and uracil. GDH assays were performed as described in Materials and Methods. Specific activity is expressed in nanomoles of reduced coenzyme oxidized per minute per milligram of protein.

[d] When the pGDH1-UZ fusion was used, the Y406 strain was replaced by the Y404 isogenic strain which carries the *ura3-52* marker.

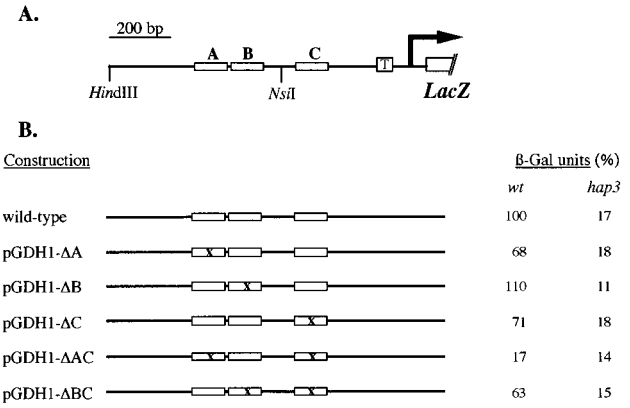

FIG. 3. Effect of point mutations located in the three putative CCAAT boxes on *GDH1-lacZ* expression. (A) Schematic representation of the *GDH1* promoter region. T, TATA box. The three open boxes labelled A, B, and C represent the three putative CCAAT boxes. The thin solid bars and the open bars correspond, respectively, to the *GDH1* gene and the *lacZ* gene sequences. The arrow designates the location of the ATG codon and the orientation of transcription. (B) Effect of the point mutations on *GDH1-lacZ* expression. Site-directed mutagenesis was performed as described in Materials and Methods. X (located in boxes) designates the mutation(s) in the corresponding CCAAT box. The constructs were named, using abbreviations as follows: ΔA, box A mutated; ΔB, box B mutated; ΔC, box C mutated; ΔAC, both boxes A and C mutated; ΔBC, both boxes B and C mutated. *wt* and *hap3* stand for the wild-type and *hap3* isogenic strains, respectively. Cells were grown in galactose minimal medium, and β-Gal assays were performed as previously described. The optimal expression level (100%) is estimated as the expression of the wild-type promoter of the *GDH1* gene in the wild-type background (strain BWG1-7a).

combinations of double mutations. While a double mutant (both box B and box C mutated) exhibits the same decrease in *GDH1-lacZ* expression as the single, box C mutant, the double box A box C mutant is much more affected than each of the two single mutants (down to 17% of wild-type expression level). Finally, if these mutations affect activation by HAP, they should not affect expression of the fusion in a *hap* background. Indeed, results presented in Fig. 3 show that neither single nor double mutants significantly lower expression of the fusion in the *hap3* mutant strain. Furthermore, the fact that the fusion is equally expressed in the double box A box C mutant and in the *hap3* mutant strain (17% of wild-type expression level) strongly suggests that these two boxes are responsible for all the HAP-dependent expression of *GDH1*.

Regulation of *GDH1* expression by carbon source. Since most of the genes activated by the HAP complex are regulated by the carbon source (13), we have studied the effect of the carbon source on expression of the *GDH1-lacZ* fusion. A plasmid carrying a *GDH1-lacZ* fusion was introduced in isogenic wild-type, *hap3*, and *hap4* mutant strains, and the carbon source-dependent expression of the *GDH1* gene was tested at different growth stages on glucose and galactose media. Several conclusions can be drawn from the results presented in Fig. 4.

(i) The rates of growth of the three strains on glucose medium are equivalent (Fig. 4B). On galactose, the *hap* mutant strains have a longer lag, but their growth rate after this lag is equivalent to that of the wild-type strain (Fig. 4D). On both media and for all strains, an OD_{600} of between 0.2 and 2 can be considered as log phase.

(ii) On glucose medium (Fig. 4A) in the wild-type strain, expression of *GDH1-lacZ* increases during log phase and then slowly decreases when the strain reaches stationary phase (compare with Fig. 4B). The situation is quite different in the *hap3* mutant strain, for which the level of expression of the fusion remains low regardless of the growth stage. Finally, in the *hap4* mutant, we observed an intermediary situation, with an increase during log phase that never reaches expression levels observed for the wild-type strain.

