Transcription factor GCN4 for control of amino acid biosynthesis also regulates the expression of the gene for lipoamide dehydrogenase

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The yeast *LPD1* gene encoding lipoamide dehydrogenase is subject to the general control of amino acid biosynthesis mediated by the GCN4 transcription factor. This is striking in that it demonstrates that GCN4-mediated regulation extends much farther upstream than simply to the direct pathways for amino acid and purine biosynthesis. In yeast, lipoamide dehydrogenase functions in at least three multienzyme complexes: pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase (which function in the entry of pyruvate into, and metabolism via, the citric acid cycle) and glycine decarboxylase. When wild-type cells were shifted from growth on amino acid-rich to amino acid-deficient medium, the expression of lipoamide dehydrogenase was induced

approx. 2-fold. In a similar experiment no such induction was observed in isogenic *gcn4* mutant cells. Northern analysis indicated that amino acid starvation affected levels of the *LPD1* transcript. In the upstream region of *LPD1* are three matches to the consensus for control mediated by GCN4. Directed mutagenesis of each site, and of all combinations of sites, suggests that only one site might be important for the general control response under the conditions tested. Gel-retardation analysis with GCN4 protein synthesized *in vitro* has indicated that GCN4 can bind *in vitro* to at least two of the consensus motifs.

Key words: gene expression, metabolic control, transcription.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, starvation for amino acids, or a sudden decrease in their availability, leads to a co-ordinated induction of at least 40 genes in 12 pathways required for the biosynthesis of amino acids [1] as well as of genes involved in tRNA charging [2,3]. Furthermore, this 'general control of amino acid biosynthesis' response has been shown to extend to genes involved in the biosynthesis of purines [4,5]; limitation for essential purines also triggers the general control response [6]. This co-ordinated response to nutrient status is mediated by the GCN4 protein, a member of the highly conserved eukaryotic AP-1 transcription factor family [7]. A common feature of all of the genes under general control is the presence of one or more GCN4-binding sites in their promoters [8]. GCN4 protein binds as a homodimer to the consensus sequence rrTGASTCA(T)_n to activate the transcription of reporter genes in either direction at distances of up to 600 bp [7]. The synthesis of GCN4 protein itself is regulated in response to changes in amino acid availability, primarily at the translational level through the use of four short upstream open reading frames that under normal (non-stressed) conditions inhibit the translation of the downstream GCN4 open reading frame [1]. GCN4 protein is one of the best characterized transcription factors and has served as a model in numerous structural and function studies [7]. Here we show that GCN4 directly regulates the expression of the *LPD1* gene, which encodes lipoamide dehydrogenase (LPDH, EC 1.8.1.4), an essential enzyme for glycolysis, the tricarboxylic acid cycle and the glycine cleavage system. This observation is remarkable in that it demonstrates that GCN4-mediated regulation extends well beyond that thought previously, to a control point much further upstream than the direct pathways for amino acid and purine biosynthesis.

LPDH is a common component of the multienzyme complexes pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase (OGDH) [9] and glycine decarboxylase (GCV) [10]. PDH catalyses the conversion of pyruvate to acetyl-CoA and therefore the entry of pyruvate into the citric acid cycle, OGDH catalyses the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA and is needed for the operation of the citric acid cycle, and GCV catalyses the conversion of glycine to CO₂, NH₄⁺ and 5,10-methylenetetrahydrofolate for active one-carbon generation. The other subunits of the PDH complex (Pda1p, Pdb1p, Pdx1p and Lat1p), the OGDH complex (Kgd1p and Kgd2p) and the GCV complex (Gcv1p, Gcv2p and Gcv3p) are all encoded by individual genes, whereas the LPDH component of all three complexes is encoded by the single nuclear gene *LPD1* [11–13].

LPD1 is subject to multiple regulatory mechanisms [14]. This makes it an attractive model gene to study, not only to gain insights into the regulation of gene expression in eukaryotes but also to address how such an array of different regulatory mechanisms communicate at a single promoter. As with other genes for mitochondrial functions, *LPD1* is subject to a form of catabolite repression when cells are grown on glucose as carbon source [15]; this control is mediated by the HAP2/HAP3/HAP4 transcriptional activator complex [16]. There is also some degree of control mediated from sequences within the coding region of the gene [14,17]. A decrease in OGDH activity is one of the very early steps in sporulation [18]. Moreover, the multiple roles of LPDH in different enzyme complexes raise interesting questions of how its synthesis is co-ordinated with respect to the other components of these complexes.

