Yeast LEU4 Encodes Mitochondrial and Nonmitochondrial Forms of α-Isopropylmalate Synthase*

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The LEU4 gene of Saccharomyces cerevisiae is the major structural gene involved in the production of α-isopropylmalate synthase. It was recently proposed that LEU4 should be capable of encoding two forms of α-isopropylmalate synthase, based mainly on the observation that two of four major transcription start sites are located downstream from the ATG at the beginning of the LEU4 open reading frame (Beltzer, J. P., Chang, L. L., Hinkkanen, A. E., and Kohlhaw, G. B. (1986) J. Biol. Chem. 261, 5160–5167). The two forms with molecular weights of 68,000 and 65,000, respectively, would differ only in the N-terminal region, and only the latter of the two forms would be imported into the mitochondria. We have now constructed LEU4-'lacZ translational fusion plasmids that contain either a normal LEU4' portion (expected to express both the long and the short forms of the fusion protein) or a modified LEU4' portion in which productive translation is possible only from the second in-frame AUG (expected to express only the short form of the fusion protein). β-Galactosidase measurements and immunoblotting of crude mitochondrial and cytoplasmic fractions of yeast cells transformed with the fusion plasmids indicate that two forms of hybrid protein are produced and that only the larger form is targeted to the mitochondria. The distinguishing feature of the targeting sequence appears to be its ability to form an 18-residue long amphiphilic helix. Expression of the native short form of LEU4-encoded α-isopropylmalate synthase behind a strong yeast promoter has enabled us to show that the short form is functional in leucine biosynthesis, is inhibited by leucine with an apparent inhibitor constant of approximately 0.4 mM, and exists as a cytoplasmic dimer.

The first committed step in the de novo biosynthesis of leucine is catalyzed by α-isopropylmalate synthase. In the yeast Saccharomyces cerevisiae, at least three genetic loci are involved in α-isopropylmalate synthesis. The vast majority of the α-isopropylmalate synthase activity present in wild type cells (75–95%) is provided by the LEU4 gene product (1–3). The remainder is provided by an isoenzyme whose synthesis is governed by at least two genes, designated LEU5 and LEU6 (2,3). Much of the LEU4 gene product and, correspondingly, of the α-isopropylmalate synthase found in wild type cells is located in the mitochondrial matrix (1, 4). The LEU4 gene has been cloned and characterized (1, 2), and the nucleotide sequence of LEU4 and flanking regions has been determined (5). In mapping the 5′ ends of the LEU4 transcripts, four major (and several minor) transcription start sites were detected (5). Two of the major sites were located downstream from the ATG at the beginning of the long open reading frame. Translation of the shorter transcripts was therefore postulated to begin at the next in-frame ATG (AUG), located 90 nucleotides downstream from the first one. The two proteins potentially encoded by LEU4 have calculated molecular weights of 68,416 and 65,169. Two anti-yeast α-isopropylmalate synthase antibody-reactive polypeptides with apparent molecular weights of 65–67,000 and 63–64,000, respectively, had been observed earlier during experiments concerned with the mitochondrial import of α-isopropylmalate synthase (4). Those experiments had shown that (i) only the larger species was taken up by isolated mitochondria; (ii) uptake occurred apparently without proteolytic cleavage; and (iii) the larger species was not a precursor of the smaller one. Based on these results, we proposed that LEU4 should be capable, by selective transcription, of producing two forms of α-isopropylmalate synthase, a long one which is targeted to the mitochondria and one which lacks the 36 N-terminal amino acid residues of the long form and remains in the cytoplasm (5). However, since the mitochondrial uptake experiments had been performed with wild type cells, it was not possible to state conclusively that the two protein species seen in those experiments were both products of LEU4.

Here we report the construction of translational LEU4-'lacZ fusions, including one which by design should allow only short form fusion protein to be made. We show that the short form of the hybrid protein is cytoplasmic, whereas the long form is targeted to the mitochondria. An important feature of the targeting sequence of the long form appears to be the ability of the first 18 amino acid residues to generate an amphiphilic α-helix. We also show that the native short form of LEU4-encoded α-isopropylmalate synthase is catalytically active, is subject to inhibition by leucine, and exists as a cytoplasmic dimer.