(iii) On galactose medium (Fig. 4C), wild-type and *hap4* mutant strains behave similarly: expression of the fusion increases during log phase and decreases when the plateau is reached. The *hap3* mutant expresses the fusion at a low level, slightly increasing when cells reach the stationary phase.

(iv) Expression of the fusion on galactose and expression on glucose are compared in Fig. 4E, where the β-Gal activity according to OD_{600} is reported. Despite differences in the growth rate (Fig. 4B and D), all the strains are in log phase between an OD_{600} of 0.2 to 2, and levels of expression of the *GDH1-lacZ* fusion can be compared in this window. As reported for other genes regulated by HAP, in the wild-type strain, the level of expression of *GDH1* is always higher on galactose than on glucose (two- to threefold). No regulation between glucose and galactose can be observed with the mutant *hap3* strain, suggesting an important role for HAP in this process. Much more surprisingly, the expression of the fusion is still regulated by the carbon source (less expressed in glucose) even when Hap4p is absent, suggesting that the Hap4p subunit does not play a critical role in this regulation. Such a result was unexpected, since Hap4p has been proposed to be the subunit responsible for the carbon source response of the HAP complex (20, 45).

Expression of *GDH1* on nonfermentable carbon sources was tested by using a *GDH1-lacZ* fusion integrated at the *GDH1* locus. As shown in Table 2, the level of expression of the fusion is low when the carbon source is glucose, galactose, glycerol, or ethanol, and it is 10-fold higher on lactate. The reason for such a higher level of expression on lactate is not yet known.

Finally, enzymatic assays confirm the regulation of NADP-GDH activity by the carbon source, but the effect is smaller than the one observed with the integrated fusion (Table 2). As discussed previously, we cannot rule out the possibility that part of this activity could be due to Gdh3p, a potential second NADP-GDH (5). In this experiment, we also found an elevated level of the NAD-GDH activity (encoded by the *GDH2* gene) when cells were grown on a nonfermentable carbon source (Table 2). This observation had been previously reported by others (7).

The HAP activation of ammonium assimilation is specific for NADP-GDH. As reviewed above, the major pathway for assimilation of ammonium is a combination of two successive reactions catalyzed by NADP-GDH and glutamine synthetase, respectively. The latter enzyme is encoded by the *GLN1* gene (41), whose expression is subject to multiple regulatory systems (1, 41). We have therefore tested whether expression of the *GLN1* gene is under control of the yeast CCAAT box-binding factor. We have also looked at the effect of the HAP complex on expression of the *GLN3* gene, encoding a transcription factor required for optimal expression of several nitrogen metabolism genes, including *GLN1*, because a potential CCAAT box has been previously described at position −480 upstream of the *GLN3* gene (18). As presented in Fig. 2, Northern blot analysis shows that, unlike *GDH1*, the transcription of these two genes is not diminished in the absence of Hap2p and might even be slightly increased.

DISCUSSION

In this report, we have provided evidence that the yeast CCAAT box-binding factor (the HAP complex) plays an important role in the control of ammonium utilization. This reg-

