Purification and Structural Characterization of Transcriptional Regulator Leu3 of Yeast*

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The transcriptional regulatory protein Leu3 of Saccharomyces cerevisiae was enriched approximately 70-fold above wild type level in yeast cells carrying a pGAL1-LEU3 expression vector. Sustained overproduction of Leu3 following induction by galactose required elevated intracellular levels of α-isopropylmalate, a leucine nitrogen intermediate known to act as transcriptional co-activator. Starting with galactose-induced cells, the Leu3 protein was purified about 3,500-fold (i.e., 245,000-fold over wild type level) by a procedure that included treatment of the cell-free extract with polyethyleneimine, fractionation with ammonium sulfate, heat treatment, and DNA affinity chromatography. Highly purified preparations still showed two protein bands when subjected to polyacrylamide electrophoresis under denaturing conditions. Their apparent molecular masses were about 104,000 and 110,000 kDa. The smaller of these values was very close to the maximum molecular weight obtained previously for Leu3 protein translated in vitro in a rabbit reticulocyte lysate. (The molecular weight deduced from the open reading frame of the LEU3 gene is 100,162.) Both protein bands reacted with antibodies raised against different portions of the Leu3 molecule and were, therefore, likely to represent two forms of Leu3. Treatment with calf intestinal phosphatase quantitatively converted the slower moving band into the faster moving one. Conversion was prevented by inorganic phosphate, a phosphatase inhibitor. These experiments showed that the two bands very likely correspond to phosphorylated and nonphosphorylated forms of Leu3. Phosphorylation did not appear to affect the DNA binding function of Leu3, but (indirect) effects on the activation function or effects on the modulation by α-isopropylmalate have not been ruled out.

Electrophoretic mobility shift assays were used to estimate the apparent dissociation constants of the two specific Leu3-DNA complexes routinely seen in these assays. The values obtained were 1.1 and 2.6 nM. Finally, using size exclusion chromatography, native Leu3 protein was shown to have dimeric structure, irrespective of the state of phosphorylation.

The Leu3 protein of yeast is a transcriptional regulator whose principal effect is on branched chain amino acid biosynthesis (1-4). It interacts with a specific 12-base pair DNA sequence (UAS)1 located upstream of the regulated genes (5-9). The Leu3 protein has several outstanding features. First, it is unique among known eukaryotic regulators in that its function as activator depends entirely on a low molecular weight metabolite (10). The metabolite, α-isopropylmalate (α-IPM), is an early intermediate in leucine biosynthesis whose intracellular concentration is influenced by the branched chain amino acid pools, the general amino acid control system, and other factors (1, 3, 4). Second, in the absence of α-IPM, Leu3 actually functions as a repressor of transcription (3, 10). Third, a recently developed, yeast-derived in vitro transcription system (11) faithfully executes Leu3-dependent activation, repression, and modulation by α-IPM (10), opening the door for a more definitive study of the mechanisms by which Leu3 operates.

Manipulation of the LEU3 gene has defined several functional regions within the 886 amino acid residues of the Leu3 protein (12-15): a DNA binding region (residues 17-110) that contains a Zn(II)2Cyss binuclear cluster typical of an entire family of lower eukaryotic transcriptional regulators whose members include the gene products of GAL4, PPRI, HAPI, PDR1, PUT3, Lac9, and qa-1F (12, 13); an acidic-hydrophobic transcriptional activation region (residues 855-886) (14); and a “modulation region,” which encompasses a large central portion of the Leu3 protein (12, 14, 15). Removal of the central portion creates a strong constitutive activator that no longer responds to α-IPM. Evidence has been obtained showing that certain individual residues of the activation region (e.g., Trp-864, Trp-861) are also involved in modulation, and a model has been proposed, which postulates that interaction of the Leu3 protein with α-IPM results in the disruption of contacts between the activation region and the central portion of the protein (14).