EXPERIMENTAL PROCEDURES

Materials—The yeast strains used in this work were S. cerevisiae CG48 (MATα ura3-52 trpl-289 cyh2 gal2, obtained from T. D. Petes and C. Giroux, University of Chicago), XK110-5B (MATα leu4-Δ2:HSIS leu5 met4 ura3-52 trpl-289; this laboratory), and HB190 (MATα leu5 leu2, Ref. 1). Escherichia coli strains used as hosts in transformations were MC1061 (Δ[lacIPOZYA]X74 Δ[ara leu] galU galK rpsL), Ref 6) and JM105 (Δ[lacIPOZYA] Δ[ara leu] galU galK rpsL; Ref 6). Plasmid pMC1790, a yeast replicon-'lacZ E. coli-yeast shuttle vector (8), was obtained from H. Zalkin, Purdue University. Plasmid pJ36 is identical to the previously isolated pLFC6 (1) except that the LEU4 gene is in the opposite replicon of the parental vector, and the 3′ end of the LEU4 gene is replaced by 'lacZ.

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orientation. Plasmid pLC2 is a LEU4-containing multi-copy vector (1). Plasmid pVE8, which contains the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (9) was a gift from R. A. Kramer, Roche Research Center, Nutley, NJ. Cloning vectors M13mp11 and M13mp18 were from Pharmacia LKB Biotechnology Inc. DNA polymerases I (Klenow enzyme) and T4 DNA ligase were from Boehringer Mannheim. All other biochemicals, including restriction endonucleases, were purchased from various national suppliers.

Construction of Fusion Plasmids—To construct the LEU4- lacZ fusion plasmid pJB2 (Fig. 1), a PuuII-BamHI fragment of the LEU4 region was purified from a digest of the large xanthine oxidase activity clone containing a 900-bp long XbaI-BamHI piece of LEU4. The PuuII-BamHI fragment consisted of 679 bp of the 5'-noncoding region of LEU4 and 228 bp (plus a four-base overhang) of the beginning of the long open reading frame. It was ligated (T4 DNA ligase, 2 units/pg of DNA) into vector pMC1790 which had been cut with restriction endonucleases BamHI and SmaI. Joining the BamHI-generated overlaps will produce an in-frame fusion between LEU4 and lacZ that encodes the first 78 N-terminal amino acids of α-isopropylmalate synthase (ending in Pro-Asp-Pro), joined to resi-
due 9 of β-galactosidase (Val). The ligation mixture was used to transform E. coli strain MC1000 (10). Transformants were selected for growth on ampicillin and screened for blue colony color using the chromogenic substrate X-gal. Plasmid DNA was prepared from blue colony cells by the alkaline lysis method (10) and analyzed by restric-
tion endonuclease digestion. Plasmids containing the EcoRI-BamHI fragment of the LEU4 subclone that contained a 990-bp long XbaI-BarnHI piece of the original EcoRI-BamHI insert, including transcription starts both upstream and downstream of the LEU4 promoter, including transcription starts both upstream and downstream of the LEU4 promoter, were judged by restric-
tion mapping. Of particular diagnostic importance was digestion with pJB2 or pJB4 were resolved into supernatant and mitochon-
drial subcellular fractionation procedures (15, 16). Protein concentration was determined according to Bradford (17). Routine growth conditions for E. coli and yeast cells were as described previously (1). Deviations from the routine condi-
tions are noted in the table and figure legends. Subcellular fraction-
ation of yeast into crude mitochondrial and postmitochondrial frac-
tions was performed with spheroplasts as starting material, following the procedure of Daum et al. (18).

RESULTS AND DISCUSSION

Cellular Localization of the Long and Short LEU4- lacZ Fusion Products—Plasmid pJB2 contains the native LEU4 promoter, including transcription starts both upstream and downstream from the ATG at position +1 (Fig. 1; see also Ref. 5 and Fig. 8). It should therefore be capable of giving rise to both long and short form protein products. By contrast, plasmid pJB4 was designed to allow production of short form protein only. As shown in Fig. 2, the 4-bp deletion of positions +34 to +37 causes the +91 AUG to be the only one encountered by ribosomes scanning the short transcripts as well as by those (presumably few (21)) ribosomes scanning the long transcripts that have not fallen off at the stop codons terminat-
ing 22-amino acid reading frame. One would therefore expect the short fusion protein to be the only one made by cells harboring plasmid pJB4.