Within the 5' upstream promoter region of *LPD1* there is a complex and overlapping set of potential control motifs, including three sequences showing similarity to the GCN4 consensus binding motif [19]. In this study we have focused on the

Abbreviations used: GCV, glycine decarboxylase; LPDH, lipoamide dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactose.

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significance of these motifs and show that the product of the *GCN4* locus is essential for inducing *LPD1* gene transcription under conditions of amino acid starvation. This represents an extension of the range of genes under general amino acid control beyond those solely concerned with amino acid biosynthesis to one that functions in central metabolism. Further, this finding suggests how cells might mediate long-range inter-promoter communication to fulfil their metabolic requirements and respond to adverse environmental conditions at the level of gene transcription.

EXPERIMENTAL

Yeast strains and media

The yeasts used in this study were: strain F113 (*MATa ino1-113 ura3-52 can1*) and strain F212 (*MATa ino1-113 ura3-52 can1 gcn4Δ-103*) supplied by A. Hinnebusch; strain 361 (*MATa leu2-112 gcn4*) supplied by F. Messenguy; and strain BWG1-7A (*MATa ade1-100 his4-519 leu2-3 leu2-112 ura3-52*) supplied by L. Guarente. Minimal medium consisted of 1.7 g/l Difco yeast nitrogen base without amino acids and $(\text{NH}_4)_2\text{SO}_4$, with the addition of 20 g/l raffinose, 5 g/l $(\text{NH}_4)_2\text{SO}_4$ and 50 µg/ml auxotrophic requirements. Non-inducing medium consisted of minimal medium supplemented with all amino acids as described by Penn et al. [20]. Inducing medium consisted of minimal medium supplemented with 3-amino-1,2,4-triazole as described by Penn et al. [20]. Solid medium consisted of either non-inducing or inducing medium supplemented with 20 g/l agar and 250 µl/l of a solution of 5-bromo-4-chloroindol-3-yl β-D-galactose (X-Gal, 20 mg/ml) in dimethylformamide as required.

Plasmids

Plasmid pGP1 contains the intact *LPD1* gene in the YEpl3 vector [15], and pZZD-2µ used for site-directed mutagenesis has been described previously [16]. The plasmids pSPACT9 containing the yeast actin gene [21] and pMC1871 carrying the *Escherichia coli lacZ* gene [22] were used for probing Northern analyses. The reporter plasmid YIp358 was kindly provided by A. Tzagoloff [23]. Plasmid pSP64-GCN4 and related plasmids for the synthesis of GCN4 protein *in vitro* [24] were supplied by I. Hope.

Growth of cultures and sampling

For induction experiments, yeast cell cultures growing at 30 °C in the exponential phase (D_{600} 0.3) were harvested rapidly by filtration on 0.45 µm membranes (Millipore), washed briefly in prewarmed water (30 °C) and resuspended in appropriate volumes of prewarmed (30 °C) medium. Samples (50 ml) of culture were withdrawn at intervals after the transfer of medium and harvested by centrifugation (5000 g, 5 min, 4 °C). For RNA preparations the cell pellet was washed once in RNA extraction buffer [0.01 M Tris/HCl (pH 7.5)/0.1 M LiCl/0.01 M dithiothreitol], pelleted and stored at –80 °C until used. For enzyme assays the pellet was washed once in cell breakage buffer [12], pelleted and stored at –80 °C until used.

Enzyme assays and protein concentration

Cultures were grown to exponential phase and assayed for LPDH activity with the substrate 2-acetylpyridine adenine dinucleotide as substrate, as described by Dickinson et al. [12]. Units are expressed as µmol of 2-acetylpyridine adenine dinucleotide reduced/min. Quantitative assays for β-galactosidase activity with *o*-nitrophenyl-β-D-galactose as substrate were

performed as described by Guarente [25]. Protein concentration was estimated by the Bio-Rad assay method as described by the manufacturer.

RNA preparation

Cell pellets were resuspended in RNA extraction buffer (5 ml) [14] and transferred to a cocktail of glass beads (14 g, 0.5 mm diameter), 0.5 ml SDS [10 % (v/v)], phenol (5 ml; equilibrated with 1.0 M Tris/HCl, pH 7.5, containing 8-hydroxyquinoline) and chloroform (5 ml). The suspension was vortex-mixed continuously for 5 min and centrifuged at 5000 g for 5 min; the aqueous phase was removed and extracted twice with a mixture of phenol (5 ml) and chloroform (5 ml) followed by two further extractions with chloroform (5 ml). RNA was precipitated by adding 2.5 vol. of ethanol.