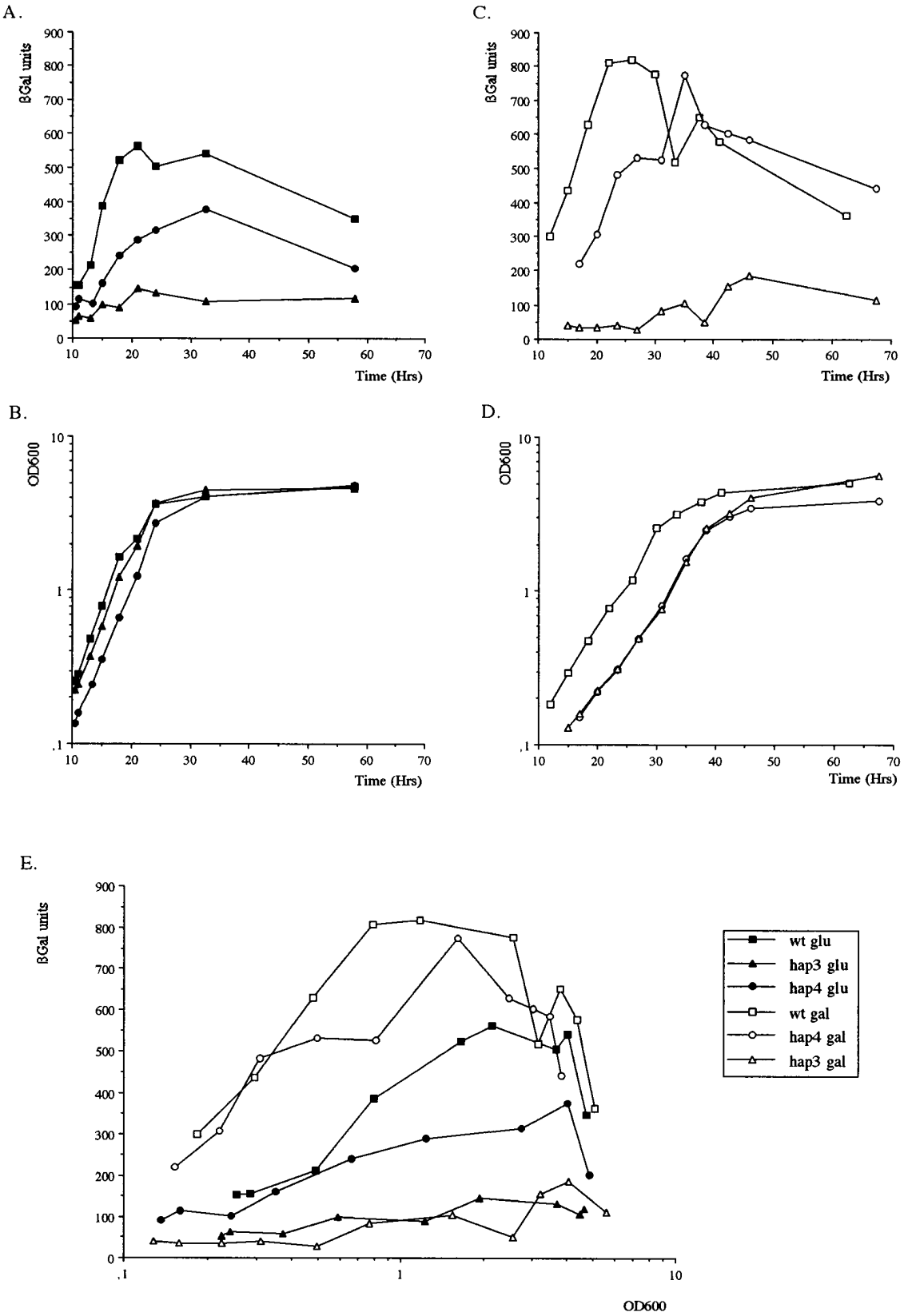

FIG. 4. Carbon source-dependent expression of the *GDH1-lacZ* fusion according to different growth stages. The fusion pGDH1-LZ was used in this experiment; therefore, no leucine had to be added to the minimal medium (Table 1 and Results). Either glucose (solid symbols) or galactose (open symbols) was used as the carbon source. (B and D) Growth of isogenic wild-type (BWG1-7a), *hap3* (SHY40), and *hap4* (Y406) strains was monitored by following OD_{600} as a function of time. (A and C) The same isogenic strains as noted above were harvested at different times, and β-Gal activity was measured as described in Materials and Methods. (E) β-Gal activities of the three strains on the two carbon sources are reported as a function of OD_{600}. wt, wild type.

TABLE 2. Effect of carbon source on expression of the integrated *GDH1-lacZ* fusion and the GDH specific activities

Carbon source	Activity		
	β-Gal assay with *GDH1-lacZ*[a]	GDH assay[b]	
		NADP-GDH	NAD-GDH
Glucose	8	459	53
Galactose	11	665	79
Glycerol	17	559	1,078
Ethanol	20	559	470
Lactate	107	1,384	769

[a] β-Gal assays were carried out with the *GDH1-lacZ* fusion integrated at the *GDH1* locus of the wild-type strain BWG1-7a. Cells from an overnight culture in rich glucose medium were harvested, washed, and then shifted for 6 h in different rich media containing either glucose, galactose, glycerol, ethanol, or lactate as the carbon source at a final concentration of 2%. β-Gal activity was measured as described in Materials and Methods.