Table I shows that yeast cells containing multiple copies of either pJB2 or pJB4 expressed β-galactosidase activity. The level of expression was about 10-fold lower for pJB4 than pJB2, assuming that the plasmid copy numbers were not appreciably different. The expression of β-galactosidase by pJB4 demonstrates that the second in-frame AUG (the +91 AUG) can be utilized as translation start point.

Spheroplast preparations from yeast strain CG48 carrying either pJB2 or pJB4 were resolved into supernatant and mi-

The abbreviations used are: bp, base pairs; HEPES, 4-(2-hydroxy-
ethyl)-1-piperazineethanesulfonic acid, X-gal, 5-bromo-4-chloro-3-
indolyl-β-d-galactopyranoside.

The relatively low ratio of mitochondrial to nonmitochondrial fusion protein may be attributable to incomplete incorpo-

Fractionation—The activity of β-galacto-

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Fig. 1. Features of plasmids pJB2 and pJB7. At the top of the figure is a linear drawing (to scale) of yeast LEU4 and flanking regions. ORF, open reading frame; the horizontal arrow indicates the direction of transcription. I and II signify the positions of the +1 ATG and the +91 ATG (see text). Restriction site abbreviations are: X, Xbal; P, PvuII; S, SphI; N, NheI; B, BamHI. Restriction sites in parentheses have been destroyed by various means (see “Experimental Procedures”). The lower part of the figure shows the principal features of plasmids pJB2 and pJB7 (not to scale). LEU4, ARS1, TRP1, URA3, the glycerol-dehydrogenase promoter (GAPDHp), and 2 \( \mu \)m are yeast elements; lacZ, ori (origin of replication), and ampicillin resistance gene are bacterial elements. The thin lines represent additional bacterial DNA.

Fig. 2. Construction of a 4-bp deletion in LEU4. Details are given under “Experimental Procedures” and “Results and Discussion.” Shown in the figure is the noncoding strand. The positions labeled \( \gamma \) and \( \delta \) are the two major transcription start points that are located downstream from the +1 ATG (\( \gamma \)). The top portion of the figure shows the native LEU4 sequence. The +1 ATG, +35 ATG (out-of-frame), and +91 ATG (in-frame) are in boldface. The lower part of the figure shows the sequence obtained after the 4-bp deletion at the SphI site. The +1 ATG (AUG) has now become the start for a short (22-codon) open reading frame.

![Diagram of Yeast LEU4](image)

**Table I**

Expression of LEU4::lacZ fusions in yeast

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Plasmid</th>
<th>Gene fusion</th>
<th>( \beta )-Galactosidase (specific activity)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG48</td>
<td>pJB2</td>
<td>LEU4::lacZ</td>
<td>71.7</td>
</tr>
<tr>
<td>CG48</td>
<td>pJB4</td>
<td>LEU4'::lacZ</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* Specific activity is expressed as nmol of o-nitrophenol formed per min per mg of protein. The experimental error was \( \pm 10\% \) of the mean. No activity was detected in CG48 cells transformed with the parent plasmid pMC1790.

m mannitol). The presence of the detergent at a concentration of 0.1% caused a 6-fold increase in measurable \( \beta \)-galactosidase activity. Triton X-100 had no effect on nonsequestered enzyme.