RNA gel electrophoresis

RNA was resuspended in aqueous solution and the concentration of nucleic acids was determined by comparing A_{260} and A_{280} . Total RNA (approx. 20 µg per lane) was electrophoresed in a formaldehyde [6 % (v/v)]-based agarose [1.5 % (w/v)] gel and transferred to a Hybond-N nylon membrane (Amersham). RNA was fixed on the membranes as recommended by the supplier. Gels were stained with ethidium bromide before transfer to assess the qualitative integrity of RNA by detecting the 18 S and 25 S ribosomal RNA bands under UV illumination.

Northern blot analysis

Membranes were prehybridized for a minimum of 3 h and hybridized for 24–38 h. The prehybridization solution consisted of 5 × SSPE (175.3 g of NaCl, 26.7 g of NaH_2PO_4 and 7.4 g of EDTA in 4 litres of water, adjusted to pH 7.4 with NaOH), formamide (50 %), 5 % (v/v) Denhardt's solution (5 g of Ficoll, 5 g of poly(vinylpyrrolidone) and 5 g of BSA in 500 ml of water) and SDS [5 % (w/v)]. Hybridization solution was similar to the prehybridization solution but contained the radioactively labelled probe. All hybridizations were performed at 42 °C in a shaking water-bath. Membranes were washed as described by the manufacturer (Amersham) and were exposed at –70 °C to Kodak X-ray film with an intensifying screen. The membranes were stripped of probe in accordance with the manufacturer's instructions and rehybridized as described above.

Labelling of DNA probes with ^{32}P was performed either by nick translation or by random priming (Amersham). The probes used to detect *LPD1* sequences were either plasmid pGP1R1 [15] or a purified 3.66 kb *XhoI* fragment derived from it. *HIS3* was detected by using the *KpnI*–*EcoRI* fragment isolated from pVecHIS3, a derivative of SCHIS3G [26] inserted into the vector pUC9. Plasmid pSPACT9 [21] was used to detect actin sequences.

Densitometry measurements of the amount of probe bound to filters after hybridization were performed with the Agfa Dusan System.

Site-directed mutagenesis

Each of the GCN4 binding motifs in the upstream region of the *LPD1* gene was modified by using the gapped duplex method on plasmid pZZD-2µm, as described by Bowman et al. [16]. The mutagenic oligonucleotides used were as follows: for site GCN4A, 5'-CGTTTTTAATGATCGATCGTTTTTAGAAT-AC-3'; for site GCN4B, 5'-CGTGAATATCACGTGATATC-GTTTTTAATGATG-3'; and for site GCN4C, 5'-CGTACC-CTTGGGCCCCACCTCGAA-3'.

Mismatches to the *LPD1* sequence are underlined. Each

mutant form was confirmed by sequencing; the wild-type and mutant promoters were cloned into plasmid YIp358 of Myers et al. [23] and the constructs were integrated at the *ura3* locus of the wild-type strain BWG1-7A as described previously [16]. The mutant HAP2/3 promoter construct was as described by Bowman et al. [16]. Multiple site mutations were generated starting from single site mutations by using the same protocol.

Gel retardation analysis

The expression vector pSP64-GCN4 was used to produce GCN4 protein as described by Hope and Struhl [24]. GCN4 protein was produced in 50 µl of TnT Wheat Germ extract (Promega) containing 1 µg of pSP64-GCN4 template DNA and 10 units of SP6 polymerase. Control reactions contained 1 µg of a pSP64-luciferase gene construct. The reaction was incubated at 30 °C for 2 h and used directly in the gel mobility-shift assay.

Complexes formed between GCN4 protein and ³²P-labelled DNA fragments from the *LPD1* gene were detected by gel retardation analysis [24]. Binding was performed in 12 µl of 16 mM Tris/HCl (pH 7.4)/50 mM KCl/3 mM MgCl₂/1 mM EDTA/10 µg/ml gelatin/5 µg/ml sonicated salmon sperm DNA. Each reaction consisted of 4 fmol of ³²P-labelled DNA fragment to be bound and GCN4 translation mixture synthesized *in vivo*; the reaction was incubated at room temperature for 15 min. Loading buffer [5 µl, containing 20% (v/v) glycerol, 1 mg/ml xylene cyanol FF and 1 mg/ml Bromophenol Blue] was added to the DNA control lanes only. The samples were immediately loaded on a 5% (w/v) polyacrylamide gel (acrylamide-to-bisacrylamide ratio 98.8:1.2) and subjected to electrophoresis at 120 V in 90 mM Tris/borate buffer, pH 8.3, containing 7.5 mM 2-mercaptoethanol until the samples entered the gel, then at 240 V until the Bromophenol Blue had migrated to 1 cm from the bottom of the gel (20 cm). Electrophoresis was performed at 4 °C with recirculation of the buffer.