[b] Cells were grown in rich media containing different carbon sources at a concentration of 2%. NADP-GDH and NAD-GDH activities were measured as described in Materials and Methods. Specific activity is expressed in nanomoles of reduced coenzyme oxidized per minute per milligram of protein.

ulation by HAP is limited to the anabolic GDH, since neither NAD-GDH activity nor *GLN1* or *GLN3* expression is affected by HAP. The regulation of *GDH1* expression by HAP defines a new example of cross-pathway regulation. The fact that a transcriptional regulator involved in the regulation of several aspects of carbon metabolism (10, 13) also regulates expression of an enzyme involved in nitrogen assimilation points out the crucial role played by GDH in the connection between nitrogen and carbon metabolisms. At this point, three regulators, Gln3p, Leu3p, and HAP, are known to activate *GDH1* expression (references 12 and 30 and results presented here). Furthermore, the dual regulation of *GDH1* expression by both leucine and the carbon source illustrates the complexity of glutamate synthesis regulation. We have shown that the growth phenotype of the *hap* mutants on ammonium sulfate is the result of a lower level of activation by both the Leu3p and HAP factors. The fact that a *hap4* mutant does not show such a phenotype (Fig. 1) is consistent with the weaker requirement of this factor for activation (Table 1). Such a weaker effect of the *hap4* mutation on expression of the HAP-regulated genes was previously reported for *CYC1* and *SDH3* (9), but in these cases, the difference between the *hap4* and *hap3* mutants was less spectacular than that found for *GDH1*. While *GDH1* expression is not totally *HAP4* independent, results presented in Fig. 4 indicate that the regulation by the carbon source (galactose versus glucose) is normal in a *hap4* mutant but is abolished in the absence of Hap3p. Since Hap4p is presumably the subunit responsible for transcriptional activation in response to the carbon source signal (20, 45), it is likely that, on the *GDH1* promoter, this transcriptional activation operates at least partly in some Hap4p-independent manner.

We have shown that expression of the *GDH1* gene is repressed in the presence of glucose, while it is at its highest level in the presence of lactate. Similarly, the level of activity of the catabolic NAD-GDH encoded by the *GDH2* gene is much higher on nonfermentable carbon sources (7). However, *GDH2* expression was shown not to be regulated by HAP (7). Why should expression of both anabolic and catabolic GDHs simultaneously increase under specific conditions? We cannot provide any clear answer to this question yet, but interestingly, it has been shown that the *GDH2* gene, when overexpressed, can suppress the growth defect on glucose caused by *pgi*Δ mutations, creating a cyclic transhydrogenase system between the NADP-GDH and NAD-GDH and providing an optimal balance for the reducing equivalents in the cytosol (2). Coactivation of both anabolic and catabolic GDHs under specific growth conditions could play a somewhat similar role.

Why should the CCAAT box-binding factor control *GDH1* expression? As mentioned above, the yeast CCAAT box-binding factor plays a critical role in the control of energy metabolism (10), and *hap* mutants exhibit a growth defect on nonfermentable carbon sources (glycerol, lactate, and ethanol) (20, 27, 38, 47). Under these conditions, activity of the Krebs cycle is required not only for energy production but also for the synthesis of heme and amino acids, including glutamate. Synthesis of several Krebs cycle enzymes is controlled by the HAP complex (3, 9, 24, 48, 49), and interestingly, at least some steps of heme (33, 34) and glutamate synthesis are activated in the presence of HAP. It is tempting to propose that HAP exerts a general control on Krebs cycle activity as well as on synthesis of its derivative products (heme, glutamate, etc.) and on the activity of the electron transfer chain. Another link between the Krebs cycle and nitrogen metabolism through lipoamide dehydrogenase has been reported. Lipoamide dehydrogenase, the product of the *LPD1* gene which is regulated by HAP (3), is a component of the Krebs cycle enzyme α-ketoglutarate dehydrogenase and is also required for catabolism of branched-chain amino acids through 2-oxoacid dehydrogenase activity (14). In order to clarify the role of HAP in the regulation of branched-chain amino acid catabolism, it would be interesting to test the effect of HAP on 2-oxoacid dehydrogenase activity.