In order to facilitate further structural and functional analyses such as complete in vitro reconstitution of the Leu3-dependent transcription process and interaction of Leu3 protein with α-IPM, we decided to purify Leu3. Here we report a method to rapidly isolate stable Leu3 protein from overproducing yeast cells. Interestingly, highly purified preparations of Leu3 display two protein species upon polyacrylamide gel electrophoresis under denaturing conditions. The apparent molecular weight of the two species, their immunoreactivity, and their response to phosphatase treatment suggest that they represent phosphorylated and nonphosphorylated forms of Leu3. This observation adds yet another layer of complexity to the structure and possibly the function of the Leu3 protein.

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1 The abbreviations used are: UAS, upstream activating sequence; IPM, isopropylmalate.
Purification and Characterization of Leu3 in yeast. See “Experimental Procedures” for details of construction.

Protein production was monitored by electrophoretic mobility shift assays using UAS probe. Each lane contained 20 μg of total protein in buffer A (see “Experimental Procedures”). Lanes 1, uninduced; lanes 2, induced for 6 h; lanes 3, induced for 8 h; lanes 4, induced for 24 h. Induction at an Aomp of about 0.5, induction of the GALI promoter was initiated by adding b-galactose to a final concentration of 2%. Cells were harvested at various times after induction. XK160/pGALI-LEU3 cells grown for the purpose of purifying Leu3 were routinely harvested 14 h after induction at an Aomp of 6–7, which yielded 20–24 g of cells (wet weight)/liter. Cell pellets, which had been washed once with deionized water, were stored at −70 °C. To prepare cell-free extract, frozen pellets were thawed on ice and suspended in extraction buffer (20 mM HEPES, NaOH buffer, pH 7.9; 400 mM (NH₄)₂SO₄; 5 mM MgCl₂; 50 μM ZnSO₄; 1 mM EDTA; 4 mM dithiothreitol; 20% (v/v) glycerol; 2 mM phenylmethylsulfonyl fluoride; 2 mM benzamidine; and 2 μg pepstatin) at a ratio of 1.5 ml/g of cells (wet weight). Suspensions were passed twice through a French pressure cell at 150 megapascals. The resulting extract was clarified by centrifugation for 70 min at 41,000 x g (4 °C).

Escherichia coli cells were grown at 37 °C in L-broth (19) containing ampicillin (100 μg/ml).

**Purification of Leu3 Protein—** Except for the heat step, all procedures were performed at 0–4 °C. Cell-free extract was prepared as outlined above from 40–50 g of XK160/pGALI-LEU3 cells (wet weight). Polyethylenimine was then added dropwise with stirring from a 5% solution to a final concentration of 0.033%. After standing on ice for 10 min, the resulting suspension was centrifuged at 12,000 x g for 10 min. The supernatant solution was adjusted to an (NH₄)₂SO₄ concentration of 46% using a saturated (NH₄)₂SO₄ solution, which also contained 25 mM HEPES-NaOH buffer, pH 7.9, and 1 mM EDTA and taking into account the amount of (NH₄)₂SO₄ present in the extraction buffer. After overnight incubation, the precipitate was collected by centrifugation at 12,000 x g for 1 h and washed twice in 25 mM HEPES-NaOH buffer, pH 7.9, containing 5 mM MgCl₂, 50 μM ZnSO₄, 1 mM EDTA, 20% (v/v) glycerol, 2 mM each of phenylmethylsulfonyl fluoride and benzamidine, and 0.02% Nonidet P-40 (“buffer A”). The solution was divided into 0.5-ml aliquots. To each aliquot, an equal volume of buffer A pre-equilibrated at 80 °C was added, and the mixture was further incubated at 86 °C for 2 min before being rapidly cooled on ice. Denatured protein was pelleted by centrifugation at 31,000 x g for 20 min. The supernatant solution was dialyzed against several changes of “buffer B” (identical to buffer A except that diethiothreitol was replaced with 0.1% β-mercaptoethanol and MgCl₂ was omitted) containing 80 mM NaCl.