RESULTS

Presence of the GCN4 binding consensus sequence in the *LPD1* gene

Figure 1 shows the relevant sequence 5' to the open reading frame of the *LPD1* gene. At -264 and -247 there are two very

Figure 1 Sequence of part of the control region of the *S. cerevisiae* *LPD1* gene

Potential core motifs for the binding of GCN4 and HAP2 proteins are indicated in bold. The three GCN4 sites have been arbitrarily assigned letters. Binding sites for the transcription factor ABF1 are underlined. The start codon for *LPD1* is indicated in bold and italics.

close matches to the consensus sequence for GCN4-mediated control. At -114 there is a third TGACTC sequence that lacks the adjacent run of T residues that is considered necessary for maximal activation by GCN4 [26]. This motif is thus more like the canonical yAP1 motif [27], the BAS1 motif [28] or the GCN4 motifs found in the *ADE* gene promoters [6]. The insertion of a single copy of a 14 bp sequence containing the GCN4-binding consensus is sufficient to bring the *CYC1* gene (encoding isocytochrome *c*) under general amino acid control [29], indicating that the sequence context of a GCN4-binding site need not be critical for activity. Therefore the presence of repeated copies of this motif in the upstream region of the *LPD1* gene indicated that it might be subject to general amino acid control. Figure 1 also indicates other potential motifs for DNA-binding proteins that occur within this region of the gene.

Amino acid starvation induces *LPD1* gene expression

The effect of amino acid starvation on the expression of the *LPD1* gene was investigated in isogenic wild-type and *gcn4* mutant cells (dysfunctional for GCN4 activity). Amino acid limitation can be imposed on yeast either by growth of an amino acid auxotroph in the absence of the required amino acid or by growth of wild-type cells in the presence of an amino acid analogue such as 3-amino-1,2,4-triazole [20], which is a competitive inhibitor of imidazoleglycerol-phosphate dehydratase, and can be used to mimic histidine starvation and induce the general control response [20,31]. Amino acid starvation by either method leads to the induction of enzymes subject to general control. Moreover, although the presence of an amino acid analogue presents the most severe starvation state, a change in external amino acid concentration is sufficient to trigger induction [20].

To study the regulation of the *LPD1* gene we used an *LPD1::lacZ* reporter gene integrated chromosomally as a single copy at the *ura3* locus in yeast. This reporter comprised 1.47 kb of the *LPD1* gene containing the upstream promoter region, the ATG start codon and downstream sequences known to affect transcription of the gene, in frame to a truncated *E. coli* *lacZ* gene. We have previously demonstrated that levels of β -galactosidase activity in cells carrying this reporter accurately reflect the transcriptional control and activation of the *LPD1* gene [14,16,17].

Figure 2 shows *LPD1::lacZ* gene expression in wild-type and *gcn4* mutant cells switched from non-inducing medium (containing all amino acids) to inducing medium (amino acid starvation). After a shift to amino acid starvation, expression of the *LPD1::lacZ* reporter was induced approx. 2-fold above basal level as measured by the specific activity of β -galactosidase. This 2-fold induction began 30 min after the shift and continued to increase gradually for up to 8 h (Figure 2A). This compares with the levels of induction seen for other enzymes directly under general control. For example, enzyme activities encoded by the *TRP2*, *HIS2* and *HIS4* genes are induced between 1.6-fold and 3.0-fold under similar conditions [31,32]. In control experiments no change in the specific activity of β -galactosidase was observed on shifting cells from a non-inducing medium to a medium of the same composition, indicating that the operations used to change growth medium did not lead to significant effects on the expression of the reporter gene. In *gcn4* cells there was no significant difference between cultures shifted from a non-inducing medium to either inducing or non-inducing media (Figure 2B), indicating a requirement for GCN4 in response to general control.

Figure 2 Induction of *LPD1::lacZ* reporter in wild-type and *gcn4* mutant cells

GCN4 wild-type cells (**A**) and *gcn4* mutant cells (**B**), each carrying an *LPD1::lacZ* reporter gene, were grown to exponential phase on non-inducing medium, harvested, washed and transferred at zero time to non-inducing medium (○) or inducing medium (●). Cells were harvested at intervals and assayed for β -galactosidase. Each point is the average for triplicate assays.

