The Saccharomyces cerevisiae Leu3 Protein Activates Expression of GDH1, a Key Gene in Nitrogen Assimilation†

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The Leu3 protein of Saccharomyces cerevisiae has been shown to be a transcriptional regulator of genes encoding enzymes of the branched-chain amino acid biosynthetic pathways. Leu3 binds to upstream activating sequences (UASLEU) found in the promoters of LEU1, LEU2, LEU4, ILV2, and ILV5. In vivo and in vitro studies have shown that activation by Leu3 requires the presence of α-isopropylmalate. In at least one case (LEU2), Leu3 actually represses basal-level transcription when α-isopropylmalate is absent. Following identification of a UASLEU-homologous sequence in the promoter of GDH1, the gene encoding NADP⁺-dependent glutamate dehydrogenase, we demonstrate that Leu3 specifically interacts with this UASLEU element. We then show that Leu3 is required for full activation of the GDH1 gene. First, the expression of a GDH1-lacZ fusion gene is three- to sixfold lower in a strain lacking the LEU3 gene than in an isogenic LEU3⁺ strain. Expression is restored to near-normal levels when the leu3 deletion cells are transformed with a LEU3-bearing plasmid. Second, a significant decrease in GDH1-lacZ expression is also seen when the UASLEU of the GDH1-lacZ construct is made nonfunctional by mutation. Third, the steady-state level of GDH1 mRNA decreases about threefold in leu3 null cells. The decrease in GDH1 expression in leu3 null cells is reflected in a diminished specific activity of NADP⁺-dependent glutamate dehydrogenase. We also demonstrate that the level of GDH1-lacZ expression correlates with the cells' ability to generate α-isopropylmalate and is lowest in cells unable to produce α-isopropylmalate. We conclude that GDH1, which plays an important role in the assimilation of ammonia in yeast cells, is, in part, activated by a Leu3-α-isopropylmalate complex. This conclusion suggests that Leu3 participates in transcriptional regulation beyond the branched-chain amino acid biosynthetic pathways.

The role of the LEU3 gene product as a transcriptional regulator in the biosynthesis of branched-chain amino acids is well established (7, 14, 33, 34, 36, 38). Leu3 is a DNA-binding protein of the Zn(II)₂-Cys₆ binuclear cluster type that interacts with an upstream activating sequence (UASLEU) in the promoter regions of LEU1, LEU2, LEU4, ILV2, and ILV5 (7, 14). Once bound to its target DNA, Leu3 can act either as a transcriptional activator or as a repressor. Leu3 mediates transcriptional activation only when α-isopropylmalate (α-IPM), the reaction product of the first committed step in leucine biosynthesis, is present (34). This dependence on α-IPM ties activation by Leu3 to the highly regulated LEU4 gene product, α-IPM synthase (4, 27). In the absence of α-IPM, Leu3 reduces basal-level transcription four- to fivefold (34). α-IPM-dependent transcriptional activation and repression in the absence of α-IPM have been observed both in vivo (3, 7) and in a yeast-derived in vitro transcription system (33, 34). In the latter, the UASLEU of the LEU2 promoter served as the Leu3 binding site.

The present investigation was prompted by the results of an earlier search for homologies to the UASLEU consensus sequence (5'-GCGGNNCCGGG-3') in the yeast nucleotide sequence database (36). The search produced one perfect match located upstream of the GDH1 gene (25). The sequence, 5'-GCGGGAACCCGGC-3', is identical to that of the UASLEU element of LEU2 (1). It is located between positions −405 and −394, with +1 signifying the beginning of the open reading frame of GDH1.

The GDH1 gene encodes NADP-dependent glutamate dehydrogenase (NADP-GDH) (17, 24, 25). In Saccharomyces cerevisiae, NADP-GDH occupies a key position in anabolic nitrogen metabolism: the combination of the NADP-GDH-catalyzed reaction (\( \text{NH}_3 + \alpha\text{-ketoglutarate} + \text{NADPH} + \text{H}^+ \rightarrow \text{glutamate} + \text{NADP}^+ \)) with the glutamine synthetase reaction (\( \text{NH}_3 + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{P}_i \)) constitutes the major pathway for the assimilation of ammonia (22). A second pathway, combining the glutamine synthetase reaction with the glutamate synthase-catalyzed reaction (glutamine + α-ketoglutarate + NADH + H⁺ → 2 glutamate + NAD⁺), is of minor importance, as shown by the fact that mutants lacking glutamate synthase grow as well as wild-type cells in media containing ammonium sulfate as the sole nitrogen source; in the same media, mutants lacking NADP-GDH grow at only about half the rate of wild-type cells (22). In spite of the key position of GDH1, our knowledge of what regulates the expression of this gene is scant. The specific activity of NADP-GDH was twofold higher in extracts from cells grown with ammonium sulfate as the sole nitrogen source when other nitrogen sources such as glutamate, aspartate, glutamine, or asparagine were used instead (29). The initial ammonium sulfate concentration in these experiments was 10 mM. Bogonez et al. (5) confirmed the induction by ammonium sulfate and added the observation that at much higher initial concentrations of ammonium sulfate (50 to 360 mM), the specific activity of the enzyme actually decreased about threefold. The authors showed that the decrease was not caused by accelerated degradation of the enzyme. They proposed the existence of some type of repression mechanism under these conditions. GDH1...
TABLE 1. Strains and plasmids used