To trap proteins that react with DNA nonspecifically, sonicated salmon sperm DNA was added at 12.5 μg/ml dialysate. The mixture was incubated for 20 min at 0 °C and then loaded on an AUS₁₂-Sepharose column (at a ratio of up to 100 mg of total protein/ml of column volume) that had been equilibrated with “buffer C” (identical to buffer A except that MgCl₂ was omitted) containing 80 mM NaCl. The column material was prepared by first ligating pieces of UAS probe DNA (see below, section on “Electrophoretic Mobility Shift Assays”) to form concatemers (ranging from 3 to 23 units), which were then coupled to CNBr-activated Sepharose 4B (Pharmacia-LKB Biotechnology Inc.). The affinity matrix contained approximately 34 μg of DNA/ml of resin. After allowing the probe to interact with the affinity matrix (1 h at 4 °C), the column was eluted successively with 10 column volumes of 80, 200, and 300 mM NaCl in buffer C, followed by 6 column volumes each of 1 and 2 M NaCl in buffer C. The appearance of Leu3 protein was monitored by electrophoretic mobility shift assays and by immunoblotting. Fractions containing the major-
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TABLE I

Purification of the Leu3 protein

| Step                        | Total protein | Total units | Specific activity | Purification | Yield |
|-----------------------------|---------------|-------------|-------------------|--------------|-------|
| Crude extract              | 1.452         | 5.3 x 10^9  | 3.65 x 10^2       | 1            | 100   |
| Polyethyleneimine treatment| 1.557         | 5.2 x 10^9  | 3.34 x 10^2       | 0.9          | 98    |
| Ammonium sulfate fractionation | 276       | 4.4 x 10^9  | 1.59 x 10^4       | 4.3          | 83    |
| Heat treatment              | 164           | 6.6 x 10^8  | 4.00 x 10^7       | 1.1          | 12.5  |
| Affinity chromatography (pool) | 0.026    | 3.3 x 10^7  | 1.27 x 10^11      | 3.479        | 6.3   |

* Protein concentration was determined by the method of Bradford (38) except for affinity-purified preparations whose protein concentration was estimated from silver stained gels (59) using bovine serum albumin as a standard.

* Activity units were measured by performing electrophoretic mobility shift assays on polyacrylamide gels (see "Experimental Procedures").

Material from the different purification steps was incubated with the same batch of 38-P-labeled DNA probe, and aliquots were applied to the same gel slab. Following electrophoresis, the two Leu3-specific bands were excised from the gel, combined, and subjected to scintillation counting. One unit is defined as 1 cpm detected under the conditions of the experiment.

![Cell-free extract](image)

**FIG. 3. UASL-Sepharose affinity chromatography.** Ten ml of a heat-treated, dialyzed preparation of Leu3 containing 164 mg of total protein were loaded onto a UASL-Sepharose affinity column (diameter, 17 mm; height, 20 mm). After equilibration and collection of approximately 10 ml of "flow-through," 33 fractions of 3 ml each were collected while increasing the NaCl concentration in a stepwise fashion (see "Experimental Procedures"). The figure shows electrophoretic mobility shift assay results obtained with the material loaded on the column, the flow-through, and fractions representing the stepwise elution. Strong, Leu3-specific band shifting was seen only after the addition of 1 M NaCl, with insignificant amounts eluting at the 300 mM step. The designations a, b, and f refer to the positions of shifted bands a and b and to free DNA probe, respectively.

**Scheme I. Procedure for the purification of Leu3 protein.**

by the Laboratory for Macromolecular Structure, Purdue University. Purification was done as described (3).