Table 1 Specific activities of lipoamide dehydrogenase in wild-type and *gcn4* mutant cells

Cells were grown to exponential phase on non-inducing medium and then shifted to inducing or non-inducing medium as described in the legend to Figure 2. Cell cultures were sampled for enzyme assays while still in the exponential phase. Specific activities are expressed relative to that in each culture before the growth conditions were shifted. Values are averages for assays in triplicate. Before the shift the specific activities of lipoamide dehydrogenase in minimal medium were 0.03 unit/mg of protein for the wild-type and 0.012 unit/mg of protein for the *gcn4* mutant.

Strain	Specific activity of lipoamide dehydrogenase		Induction ratio
	Non-inducing	Inducing	
Wild-type	1.8	3.7	2.1
<i>gcn4</i> mutant	1.9	1.7	0.9

The activity of the *LPDH* enzyme encoded by the intact *LPD1* gene in wild-type cells was also found to be induced under conditions of amino acid starvation (Table 1). The level of induction under these conditions was approx. 2-fold in wild-type cells but no change was observed in the *gcn4* cells, in agreement with the results obtained from the *LPD1::lacZ* fusion. Taken

Figure 3 Steady-state *LPD1* mRNA levels in wild-type and *gcn4* mutant cells

Total RNA was prepared from cell cultures grown to exponential phase in inducing medium. RNA (20 μ g) was separated on formaldehyde-containing agarose gels, then transferred to a Hybond-N nylon membrane. The membrane was probed for *LPD1* and actin mRNA by using 32 P-labelled plasmids or DNA fragments containing appropriate sequences. Before transfer, the gel was stained with ethidium bromide to reveal the 18 S and 25 S ribosomal RNA bands.

together, these results indicated that expression of the *LPD1* gene is subject to regulation by general control that is mediated by the product of the *GCN4* gene.

***GCN4* is required for maintenance of steady-state *LPD1* mRNA levels**

Figure 3 shows a direct role for the product of the *gcn4* locus in maintaining elevated steady-state *LPD1* mRNA levels in response to general control. *LPD1* mRNA levels were determined in wild-type and *gcn4* cells growing in exponential phase in inducing medium. Actin gene expression is not subject to the general control response (see below) and was therefore used as an internal control. The loading and integrity of RNA was initially assessed by examining the 18 S and 25 S ribosomal RNA bands. Steady-state *LPD1* mRNA levels in wild-type cells were approx. 5-fold above those in the *gcn4* cells as measured by densitometry and normalized to actin mRNA. The difference between *LPD1* mRNA levels and the enzymic activities (see Table 1 and Figure 2) might be attributable to some form of post-transcriptional regulation or processing of RNA. However, both sets of results confirm that *LPD1* gene expression in response to general control requires *GCN4*.

The protracted response of wild-type cells to continuous growth in inducing medium is the maintenance of induced *LPD1* mRNA levels. This induction is similar to that seen for other genes regulated by the general response [1,20], suggesting an important role for *LPD1* in the *GCN4*-mediated biosynthetic

Figure 4 Induction kinetics of *LPD1* mRNA

Wild-type cells were pre-grown to exponential phase on non-inducing medium. At zero time a sample of cells was removed and harvested by centrifugation. The remainder of the culture was filtered and resuspended in either inducing or non-inducing medium. Samples of the culture were withdrawn at intervals and harvested by centrifugation. RNA was prepared from each sample; 20 µg was separated on formaldehyde-containing agarose gels and transferred to a Hybond-N nylon membrane. The membrane was probed for *LPD1*, *HIS3* and actin mRNA species by using ³²P-labelled plasmids or DNA fragments containing appropriate sequences. Before transfer, the gel was stained with ethidium bromide to reveal the 18 S and 25 S ribosomal bands.

pathways. Elevated levels of *LPD1* gene expression might reflect not only a continued need for the metabolic precursors of amino acid biosynthesis but also a refined rapid feedback mechanism (via GCN4 and possibly other factors) to maintain general control extending beyond those enzymes directly involved in the pathway.

General control mechanism stimulates rapid induction of *LPD1* mRNA

The sensitivity and response to changes in the amino acid environment might prove important in establishing the physiological significance of a gene product to general control. Kinetic studies of the general control response of *TRP5* transcription revealed that the *TRP5* mRNA peaked at approx. 1.5 h after transfer to amino acid-starvation medium [31]. More detailed studies by Penn et al. [20] showed that a number of genes under general control, including *HIS4*, *HIS3*, *ARG4* and *TRP5*, responded within 5 min of a shift from growth in amino acid-containing medium to growth in starvation medium, with peak mRNA levels occurring after 1–2 h.