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E. coli

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<tr>
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Plasmids

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<tr>
<td>pSEYC102</td>
<td>CEN4 plasmid containing a lacZ gene preceded by a polylinker region</td>
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<tr>
<td>pYH100</td>
<td>See Fig. 1 and text</td>
<td>This work</td>
</tr>
<tr>
<td>pYH101</td>
<td>See Fig. 1 and text</td>
<td>This work</td>
</tr>
<tr>
<td>pKZ5</td>
<td>2μm plasmid carrying the LEU3 gene under the control of the PHO5 promoter</td>
<td>37</td>
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</table>

FIG. 1. Partial restriction maps and identification of important elements of plasmids pYH100 and pYH101. The position designation –390 refers to the GDH1 promoter (GDH1-p), with +1 signifying the beginning of the open reading frame of GDH1. The UASLEU consensus sequence begins at –394. See Materials and Methods (construction of GDH1-lacZ fusions) for further details.

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 Yeast nitrogen base without amino acids and ammonium sulfate (Difco Laborato-
ries), 1% glucose, and 0.1% nitrogen sources as specified plus 0.15 mM leucine, 0.18 mM uracil, and 0.1 mM each of tryptophan and histidine when required. E. coli TG1 was routinely used for DNA manipulations. Strain C236 was used for the isolation of uridine-containing single-stranded DNA. E. coli cells were grown in LB (9) containing ampicillin (100 μg/ml) alone or with chloramphenicol (50 μg/ml), as needed.

Construction of GDH1-lacZ fusions. Plasmid pCYG4, which contains the GDH1 gene (25), was a gift from Benjamin Hal, University of Washington. Plasmid pSEYC102 was obtained from S. Emr, California Institute of Technology. To construct a GDH1-lacZ fusion, the termini of the 0.95-kb HindIII fragment from plasmid pCYG4 were filled in with Klenow fragment of DNA polymerase. This fragment was then ligated to Smal-digested plasmid pSEYC102. The resulting plasmid, pYH100 (Fig. 1), expresses a fusion protein containing nine amino acids from the GDH1 gene, three amino acids from the linker region, and amino acids 10 to 1024 from the lacZ gene. To construct a GDH1-lacZ fusion lacking a functional UASLEU element, the 0.95-kb HindIII fragment from pCYG4 was cloned into plasmid pUC118. The subclone was transformed into strain C236, and uridine-containing single-stranded DNA was isolated. An oligonucleotide (5′-GAAAATTCAGGCGCGGATTTTTTC GCCGCT-3′) was synthesized, and site-directed mutagenesis was carried out by the procedure of Kunkel et al. (20). The mutation was identified by the presence of a new Apal site and confirmed by sequencing. The mutated HindIII fragment was recovered, the termini were filled in, and the fragment was ligated to Smal-digested plasmid pSEYC102. The resulting plasmid was designated pYH101 (Fig. 1).

Yeast transformation, cell growth, and enzyme assays. Plasmids pYH100 and pYH101 were introduced into XK157-R and XK157-3C cells by a modified lithium acetate method (15). The transformants were purified once, and single colonies were inoculated into 10 ml of SD medium (13). After overnight growth at 30°C yeast cultures were harvested by centrifugation and the cell pellets were washed once with sterile double-distilled water. The cells were inoculated into 50 ml of yeast nitrogen base-glucose medium containing 0.1% ammonium sulfate, 1% asparagine, 1% glutamine, or 1% glutamate as the sole nitrogen source. The cells were harvested at an optical density at 600 nm (OD600) of between 0.9 and 1.0. They were washed once with double-distilled water and then with Z buffer (100 mM Na2CO3, pH 7.0), 10 mM KC1, 1 mM MgSO4, 25 mM β-mercaptoethanol, and resuspended in Z buffer (final volume, 1 ml). Twenty microliters of 0.1% sodium dodecyl sulfate (SDS) and 50 μl of chloroform were added, and the mixture was vortexed for 30 s. After a 15-min preincubation at 30°C, 0.2 ml of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml in Z buffer) was added, and the reaction mixture was further incubated until visible color developed. The reaction was stopped by adding 0.5 ml of 1 M Na2CO3. Cell debris was removed by centrifugation, and the OD420 of the supernatant solution obtained after centrifugation at 27,000 × g for 20 min was used to assay NADP-GDH by the method of Doherty (10). The protein concentration was determined by the method of Bradford (6).