Electrophoretic Mobility Shift Assay—DNA binding was monitored as follows. A given amount of protein was incubated with 25 mM HEPES-NaOH buffer, pH 7.9, containing 80 mM NaCl, 1 mM EDTA, 4 mM dithiothreitol, 5% (v/v) glycerol, 1 µg of poly(dI-dC), and 0.8 ng of DNA probe (unless indicated otherwise) for 15 min at 30 °C in a total volume of 30 µl. The DNA probes were (the sequence of only one strand is shown): (i) UASL 30-mer containing the UASL of the LEU2 promoter (5'-AGGTGAGAGGCCGGAACCGGC TTTTCATAT-3'); (ii) UASL 30-mer, a 30-mer containing the UASL of the LEU2 promoter and also containing 5' overhangs for the generation of a BamHI and a SalI site (iii) and (iv) could be used interchangeably); and (iii) UASL 30-mer, a nonbinding 24-mer used as a control (5'-AGGTGAGAGGCCGGAACCGGC TTTTCATAT-3'). Prior to annealing, complementary strands were 5' end-labeled using [γ-32P]ATP with a specific radioactivity of about 5,000 Ci/mmol. Following incubation, reaction mixtures were loaded onto a 4% polyacrylamide gel (cross-linking ratio, 40:1) containing 80.7 g of Tris base, 36.7 g of boric acid, and 372.2 g of Na2EDTA·2H2O/liter. Following electrophoresis, gels were dried and subjected to autoradiography. When needed, shifted bands were excised individually, and the radioactivity in each slice was determined by scintillation counting, using a Beckman model LS8100 counter and ICN Ecolume as scintillation fluid.

Antibody Preparation and Immunoblotting—In order to prepare antibodies against the COOH-terminal "activation domain" of Leu3 (14), a peptide corresponding to the 28 COOH-terminal amino acid residues of the Leu3 protein (859-AGWDNWSDMVWDRVDLIM-NFAPNPKV-886) was synthesized by the Laboratory for Macromolecular Structure, Purdue University. It was purified by reverse-phase high performance liquid chromatography, and its structure was confirmed by mass spectrometry and partial amino acid sequence analysis.
The designations plasmid carrying the GAL1-LEU3 construct was expressed in and used by Buch and Audran (23) as modified by Dankert after the second boost was partially purified by the method of Stein-donkey using the enhanced chemiluminescence system of Amersham by horseradish peroxidase-conjugated anti-rabbit antibodies from peptide was conjugated to maleimide-activated keyhole limpet he- would be the amino terminus of the peptide. Next, the modified analysis. The purified peptide was then modified with Traut’s reagent, 2-iminothiolane HCl, to introduce sulfhydryl groups (22). At pH 9, the pH at which the reaction was performed, the preferred target was the amino terminus of the peptide. Next, the modified peptide was conjugated to maleimide-activated keyhole limpet hemocyanin, following the protocol of the supplier (Pierce Chemical Co.). After purification by gel filtration, the peptide-hemocyanin conjugate was used directly to immunize rabbits. Antiserum obtained from each of the preparations (prepared as described under “Experimental Procedures”) was used to elute stepwise with increasing concentrations of NaCl. Leu3 protein appeared after raising the NaCl concentration to 1 M. Recovery of Leu3 protein after heat treatment fluctuated from preparation to preparation. It was nevertheless essential to retain the heat step as part of the purification procedure, since it significantly improved the subsequent stability of the Leu3 protein. The overall purification factor was close to 3,500.

Electrophoretic mobility shift assays showed that material from all stages of purification was capable of forming the two UASL-Leu3 complexes a and b observed before with crude yeast preparations (Fig. 4). The relative mobility of the complexes formed with affinity-purified material was indistingui-shable from that of less purified preparations.

Affinity of Leu3 Protein for UASL—Since the mobility shift assays clearly separated complexes a and b from each other and from DNA, attempts were made to determine the apparent dissociation constants (Fig. 5). Under the experimental conditions chosen, $K_{d,app}$ was 2.6 nM for complex a and 1.1 nM for complex b. The range of these values is very close to that reported for estrogen and thyroid hormone receptors (26, 27) and for the yeast transcriptional factors Gcn4 and Gal4 (28, 29). The finding that the protein component of complex a has a slightly lesser affinity for UASL than the protein component of complex b is consistent with previous estimations of relative association and dissociation rate constants, which suggested that complex a was somewhat less stable than complex b and formed much more slowly (13).