To assess the response time for *LPD1*, *LPD1* mRNA levels were examined at several time points after a shift of cells from growth on non-inducing medium to inducing medium (Figure 4). *LPD1* mRNA levels normalized to actin mRNA were elevated 2–5-fold with respect to zero-time levels after a shift from growth on non-inducing medium to growth on inducing medium. A similar induction pattern was seen with the *HIS3* mRNA, confirming that the experiment mimicked conditions for a general control response because *HIS3* gene expression is known to be

Figure 5 Effect of each GCN4-binding site on the induction of *LPD1*

LPD1::lacZ reporter genes containing site-directed mutations that eliminated the binding of GCN4 to each binding site were generated and integrated independently in wild-type cells. Reporter cells were streaked on non-inducing medium plates, grown for 3 days and then replica-plated on inducing and non-inducing media plates containing X-Gal. The plates were incubated at 30 °C and examined at intervals for the blue colony colour conferred by hydrolysis of the X-Gal by β-galactosidase expression. Activities were scored as follows: —, white colonies; +, weakly blue colonies; ++, blue colonies; +++, strongly blue colonies.

subject to this type of regulation [33]. The results also reveal that, like the *URA3* gene [34], the actin gene was not subject to this control (confirmed by using the 18 S and 25 S ribosomal bands as controls for the integrity and amount of RNA loaded in each lane). For *LPD1* a smaller degree of induction was also observed for a shift of cells from non-inducing medium to fresh non-inducing medium, which was not observed for the *HIS3* transcript. This probably reflects the greater complexity of control elements affecting *LPD1* gene expression [14]. The *HIS3* response reached a maximum within 30 min of a shift to starvation conditions, then gradually declined, as seen by Penn et al. [20]. The *LPD1* mRNA levels, however, showed a marked increase in the first 30 min after the shift (approximately 2-fold with respect to zero time) but continued to increase at a decreasing rate throughout the course of the experiment, reaching nearly 5-fold levels 6–10 h after induction. These results indicate that the general control mechanism stimulates a rapid induction of *LPD1* mRNA to conditions of amino acid starvation and that induction levels are maintained under continuous starvation.

Central GCN4-binding motif in the *LPD1* promoter is essential for induction

Several gene promoters co-regulated by general control contain repeated copies, with minor variations, of the core consensus sequence TGACTC for the binding of GCN4 by DNA [7]. Although not all binding sites seem to be required for induction, a minimal set of functional copies is required for maximal induction in conditions of amino acid starvation [29,35]. *LPD1* has three potential GCN4-binding motifs (designated sites A, B and C in Figure 1) that might contribute to its regulation by the general control mechanism. To assess the functional significance of each site, site-directed mutagenesis was used to alter the core binding motifs in each of the A, B and C sites, and in all combinations of sites. Mutant sites were generated such that they would abolish potential GCN4 binding as suggested by saturation mutagenesis of the core consensus sequence [36]. Wild-type cells

Figure 6 Retardation analysis of GCN4 protein binding to *LPD1* promoter sequences

Upper panel: gel retardation analysis with the ^{32}P -labelled 114 bp DNA fragment from -217 to -103 of the *LPD1* gene with GCN4 protein synthesized *in vitro*. Each lane contained 4 fmol of fragment DNA. Lane 1, DNA alone; lanes 2–6, 0.5, 1, 2, 5 and 10 μl of protein extract respectively; lane 7, 10 μl of luciferase protein synthesized *in vitro* as control. Lower panel: gel retardation analysis with ^{32}P -labelled 165 bp DNA fragment from -383 to -218 of the *LPD1* gene with GCN4 protein synthesized *in vitro*. The lane profile is identical with that in the upper panel.

with chromosomally integrated *LPD1::lacZ*. reporter genes containing these GCN4-binding site mutations were tested for their ability to respond to general control. Cells were scored for *lacZ* expression on either inducing or non-inducing medium containing X-Gal as indicator, as described by Hope and Struhl [24] (Figure 5).

The results depicted in Figure 5 show that site A is essential for the general response. Mutations in site A, alone or in combination

with the other two sites, completely abolish a general control response (compare non-inducing with inducing). Sites B and C do not seem to be required for a response under these conditions. However, site C might have a role in general control in lactate-based medium, possibly in concert with factors at the HAP2/3/4/5 DNA-binding site (results not shown).