Electrophoretic mobility shift assays. A 510-bp fragment containing nucleo-
tides – 485 to +25 of the GDH1 gene (25) was excised from plasmid pCYG4 and purified. The termini were then filled in with the Klenow fragment of DNA polymerase in the presence of [α-32P]dCTP. After passage through a Sephadex G-50 column, the labeled DNA was digested with RsaI. Two labeled DNA fragments (309 and 205 bp) were recovered and used as probes. Synthetic oligonucleotides containing the UASLEU from the GDH1 promoter (5′-[−409]ATGCCCGGACAGCGGCCCA[−391]−3′) and the LEU2 promoter (7) were also used as probes. The electrophoretic mobility shift assays were performed as described previously (7, 37), using a partially purified Leu3 preparation (a gift from D. Wang of this laboratory).

Northern (RNA) blot analysis. Yeast strains XK157-3C and XK157-3C/pKZ5 were grown in medium containing either 0.1% 1-t-asparagine or 0.1% ammonium sulfate and harvested at an OD600 of between 0.9 and 1.0. Total RNA was isolated by the hot phenol-glass bead method (19). Twenty micrograms of total RNA was fractionated on a 1.4% agarose gel containing 0.7 M formaldehyde. The RNA was transferred to a nylon membrane by using a downward alkaline transfer apparatus (Turboblotter; Schleicher & Schuell). The blot was first hybridized to a 32P-labeled actin oligonucleotide probe (5′-CGAGGAGCGTCGT-CACCGG-3′) in the rapid hybridization buffer supplied by Amersham. The blot was then exposed for several hours.

RESULTS

Leu3 interacts with a UAS LEU element present in the GDH1 promoter. To ascertain whether the UAS LEU element located between positions −405 and −394 of the GDH1 promoter interacted with Leu3, we conducted a series of electrophoretic mobility shift assays (Fig. 2). First, dividing the promoter region into a distal and a proximal portion, we found that only the distal portion, extending from positions −283 to −487, was capable of forming a specific complex with Leu3 (Fig. 2A). The more slowly moving complex in lanes 1 and 3 of Fig. 2A represented specific binding since its formation was competed against by a DNA fragment containing the UAS LEU element of the LEU2 promoter (Fig. 2A, lane 4). That fragment had been shown previously to interact specifically with Leu3 (7). We also observed specific interaction between a synthetic 19-bp fragment consisting of positions −409 to −391 of the GDH1 promoter and Leu3 (Fig. 2B). Competition for binding was seen both with unlabeled GDH1-UAS LEU DNA and with unlabeled LEU2-UAS LEU DNA (Fig. 2B, lanes 2 and 3) but not with unlabeled LEU2 DNA whose UAS LEU had been destroyed by the deletion of six core base pairs (Fig. 2B, lane 4). These results are consistent with the idea that the UAS LEU-related sequence identified by the database search is the only segment within the GDH1 promoter that Leu3 will interact with.

Both the LEU3 gene product and the UAS LEU element are required for enhanced transcription from the GDH1 promoter. To determine whether LEU3 affected the expression of GDH1 and to evaluate any effects that LEU3 might have, we constructed plasmid pYHI100 (Fig. 1), in which the promoter region of GDH1 (−935 to +27) was fused to the bacterial lacZ gene. The presumptive UAS LEU core sequence is located between positions −405 and −394, and a TATA sequence (TAT ATAAA) is located between positions −149 and −142. Transcription start sites are present at positions −69, −65, and −56 (24, 25). The GDH1-lacZ fusion plasmid was used to transform a wild-type (with respect to LEU3) (strain XK157-R), an isogenic leu3 deletion strain (XK157-3C), and the same leu3 deletion strain that had been made LEU3 + by transformation with plasmid pKZ5. The transformed strains were grown with different nitrogen sources, and β-galactosidase activities were determined (Table 2). Irrespective of the nitrogen source, the activities obtained with the leu3 deletion strain were significantly below those obtained with the LEU3 + strains, the difference ranging from three- to sixfold. Upon transformation of the leu3 deletion strain with a LEU3-carrying plasmid, the β-galactosidase activity returned to levels similar to those seen with the wild-type strain. These results suggest that the differences in specific activities for a given nitrogen source are related to the availability to the Leu3 protein and that Leu3 is partially required for the expression of GDH1.