Highly Purified Preparations Contain Two Forms of Leu3 Protein That Can Be Converted into One by Phosphatase Treatment—Polyacrylamide gel electrophoresis under denaturing conditions revealed that affinity-purified Leu3 protein contained two protein species, designated α and β (Fig. 6A). The apparent molecular weights of these two proteins were about 104,000 and 110,000. The smaller of these values was within 2% of the maximum molecular weight obtained previously for Leu3 protein translated from full-length LEU3 mRNA in a rabbit reticulocyte lysate (41). In that experiment,
FIG. 5. Determination of apparent dissociation constants for the two Leu3-UASL complexes a and b. Values of $K_d$ were obtained by quantitation of electrophoretic mobility shift assays (see “Experimental Procedures” for general method). A constant concentration of affinity-purified Leu3 protein (0.8 ng/30 µl) was incubated with radio-labeled UASL DNA at concentrations ranging from 0.16 to 9.30 nM. Following electrophoresis, gel segments corresponding to the positions of complexes a and b and free DNA were excised and their radioactivity determined by scintillation counting. Data were plotted as total DNA versus bound DNA (panel A, complex a (●) and complex b (○)) and as free DNA versus free DNA/bound DNA (panels B and C (symbols as in panel A)). Lines in panels B and C were drawn based on an unweighted least squares analysis. With this plot, the apparent dissociation constants are given by the negative abscissa intercepts. The values obtained were 2.6 nM for complex a and 1.1 nM for complex b.

FIG. 6. Polyacrylamide gel electrophoresis under denaturing conditions and immunoblotting of highly purified Leu3 protein. Polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (40) using 7.5% acrylamide and a ratio of acrylamide:N,N'-methylenebisacrylamide of 30:0.8 (panel A). Protein was visualized by silver staining (39). Lane 1, molecular weight standards: myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 97,000; bovine serum albumin, 66,000; and ovalbumin, 45,000. Lane 2, 100 ng of affinity-purified Leu3 protein. The two major species are identified as α and β Panel B, immunoblotting of affinity-purified Leu3 protein. The antibodies used were raised against a peptide containing the 28 COOH-terminal residues of the Leu3 protein (the activation domain) fused to a carrier protein. See “Experimental Procedures” for further details.

FIG. 7. Determination of apparent dissociation constants for the two Leu3-UASL complexes a and b. Values of $K_d$ were obtained by quantitation of electrophoretic mobility shift assays (see “Experimental Procedures” for general method). A constant concentration of affinity-purified Leu3 protein (0.8 ng/30 µl) was incubated with radio-labeled UASL DNA at concentrations ranging from 0.16 to 9.30 nM. Following electrophoresis, gel segments corresponding to the positions of complexes a and b and free DNA were excised and their radioactivity determined by scintillation counting. Data were plotted as total DNA versus bound DNA (panel A, complex a (●) and complex b (○)) and as free DNA versus free DNA/bound DNA (panels B and C (symbols as in panel A)). Lines in panels B and C were drawn based on an unweighted least squares analysis. With this plot, the apparent dissociation constants are given by the negative abscissa intercepts. The values obtained were 2.6 nM for complex a and 1.1 nM for complex b.

a strong single band appeared at a position corresponding to a molecular weight of 106,000. All of these apparent molecular weights are slightly above the value of 100,162 deduced for Leu3 protein monomer from the DNA sequence (2). When highly purified Leu3 preparations were subjected to immunoblotting with antibodies raised against different portions of Leu3, both species reacted. Fig. 6B shows the results of an experiment with polyclonal antibodies raised against a hemocyanin-linked peptide consisting of the 28 COOH-terminal amino acid residues of Leu3 (residues 859–886). Similar results were obtained when polyclonal antibodies raised against a peptide containing residues 17–147 of Leu3 were used (data not shown).

Incubation of highly purified Leu3 protein with calf intestinal phosphatase in the presence of 0.2% sodium dodecyl sulfate caused a dramatic disappearance of the α band with a concomitant intensification of the β band (Fig. 7). The presence of 10 mM sodium phosphate, a phosphatase inhibitor, prevented the disappearance of the α band. These results strongly suggest that the slower moving band represents a phosphorylated form of the Leu3 protein.