Results on non-inducing medium suggest that GCN4 motifs B and C affect basal activity differentially (Figure 5). Mutation of sites B and C alone (reporters 3 and 4) or in combination (reporters 7 and 8) increases basal activity on non-inducing medium with respect to the unaltered reporter. A mutation in GCN4 motif A does not affect basal activity on this same medium (compare reporters 1 and 2). Despite this, and excluding the observation of reporter 4 (see below), site A seems to mediate the general control response over and above any differences in basal activity.

Mutation of motif C seems to suppress a general response (reporter 4). However, this response is restored when site C is mutated in combination with a mutation at site B (reporter 7). The *LPD1* gene promoter contains binding sites for several known transcription factors and is therefore subject to complex regulation [14,16,17]. This is further complicated by the fact that there might be more than one functional TATA element immediately distal to motif C. Thus a mutation of motif C might not only abolish the binding of GCN4 but also weaken the binding of transcription factors that respond specifically to induction by site A. Further, because sequences flanking a GCN4 site have an important role in its binding [30,35], a mutated site B that is in close proximity to site A might have rendered site A a stronger site such that it can now overcome the influence of a mutated site C. Moreover, effects which might influence synergy or co-operativity between similar or dissimilar transcription factors regulating *LPD1* transcription cannot be ruled out [37].

Deletion and mutational analysis of several gene promoters produce a change in basal activity. The change in basal activity on the mutation of sites B and C in *LPD1* is reminiscent of effects on the basal activity of *HIS4* when certain intervening and target sequences for GCN4 are deleted or mutated [29,30]. These effects might be due to the removal of overlapping sites for repressing factors, the generation of fortuitous sites for activating factors or the remodelling of chromatin to allow more accessibility to general transcription factors. Mutations of regions close to the TATA element might alter the binding of the basal transcriptional machinery. These possibilities have yet to be addressed for *LPD1*.

GCN4 protein binds to three motifs in the *LPD1* promoter

Although Northern blotting results show that *LPD1* is induced on severe limitation for amino acids, this result could be due to the induction of the gene via a signalling pathway other than GCN4 (for example in response to the depletion of a general pool of substrates), to the increased requirement for ATP to synthesize amino acids or as a general stress response. Mutation of the three putative GCN4 motifs does support the hypothesis that these sequences are required for mediation of the response. However, two of these sequences diverge from the canonical consensus GCN4 motif. We therefore tested the ability of GCN4 protein to bind to the sequences in the upstream region of the *LPD1* gene. Binding of GCN4 protein to the DNA was demonstrated by using GCN4 protein made by transcription and translation *in vitro* from the expression vector pSP64-GCN4 [24] in a series of gel mobility-shift assays.

Binding of GCN4 protein synthesized *in vitro* was shown to occur within the upstream promoter of the *LPD1* gene by using two end-labelled restriction fragments encompassing the regions from -383 to -218 and from -217 to -103. In gel mobility-shift assays, GCN4 protein bound to the DNA in a relative concentration-dependent manner (Figure 6). Incubation of either region of DNA with a control transcription/translation reaction that did not include *GCN4* DNA did not give rise to a mobility shift. Although the GCN4-C motif does not contain a run of thymidines 3' of the core motif as occurs in most GCN4-binding sites in amino acid biosynthetic genes, GCN4 protein was able to bind *in vitro*. The larger DNA fragment contains two potential binding sites directly repeated with a phasing of 17 bp that could not be separated without removing the flanking sequence around the motifs. At the relative protein concentrations tested, only one distinct shift was seen for this fragment. GCN4 protein interactions were abolished when the DNA was first incubated with the restriction enzyme *HinfI* (results not shown). This enzyme cleaves at the sequence GATC, a element central in the GCN4 DNA-binding motif, confirming the localization of the GCN4 protein-binding sites in the *LPD1* promoter region.

DISCUSSION

Until recently, all genes shown to be subject to general amino acid control were directly involved with the synthesis of amino acids, purines and the charging of tRNA species [1-5]. Here we have shown that expression of the *LPD1* gene is also subject to the same general control mechanism. *LPD1* encodes LPDH, an enzyme central to respiratory metabolism but not directly involved in purine or amino acid biosynthesis.