Previous determinations of NADP-GDH1 activity in a wild-type strain had shown it to be highest when cells were grown on ammonium sulfate as the sole nitrogen source. With asparagine, aspartate, glutamine, or glutamate as the sole nitrogen source, the specific activity dropped to about half the value obtained with ammonium sulfate (29). The expression of GDH1-lacZ as a presumptive nitrogen source yielded a similar pattern except that cells grown on asparagine as the sole nitrogen source showed a fourfold decrease in β-galactosidase activity compared with ammonium sulfate-grown cells (Table 2).

Further evidence for the involvement of Leu3 in the regulation of GDH1 came from studying the effect caused by eliminating the UAS LEU site from the GDH1 promoter. The UAS LEU site of the GDH1 promoter was mutated by deleting 8 bp of the 12-bp core sequence (Fig. 1). The resulting con-
TABLE 2. Expression of a GDH1-lacZ fusion in LEU3+ and leu3 deletion strains grown on different nitrogen sources and effect of deleting the UASLEU element from the GDH1 promoter

<table>
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<tr>
<th>Nitrogen source</th>
<th>Mean β-galactosidase sp act (Miller units) ± SD</th>
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<tbody>
<tr>
<td></td>
<td>XK157-RpYH100 (WT/WT UAS&lt;sub&gt;LEU&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>581 ± 32</td>
</tr>
<tr>
<td>Asparagine</td>
<td>146 ± 37</td>
</tr>
<tr>
<td>Aspartate</td>
<td>252 ± 17</td>
</tr>
<tr>
<td>Glutamine</td>
<td>226 ± 20</td>
</tr>
<tr>
<td>Glutamate</td>
<td>333 ± 38</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three independent colonies were used for each condition. Assays were performed in quadruplicate. See Table 1 for genotypes of strains used.

† WT, wild type.

‡ ND, not determined.

as the sole nitrogen source. In a wild-type strain and in a strain producing feedback-resistant α-IPM synthase (LEU4<sup>res</sup>), expression of the GDH1-lacZ fusion was high. When the two strains were grown in the presence of leucine, a condition expected to lower α-IPM production in the wild-type but not the LEU4<sup>res</sup> strain, the level of GDH1-lacZ expression dropped by more than twofold in the wild-type strain and by less than 20% in the LEU4<sup>res</sup> strain. Furthermore, in a strain devoid of α-IPM synthase activity and hence α-IPM, the expression of GDH1-lacZ was between 7 and 10% of what was seen with the other two strains. Taken together, these results are consistent with the idea that α-IPM is a coactivator of GDH1 gene expression.

**DISCUSSION**

In this report, we have provided evidence that expression of the GDH1 gene of *S. cerevisiae* possesses a partial requirement for Leu3, a regulator previously reported to be involved only in the control of branched-chain amino acid biosynthesis. Eliminating either Leu3 itself or the cis element to which Leu3 binds reduces the expression of the GDH1-lacZ fusion and the mRNA level by three- to sixfold. We conclude that Leu3 participates in transcriptional activation of GDH1. Leu3 activation of GDH1 requires α-IPM, correlating with the results obtained in other studies of this activator protein.

Is a regulation of GDH1 expression by Leu3–α-IPM physiologically significant? We believe that it is since it would establish a link between the cellular pool of a relatively abundant reporter amino acid, i.e., leucine, and a key reaction in the early stages of assimilatory nitrogen metabolism. Leucine

![FIG. 3. Northern (RNA) blotting analysis of GDH1 mRNA steady-state levels in cells grown on ammonium sulfate or asparagine as the sole nitrogen source. See Materials and Methods for experimental details. Lanes: 1, RNA from XK157-3C cells (pertinent genotype, leu3-32) grown on asparagine as the sole nitrogen source; 2, same as lane 1 except that cells were grown on ammonium sulfate as the sole nitrogen source; 3, RNA from XK157-3CpKZS cells (pertinent genotype, LEU3+) grown on asparagine as the sole nitrogen source; 4, same as lane 3 except that cells were grown on ammonium sulfate as the sole nitrogen source. GDH1, blotting with a GDH1 probe; ACT1, blotting with an actin gene probe. The blots were quantitated by densitometry. After normalization against actin mRNA, the GDH1 blots had the following relative values: lane 1, 0.32; lane 2, 1.00; lane 3, 1.15; lane 4, 2.76.](http://mcb.asm.org/)