In *S. cerevisiae*, NADP-GDH activity is cytosolic (46), and therefore, the question arises as to how the substrate (α-ketoglutarate) is supplied to this enzyme. One possibility is that α-ketoglutarate produced from the Krebs cycle is transported from the mitochondria to the cytosol. Alternatively, some α-ketoglutarate could be produced in the cytosol, since all the enzymes necessary for such a synthesis have been detected in the cytosol. Indeed, pyruvate carboxylase is a cytosolic enzyme in *S. cerevisiae* (51, 60), and citrate synthase, aconitase, and isocitrate dehydrogenase activities have been also detected in the cytosolic compartment (17, 36, 50). It is possible that both sources of α-ketoglutarate coexist, since mutations in the two genes encoding cytosolic and mitochondrial citrate synthases are required in order to obtain auxotrophy for glutamate (35). Similarly, disruption of the three genes encoding mitochondrial isocitrate dehydrogenase leads to glutamate auxotrophy only under conditions in which the cytosolic isozyme is not expressed (28, 36). In this respect, it is noteworthy that expression of citrate synthase (35), aconitase (24), and isocitrate dehydrogenase (28) is repressed by both glucose and glutamate. Such a regulation allows adaptation of the α-ketoglutarate supply both to the tricarboxylic acid cycle carbon flux and to glutamate synthesis. Regulation by glucose is sustained at least partly by the HAP complex, which activates the expression of the *CIT1* and *ACO1* genes (24, 53), while the mechanism of repression in the presence of glutamate is not yet understood.