Studies on *LPD1::lacZ* reporter genes along with LPDH enzyme assays and mRNA analysis revealed that *LPD1* gene expression was induced in cells starved of amino acids. The induction level was equivalent to that for other enzymes subject to general control in response to amino acid starvation [1,20,30-32]. A comparison of induction levels in wild-type and *gcn4* mutant cells indicated that the GCN4 transcription factor was necessary for *LPD1* gene expression in response to general control (Table 1, Figures 2 and 3). The *LPD1* gene promoter contains putative DNA-binding sites for GCN4 (Figure 1). Studies *in vivo* on reporter genes harbouring site-directed mutations in the *LPD1* promoter indicated a need for intact GCN4 DNA-binding sites for a response to general control (Figure 5). Taken together, these results demonstrated unequivocally the regulation of *LPD1* by the general control mechanism mediated by GCN4.

Of the three possible GCN4-binding sites within the *LPD1* promoter, the results in Figure 5 suggest that only one particular site, site A (Figure 1), seems to be necessary for the general control response. This is analogous to the *HIS3* gene promoter, which also contains multiple GCN4 DNA-binding sites with only one site being important [30]. However, results from gel-shift analyses suggest that GCN4 can bind to at least two of the three potential GCN4-binding sites in the *LPD1* gene promoter. Whereas such studies *in vitro* add credence to the genetic data, they do not necessarily correspond to activity *in vivo*. *LPD1* is subject to complex regulation and a role for the other two sites under a different set of conditions cannot be ruled out. The GCN4 mechanism has a role in response to many stress types [38]. The presence of multiple GCN4 DNA-binding sites in the *LPD1* promoter might allow the interplay of several transcription factors that compete for the same site and stimulate transcription in response to different types of stress. For example, yAP1 and

GCN4 are required for *ATRI* gene expression and bind at the same site within the promoter [39].

A time-course analysis of enzyme and mRNA levels demonstrated that *LPD1* gene expression responds rapidly to changes in the amino acid environment of the cell (Figures 2 and 4). This behaviour is similar to that of genes involved in the histidine, tryptophan and arginine biosynthetic pathways [20,30]. However, over long periods of amino acid deprivation, a comparison of *LPD1* and *HIS3* mRNA species indicated differences in induction profiles: whereas *LPD1* mRNA levels continued to rise over time, *HIS3* mRNA levels peaked and then showed a decline over the same period. This difference might reflect the differences in the complexities of the two promoters. Further, the metabolic demand for LPDH under conditions of continued stress (such as starvation for amino acids or the demand for central metabolites) differs from those of pathway-specific enzymes for amino acid biosynthesis showing a classical response to general control; there might be some overlap between the different responses.

The finding that LPDH is under general amino acid control might be surprising, given the known role of this control system in the synthesis of amino acids and purines as a response to amino acid starvation. However, LPDH as a component of the two multienzyme complexes PDH and OGDH has an important role in the flow of metabolites into and through the tricarboxylic acid cycle. During growth on minimal medium, PDH is needed to generate acetyl-CoA, which is required for the biosynthesis of several amino acids including methionine and cysteine, and the tricarboxylic acid cycle (together with anaplerotic pathways) is needed for the synthesis of precursors of the glutamate family of amino acids and for purine biosynthesis [40]. Because LPDH acts in concert with the other components of the PDH and OGDH complexes, one might expect to find GCN4 elements in some of the genes encoding the other subunits of these complexes, and possibly in other tricarboxylic acid cycle and glyoxylate cycle enzymes. Delforge et al. [41] have reported that fumarase activity is depressed in arginine bradytrophs and have suggested that this tricarboxylic acid cycle enzyme might also be under general amino acid control. Of the other genes encoding the subunits of the PDH complex, *PDA1* (encoding the E1 α subunit), *PDB1* (encoding the E1 β subunit) and *PDX1* (encoding the protein X subunit) have GCN4 motifs in the upstream control regions, and that in *PDA1* is active [42]. *PDA1* is expressed during anaerobic growth, when the PDH complex is not required for catabolism, and disruption of *PDA1* results in a partial leucine deficiency, indicating that the gene might be regulated in response to precursors of amino acid biosynthesis [42a]. A search of the available sequences for *KGD1* and *KGD2* encoding the two specific OGDH subunits [43,44] has not identified any GCN4-binding motifs; this is consistent with the hypothesis that the control of GCN4 over *LPD1* is relevant to its role in amino acid synthesis because OGDH is not needed for the synthesis of amino acids of the glutamate family, for the glyoxylate cycle or for the generation of acetyl-CoA.

The physiological role of the general control response of the *LPD1* gene might therefore be to fine-tune the synthesis of this enzyme when it is required for the production of central metabolites acting as precursors for amino acid biosynthesis. General amino acid control might therefore have a wider role than in regulating the expression of genes that are uniquely involved in amino acid biosynthetic pathways, and our results indicate that this extends to at least one gene that is involved in central metabolism.

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