Both the short and the long LEU4::lacZ fusion proteins could be visualized by immunoblotting (Fig. 3). The predicted molecular weights for the short and the long proteins are approximately 120,300 and 123,600, respectively, based on the presence of either a 48-residue peptide (molecular weight about 5,300) or a 78-residue peptide (molecular weight about 8,600) in front of the truncated \( \beta \)-galactosidase molecule (molecular weight about 115,000). The immunoblot shows that anti-\( \beta \)-galactosidase antibody-reactive proteins within the expected molecular weight range were present in extracts from CG48/pJB2 as well as CG48/pJB4 cells. It appears that CG48/pJB2 cells produce both the long and the short form of the fusion protein. While complete resolution was not achieved with whole cell extract of CG48/pJB2, the largest fusion proteins detected in the mitochondrial fraction of CG48/pJB2 cells and whole cell extract of CG48/pJB4 cells, respectively, clearly migrated to two different positions that
Comparison of the N-terminal Regions of the Long and Short LEU4 Gene Products—The results presented in the preceding section provide strong evidence for the ability of the LEU4 gene to encode mitochondrial and nonmitochondrial gene products that differ only in the 30 N-terminal amino acids.

Both fell within the molecular weight range of the uppermost broad band seen with whole cell extract from CG48/pJB2. The position of the single band produced by CG48/pJB4 cells has been seen in other cases of hybrid proteins involving galactosidase; they appear to be proteolytic degradation products (23). Their absence from the lane containing the CG48/pJB4 preparation was probably due to the low level of hybrid protein in these cells.

TABLE II

<table>
<thead>
<tr>
<th>Enzyme assayed</th>
<th>Mitochondrial fraction</th>
<th>Postmitochondrial supernatant fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain CG48/pJB2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>37</td>
<td>63</td>
</tr>
<tr>
<td>Fumarase</td>
<td>62</td>
<td>38</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>&lt;1</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Strain CG48/pJB4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>Fumarase</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>&lt;1</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

FIG. 3. Immunoblot of LEU4′-lacZ fusion proteins. Details of the method are given under “Experimental Procedures.” Lanes 1 and 6, highly purified β-galactosidase from E. coli (10 ng of protein); lanes 2 and 5, whole cell extract of strain CG48/pJB2 (400 µg of protein); lane 3, mitochondrial fraction of strain CG48/pJB2 (400 µg of protein); lane 4, whole cell extract of strain CG48/pJB4 (400 µg of protein). The positions of two prestained high molecular weight standards (200,000 and 97,400, respectively) are indicated by arrows. Strains CG48/pJB2 and CG48/pJB4 were grown to early log phase on “rich lactate” medium (18).
Expression and Some Properties of the Native Short Form of LEU4-encoded a-Isopropylmalate Synthase—The LEU4'-lacZ and LEU4(Δ34–37)-lacZ fusions served as convenient tools for demonstrating that both the +1 AUG and the +91 AUG of the LEU4 transcripts could be used as translation starts and that the resulting long and short fusion proteins were localized to the mitochondria and the cytosol, respectively. It remained to be established, however, that both forms were functional as native, nonfusion proteins. Past experiments had provided evidence that the long, mitochondrial form of LEU4-encoded a-isopropylmalate synthase was responsible for a very large portion of the biosynthesis of a-isopropylmalate under the conditions employed (1, 2). By contrast, the short form had been elusive and may only have been seen in immunoprecipitations of radioactively labeled protein (4). The results shown in Table I and Fig. 3 are consistent with a low rate of expression of the short form, at least under the conditions of those experiments. In order to obtain enough of the short form of the LEU4 gene product to be able to assay it by conventional means and to study its properties, we decided to place an appropriately truncated LEU4 gene behind a strong yeast promoter on a multicopy plasmid (see “Experimental Procedures” and Fig. 1, plasmid pJB7). The point at which the glyceraldehyde-3-phosphate dehydrogenase promoter was fused to LEU4 was the NheI site at position +75, which is 15 bp upstream from the +91 ATG and downstream from all known LEU4 transcription starts (the location of the NheI site is shown in Fig. 2).