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REFERENCES

1. **Benjamin, P. M., J. I. Wu, A. P. Mitchell, and B. Magasanik.** 1989. Three regulatory systems control expression of glutamine synthetase in *Saccharomyces cerevisiae* at the level of transcription. Mol. Gen. Genet. **217:**370–377.
2. **Boles, E., W. Lehnert, and F. K. Zimmermann.** 1993. The role of NAD-dependent glutamate dehydrogenase in restoring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant. Eur. J. Biochem. **217:**469–477.
3. **Bowman, S. B., Z. Zaman, L. P. Collinson, A. J. P. Brown, and I. W. Dawes.** 1992. Positive regulation of the *LPD1* genes of *Saccharomyces cerevisiae* by the HAP2/HAP3/HAP4 activation system. Mol. Gen. Genet. **231:**296–303.
4. **Broach, J. R., J. N. Strathern, and J. B. Hicks.** 1979. Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene **8:**121–133.
5. **Bussey, H., et al.** 1995. The nucleotide sequence of chromosome I from *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **92:**3809–3813.
6. **Chang, L. L., P. R. Gatzek, and G. B. Kohlhaw.** 1985. Total deletion of yeast *LEU4*: further evidence for a second α-isopropylmalate synthase and evidence for tight *LEU4-MET4* linkage. Gene **33:**333–339.
7. **Coschigano, P. W., S. M. Miller, and B. Magasanik.** 1991. Physiological and genetic analysis of the carbon regulation of the NAD-dependent glutamate dehydrogenase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**4455–4465.
8. **Daignan-Fornier, B., C. C. Nguyen, P. Reisdorf, B. Lemeignan, and M. Bolotin-Fukuhara.** 1994. *MBR1* and *MBR3*, two related yeast genes that can suppress the growth defect of *hap2*, *hap3* and *hap4* mutants. Mol. Gen. Genet. **243:**575–583.
9. **Daignan-Fornier, B., M. Valens, B. D. Lemire, and M. Bolotin-Fukuhara.** 1994. Structure and regulation of *SDH3* the yeast gene encoding the cytochrome b_{560} subunit of respiratory complex II. J. Biol. Chem. **269:**15469–15472.
10. **Dang, V.-D., C. Bohn, M. Bolotin-Fukuhara, and B. Daignan-Fornier.** Submitted for publication.
11. **Dang, V.-D., M. Valens, M. Bolotin-Fukuhara, and B. Daignan-Fornier.** 1994. A genetic screen to isolate genes regulated by the yeast CCAAT-box binding protein Hap2p. Yeast **10:**1273–1283.
12. **Daugherty, J. R., R. Rai, H. M. E. Berry, and T. G. Cooper.** 1993. Regulatory circuit for responses of nitrogen catabolic gene expression to the GLN3 and DAL80 proteins and nitrogen catabolite repression in *Saccharomyces cerevisiae*. J. Bacteriol. **175:**64–73.
13. **De Winde, J. H., and L. A. Grivell.** 1993. Global regulation of mitochondrial biogenesis in *Saccharomyces cerevisiae*. Prog. Nucleic Acid Res. **46:**51–91.
14. **Dickinson, J. R., and I. W. Dawes.** 1992. The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **138:**2029–2033.
15. **Doherty, D.** 1970. L-Glutamate dehydrogenase (yeast). Methods Enzymol. **17:**850–856.
16. **Dubois, E. L., and M. Grenson.** 1974. Absence of involvement of glutamine synthetase and of NAD-linked glutamate dehydrogenase in the nitrogen catabolite repression of arginase and other enzymes in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **60:**150–157.
17. **Duntze, W., D. Neumann, J. M. Gancedo, W. Atzpodien, and H. Holzer.** 1969. Studies of the regulation and localization of the glyoxylate cycle enzymes in *Saccharomyces cerevisiae*. Eur. J. Biochem. **10:**83–89.
18. **Fondrat, C., and A. Kalogeropoulos.** 1994. Approaching the function of new genes by detection of their potential upstream activation sequences in *Saccharomyces cerevisiae*. Curr. Genet. **25:**396–406.
19. **Forsburg, S. L., and L. Guarente.** 1988. Mutational analysis of upstream activation sequence 2 of the *CYC1* gene of *Saccharomyces cerevisiae*: a *HAP2-HAP3*-responsive site. Mol. Cell. Biol. **8:**647–654.
20. **Forsburg, S. L., and L. Guarente.** 1989. Identification and characterization of HAP4: a third component of the CCAAT-bound HAP2/HAP3 heteromer. Genes Dev. **3:**1166–1178.
21. **Forsburg, S. L., and L. Guarente.** 1989. Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae*. Annu. Rev. Cell Biol. **5:**153–180.
22. **Friden, P., and P. Schimmel.** 1988. *LEU3* of *Saccharomyces cerevisiae* activates multiple genes for branched-chain amino acid biosynthesis by binding to a common decanucleotide core sequence. Mol. Cell. Biol. **8:**2690–2697.
23. **Gallwitz, D., and I. Sures.** 1980. Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:**2546–2550.
24. **Gangloff, S. P., D. Marguet, and G. J. M. Lauquin.** 1990. Molecular cloning of the yeast mitochondrial aconitase gene (*ACO1*) and evidence of a synergistic regulation of expression by glucose plus glutamate. Mol. Cell. Biol. **10:**3551–3561.
25. **Grenson, M., E. Dubois, M. Piotrowska, R. Drillien, and M. Aigle.** 1974. Ammonia assimilation in *Saccharomyces cerevisiae* as mediated by the two glutamate dehydrogenases. Mol. Gen. Genet. **128:**73–85.
26. **Guarente, L., B. Lalonde, P. Giffon, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolic repression of the *CYC1* gene of *S. cerevisiae*. Cell **36:**503–511.
27. **Hahn, S., J. Pinkham, R. Wei, R. Miller, and L. Guarente.** 1988. The *HAP3* regulatory locus of *Saccharomyces cerevisiae* encodes divergent overlapping transcripts. Mol. Cell. Biol. **8:**655–663.