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Struct (plasmid pYH101) was introduced into the wild-type and leu3 deletion strains, and β-galactosidase levels were measured in cells grown on different nitrogen sources (Table 2). With respect to the XK157-RpYH101 cells, it is obvious that under all conditions, expression of the GDH1-lacZ fusion was considerably less than in the XK157-RpYH100 cells, with the decrease ranging from three- to fivefold (Table 2). β-Galactosidase levels in the strain containing an intact GDH1 promoter but a leu3 deletion were similar to those in the strain containing a UAS<sub>LEU</sub>-less GDH1 promoter but an intact LEU3 gene (with differences ranging from essentially none to twofold) (Table 2). β-Galactosidase levels in strain XK157-3C/pYH101 (leu3 deletion, mutated GDH1 UAS<sub>LEU</sub>) were 30 to 40% below the levels in strain XK157-3C/pYH100 (leu3 deletion, intact GDH1 UAS<sub>LEU</sub>). These results raise the question of whether a protein other than Leu3 might interact with UAS<sub>LEU</sub>. So far, no evidence for such a protein has been obtained.

To further test the idea that regulation of GDH1 by Leu3 occurs at the transcriptional level, we determined the steady-state concentration of GDH1 mRNA under selected conditions. The results (Fig. 3) support the conclusions drawn on the basis of the GDH1-lacZ fusion data. The GDH1 mRNA level in the leu3 deletion strain was three- to fourfold lower than the mRNA level in the isogenic (transformed) LEU3+ strain when cells were grown on asparagine as the sole nitrogen source (Fig. 3, lanes 1 and 3); the difference was about threefold with ammonium sulfate-grown cells (Fig. 3, lanes 2 and 4). The corresponding differences in terms of β-galactosidase activities were three- to fivefold (Table 2). The mRNA levels were 3-fold higher in the leu3 deletion strain when ammonium sulfate was used as nitrogen source (compared with asparagine) and 2.4-fold higher in a corresponding wild-type strain grown under the same conditions. A similar effect had been seen with the GDH1-lacZ fusion (Table 2).

The decreased expression of the GDH1 gene in a leu3 deletion strain was reflected in the specific activity of NADP-GDH. In crude extracts prepared from cells grown with ammonium sulfate (0.1%) as the sole nitrogen source, the specific activities were 0.73 and 0.22 μmol of NADPH per min per mg of protein, respectively, for a wild-type (XK25-1B) and a leu3 deletion strain (XK157-3C).

Expression of a GDH1-lacZ fusion is also affected by the intracellular concentration of α-IPM. Gene activation by Leu3 requires the presence of α-IPM (3, 7, 27, 34). To determine whether this requirement also holds for the activation of GDH1, we measured the level of expression of our GDH1-lacZ fusion in strains with different capacities to synthesize α-IPM (Table 3). All strains were grown on 0.1% ammonium sulfate...
would act by controlling the production of α-IPM, mainly through feedback inhibition of α-IPM synthase (8). In addition, the Leu3–α-IPM connection would subject GDH1, and hence the rate by which ammonia can be assimilated, to a subtle, indirect control by the general amino acid control system since the expression of both LEU4 (the gene encoding α-IPM synthase) and LEU3 is regulated by Gcn4, the transcriptional activator that mediates the general amino acid control (17a, 27, 38). GDH1 is apparently not a direct target for Gcn4 (16, 35). Regarding the role of Leu3 as a more general regulator, it is important to recall that the Neurospora crassa leu-3 gene also pleiotropically regulates expression of genes beyond those of the branched-chain amino acid pathway (26, 28). For example, the N. crassa gene has been shown to regulate his-1, the gene encoding imidazoleglycerol-phosphate dehydratase (18), and α-IPM has been reported to mediate transient repression of overall protein biosynthesis (2). Additional work will be required to identify the other cis-acting elements and trans-acting factors that may participate along with Leu3 in controlling the expression of GDH1. It will then be possible to more completely understand the precise physiological role of Leu3 in GDH1 expression in particular and ammonia assimilation and utilization in general.

ACKNOWLEDGMENTS

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REFERENCES