Transformation of a-isopropylmalate synthase-less strain XK110-5B, which contains a total deletion of LEU4 and a mutation in LEU5 (2), with plasmid pJB7 led to leucine prototrophy, indicating that the short form of the LEU4 product was able to participate in leucine biosynthesis. The specific activity of a-isopropylmalate synthase in a cell-free extract of strain XK110-5B/pJB7 was 32 nmol of CoA produced/min/mg of protein, which is about 3–4 times the level of the total a-isopropylmalate synthase activity found in wild type cells. The short form of a-isopropylmalate synthase was inhibited by L-leucine (Fig. 6). The leucine concentration required for half-maximal inhibition at pH 7.2 was approximately 0.4 mM. The corresponding value for a preparation containing essentially only the long form of a-isopropylmalate synthase had been found to be about 0.1 mM (1). In an experiment similar to that described in Table II, a spheroplast preparation of strain XK110-5B/pJB7 was separated into a crude mitochondrial and a cytosolic fraction. The distribution among cytosol and mitochondria was >99%/<1% for a-isopropylmalate synthase, 100%/0% for glucose-6-phosphate dehydrogenase, 46%/54% for fumarase, and 23%/77% for citrate synthase. Confirmation of the presence of the short form of a-isopropylmalate synthase in the cytosol and its virtual absence from the mitochondrial fraction came from an immunoblot experiment (Fig. 7) which also showed that the transformation host XK110-5B did not produce any measurable cross-reacting material. It should be noted that plasmid pJB7 was designed to overproduce the short form of LEU4-encoded a-isopropylmalate synthase. Even so, there was almost no association of this enzyme with the mitochondria.

Gel filtration of a cell-free preparation of the short form of a-isopropylmalate synthase on a calibrated column of Sephadex G-150 resulted in the elution of a sharp peak of activity at a position corresponding to an apparent molecular weight of 130,000. This result suggests that, in its native form, the enzyme exists as a dimer.

Fig. 8 schematically summarizes the expression of the LEU4

![Fig. 5](image-url)  
**Fig. 5.** α-Helical projections of the first 18 amino acid residues of the long form of the LEU4 gene product. A, helical wheel (axial projection), 3.6 residues/turn (35). Charged residues are indicated by + or − signs, and hydrophobic residues are boxed. B, approximate cylindrical projection. Charged residues are indicated by + or − signs, hydrophobic residues are circled.

![Fig. 6](image-url)  
**Fig. 6.** Inhibition of the short form of the LEU4 gene product by leucine. Strain XK110-5B/pJB7 was grown at 30 °C on medium containing 0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, and 0.2 mM each of methionine and tryptophan. Cells were harvested at an OD600 of about 1.0 and washed twice with distilled water. The, 1.5 g of cells (wet weight) was suspended in 2.25 ml of 0.1 M potassium phosphate buffer, pH 6.9, containing 1.5 M ammonium sulfate, 29% glycerol, 0.03% NaN3, 1 mM phenylmethylsulfonyl fluoride, and 6 mg/ml benzamidine. The suspension was passed twice through a French pressure cell at 138 megapascals. The extract was clarified by centrifugation, and ammonium sulfate was added to a final concentration of 75% (4 °C). The precipitate was collected by centrifugation and resuspended in 50 mM HEPES-KOH buffer, pH 7.2, containing 1.2 M ammonium sulfate and 10% glycerol. α-Isopropylmalate synthase assays were performed in the same buffer containing, in addition, 0.8 mM each of acetyl-CoA and α-ketoisovalerate and the indicated concentrations of leucine. The values shown represent the average of at least two determinations. Experimental error was ±10% of the mean.
The localization of two forms of LEU4-encoded α-isopropylmalate synthase. The presence of α-isopropylmalate synthase in the mitochondrial matrix can be rationalized by assuming that it provides easier access to central metabolites that act as regulators of the enzyme’s activity (30) and to the substrates acetyl-CoA and α-ketoisovalerate; in yeast, enzymes involved in the de novo synthesis of α-ketoisovalerate are largely associated with the mitochondria (31). The need for having a second form of α-isopropylmalate synthase in the cytosol is not as obvious. Experiments to study the relative expression of the two forms under various environmental conditions, including anaerobiosis, are underway.

Since the ability of the LEU4 gene to encode both mitochondrial and cytosolic gene products was first proposed (5), at least two additional examples of this type have been reported for yeast. They are the HTSI1 gene encoding histidyl-tRNA synthetase (32) and the MOD5 gene believed to encode the tRNA-modifying enzyme Δ2-isopentenyl-pyrophosphate-tRNA isopentenyltransferase (33).

REFERENCES

Yeast LEU4