28. **Haselbeck, R. J., and L. McAlister-Henn.** 1993. Function and expression of yeast mitochondrial NAD- and NADP-specific isocitrate dehydrogenases. J. Biol. Chem. **268:**12116–12122.
29. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
30. **Hu, Y., T. G. Cooper, and G. B. Kohlhaw.** 1995. The *Saccharomyces cerevisiae* Leu3 protein activates expression of *GDH1*, a key gene in nitrogen assimilation. Mol. Cell. Biol. **15:**52–57.
31. **Ito, H., Y. Fukada, K. Nutara, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
32. **Kammann, M., J. Laufs, J. Schell, and B. Gronenborn.** 1989. Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). Nucleic Acids Res. **17:**5404.
33. **Keng, T., and L. Guarente.** 1987. Constitutive expression of the yeast *HEM1* gene is actually a composite of activation and repression. Proc. Natl. Acad. Sci. USA **84:**9113–9117.
34. **Keng, T., C. Richard, and R. Larocque.** 1992. Structure and regulation of yeast *HEM3*, the gene of porphobilinogen deaminase. Mol. Gen. Genet. **234:**233–243.
35. **Kim, K.-S., M. S. Rosenkrantz, and L. Guarente.** 1986. *Saccharomyces cerevisiae* contains two functional citrate synthase genes. Mol. Cell. Biol. **6:**1936–1942.
36. **Loftus, T. M., L. V. Hall, S. L. Anderson, and L. McAlister-Henn.** 1994. Isolation, characterization and disruption of the yeast gene encoding cytosolic NADP-specific isocitrate dehydrogenase. Biochemistry **33:**9661–9667.
37. **Magasanik, B.** 1992. Regulation of nitrogen utilization, p. 283–317. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces cerevisiae*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **McNabb, D. S., Y. Xing, and L. Guarente.** 1995. Cloning of yeast *HAP5*: a novel subunit of a heteromeric complex required for CCAAT binding. Genes Dev. **9:**47–58.
39. **Miller, S. M., and B. Magasanik.** 1990. Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. **172:**4927–4935.
40. **Minehart, P. L., and B. Magasanik.** 1991. Sequence and expression of *GLN3*, a positive nitrogen regulatory gene of *Saccharomyces cerevisiae* encoding a protein with a putative zinc finger DNA-binding domain. Mol. Cell. Biol. **11:**6216–6228.
41. **Minehart, P., and B. Magasanik.** 1992. Sequence of the *GLN1* gene of *Saccharomyces cerevisiae*: role of the upstream region in regulation of glutamine synthetase expression. J. Bacteriol. **174:**1828–1836.
42. **Moye, W. S., N. Amuro, J. K. Mohana Rao, and H. Zalkin.** 1985. Nucleotide sequence of yeast *GDH1* encoding nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase. J. Biol. Chem. **260:**8502–8508.
43. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45:**299–310.
44. **Nagasu, T., and B. D. Hall.** 1985. Nucleotide sequence of the *GDH1* gene coding for the NADP-specific glutamate dehydrogenase of *Saccharomyces cerevisiae*. Gene **37:**247–253.
45. **Olesen, J., and L. Guarente.** 1990. The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev. **4:**1714–1729.
46. **Perlman, P. S., and H. R. Mahler.** 1970. Intracellular localization of enzymes in yeast. Arch. Biochem. Biophys. **136:**245–259.
47. **Pinkham, J. L., and L. Guarente.** 1985. Cloning and molecular analysis of the *HAP2* locus: a global regulation of respiratory genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5:**3410–3416.
48. **Repetto, B., and A. Tzagoloff.** 1989. Structure and regulation of *KGD1*, the structural gene for yeast α-ketoglutarate dehydrogenase. Mol. Cell. Biol. **9:**2695–2705.
49. **Repetto, B., and A. Tzagoloff.** 1990. Structure and regulation of *KGD2*, the structural gene for yeast dihydrolipoyl transuccinylase. Mol. Cell. Biol. **10:**4221–4232.
50. **Rickey, T. M., and A. S. Lewin.** 1986. Extramitochondrial citrate synthase activity in bakers' yeast. Mol. Cell. Biol. **6:**488–493.
51. **Rohde, M., F. Lim, and J. C. Wallas.** 1991. Electron microscopic localization of pyruvate carboxylase in rat liver and *Saccharomyces cerevisiae* by immunogold procedures. Arch. Biochem. Biophys. **290:**197–201.
52. **Roon, R. J., H. L. Even, and F. Larimore.** 1974. Glutamate synthase: properties of the reduced nicotinamide adenine dinucleotide-dependent enzyme from *Saccharomyces cerevisiae*. J. Bacteriol. **118:**89–95.
53. **Rosenkrantz, M., C. S. Kell, E. A. Pennell, and L. J. Devenish.** 1994. The HAP2,3,4 transcriptional activator is required for derepression of the yeast citrate synthase gene, *CIT1*. Mol. Microbiol. **13:**119–131.
54. **Ruby, S. W., J. W. Szostak, and A. W. Murray.** 1983. Cloning regulated yeast genes from a pool of *lacZ* fusions. Methods Enzymol. **101:**235–269.

55. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
56. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18:**3091–3092.
57. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
58. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
59. **Sze, J.-Y., M. Wootner, J. A. Jaehning, and G. B. Kohlhaw.** 1992. *In vitro* transcriptional activation by a metabolic intermediate: activation by Leu3 depends on α-isopropylmalate. Science **258:**1143–1145.
60. **Walker, M. E., D. L. Val, M. Rohde, R. J. Devenish, and J. C. Wallas.** 1991. Yeast pyruvate carboxylase: identification of two genes encoding isoenzymes. Biochem. Biophys. Res. Commun. **176:**1210–1217.