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 gen4 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 rTGAStCA(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, NH3, 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...
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 fulfill 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 GCN4A-103) supplied by A. Hinnebusch; strain 361 (MATa leu2-112 can4) supplied by F. Messenguy; and strain BW37-7A (MATa ade1-100 his4-519 leu2-3 ade2-112 ura3-52) supplied by L. Guarente. Minimal medium consisted of 1.7 g/l Difco yeast nitrogen base without amino acids and (NH4)2SO4, with the addition of 20 g/l raffinose, 5 g/l (NH4)2SO4 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-galactoside (X-Gal, 20 mg/ml) in dimethylformamide as required.

Plasmids

Plasmid pGPI contains the intact LPD1 gene in the YEp13 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 yeast actin gene [23] were supplied by A. Tzagoloff [23]. Plasmid pSP64-GCN4 and related plasmids used for site-directed mutagenesis has been described previously [16]. The plasmids pSPACT9 containing the yeast actin gene [21] and pMC1871 carrying the yeast actin gene [23] were supplied by A. Tzagoloff [23]. Plasmid pSP64-GCN4 and related plasmids used for site-directed mutagenesis has been described previously [16].

Growth of cultures and sampling

For induction experiments, yeast cell cultures growing at 30 °C in the exponential phase (D600 0.3) were harvested 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 LDH 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-galactoside 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 A260 and A280. 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 x SSPE (175.3 g of NaCl, 26.7 g of NaH2PO4 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 %, (v/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 32P was performed either by nick translation or by random priming (Amersham). The probes used to detect LPD1 sequences were either plasmid pGPI1R1 [15] or a purified 3.66 kb Xhol fragment derived from it. HIS3 was detected by using the Kpn–EcoRI fragment isolated from pVecHIS3, a derivative of SCH133G [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 Duscan 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-2μ, as described by Bowman et al. [16]. The mutagenic oligonucleotides used were as follows: for site GCN4A, 5′-CGTGTGTAATGATCGATCGTGGTTTTTAGAATAC-3′; for site GCN4B, 5′-CGTGAATACGCTGATACGTTTTAAATGATG-3′; and for site GCN4C, 5′-CGTACCCTTGGCCACCTGCA-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/μl sonicated salmon sperm DNA. Each reaction consisted of 4 fmol of 32P-labelled DNA fragment to be bound and GCN4 translation mixture synthesized in vitro; 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 % (v/v) polyacrylamide gel (acrylamide-to-bisacrylamide ratio 98.8:1.2) and subjected to electrophoresis at 120 V in 90 mM Tris-bisacrylamide ratio 98.8:1.2). The samples were immediately loaded on a 5 % gel and electrophoresed 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 for 2 h and used directly in the gel mobility-shift assay.

RESULTS

Presence of the GCN4 binding consensus sequence in the LPD1 gene

Figure 1 shows the relevant sequence S′ to the open reading frame of the LPD1 gene. At −264 and −247 there are two very 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 iso-cytochrome 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 induction was seen 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 (C) or inducing medium (D). Cells were harvested at intervals and assayed for \( \beta \)-galactosidase. Each point is the average for triplicate assays.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Non-inducing</th>
<th>Inducing</th>
<th>Induction ratio</th>
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<tr>
<td>Wild-type</td>
<td>1.8</td>
<td>3.7</td>
<td>2.1</td>
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<tr>
<td>gcn4 mutant</td>
<td>1.9</td>
<td>1.7</td>
<td>0.9</td>
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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 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 process.
revealed that the TRP5 ribosomal bands. Before transfer, the gel was stained with ethidium bromide to reveal the 18 S and 25 S LPD1 Hybond-N nylon membrane. The membrane was probed for studies of the general control response of logical significance of a gene product to general control. Kinetic environment might prove important in establishing the physio-

The sensitivity and response to changes in the amino acid biosynthesis but also a refined rapid feedback mechanism 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 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
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 thymines 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 spacing 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 Hinfl (results not shown). This enzyme cleaves at the sequence GANTC, 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 ATR1 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 KPDI 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 Elz subunit), PDB1 (encoding the Elβ 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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