Site Exclusion Chromatography of Native Leu3 Protein—For this experiment, a partially purified preparation from induced XK160/pGAL1-LEU3 cells was placed on a calibrated fast protein liquid chromatography Superose 12 column. Fractions were collected and tested for the presence of Leu3 protein using electrophoretic mobility shift assays (Fig. 8). There was only one peak of material capable of forming specific complexes with UASL; it eluted at a position corresponding to an apparent molecular weight of about 220,000. This result suggests that DNA complex formation-competent Leu3 protein exists as a dimer in solution.

DISCUSSION

Until now, our knowledge of the structure and function of the Leu3 protein has come exclusively from molecular genetic

2 The specificity of the anti-COOH-terminal peptide antibodies was demonstrated recently by showing that they do not react with a Leu3 mutant that lacks the 32 COOH-terminal residues.
studies of the LEU3 gene and from work with crude Leu3 preparations (2, 3, 12-15). A detailed characterization of the Leu3 protein and of the mechanisms by which it makes contact with DNA, the transcription machinery, and modulating factor(s) has been hampered by the very low abundance of the Leu3 protein in wild type yeast cells on the one hand and, on the other hand, by difficulties in expressing recombinant Leu3 protein in E. coli in which it tends to be degraded. Similar difficulties were reported for other eukaryotic regulatory factors, most notably Gal4 (30-32). In this paper, we have presented a simple and rapid method for isolating Leu3 protein from a Leu3 overproducing yeast strain. The key steps in the purification procedure were heat treatment and affinity chromatography. Although the heat treatment caused an obvious loss of Leu3 binding activity, this disadvantage was far outweighed by two important advantages: (i) the heat step made possible the fast isolation of active Leu3 protein by eliminating the need for further chromatographic procedures; and (ii) it caused a dramatic increase in the stability of the Leu3 protein, which we attribute to the inactivation of contaminating proteinases. Without heat treatment, the Leu3 protein loses its activity within a few days; with heat treatment, it is stable for at least 6 months at -70°C, even when it is repeatedly thawed and refrozen. How functionally intact is the purified Leu3 protein? The electrophoretic mobility shift assays used to monitor the purification of the Leu3 protein gave practically identical results irrespective of whether crude extract or highly purified protein was assayed, indicating that no auxiliary factors were lost during purification and that the DNA binding function remained intact. We have also begun to examine Leu3 protein-dependent transcriptional activation in vitro (10). For these experiments, whole cell yeast extract capable of supporting accurate transcription by RNA polymerase II (11) was used. Extract was prepared from cells from which the genomic LEU3 gene had been deleted (3). The template used contained a Leu3 binding site upstream from the CYCI TATA box linked to a G-less cassette. In this system, affinity-purified Leu3 protein strongly activated transcription. Significantly, activation was absolutely dependent on the addition of α-IPM (10). In fact, in the absence of α-IPM, Leu3 caused repression of transcription. Taken together, these results strongly argue that the Leu3 protein purified by the protocol presented here is active with respect to DNA binding, trans-

Fig. 7. Phosphatase treatment of purified Leu3 protein. The incubation mixtures contained (in a total volume of 100 µl): 17 ng of affinity-purified Leu3 protein, 24 units of calf intestinal alkaline phosphatase where indicated (CIP, Boehringer Mannheim, molecular biology grade), 10 mM sodium phosphate where indicated (Inhibitor), 50 mM Tris-HCl buffer, pH 7.9, 0.2% sodium dodecyl sulfate, 1 mM each of EDTA, dithiothreitol, and MgCl2, 200 µM ZnSO4, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 2 µM pepstatin, 0.6 µM leupeptin, 2 µg of aprotinin, and 100 µg of bovine serum albumin. Incubation was for 2 h at 37°C. The reactions were terminated by the addition of 20 µl of 5 X standard electrophoresis buffer (40), which resulted in a final concentration of sodium dodecyl sulfate of 2%, and by heating to 87°C for 5 min. Aliquots of each reaction mixture were then subjected to immunoblotting using anti-Leu3 antibodies as described under "Experimental Procedures," and loaded on a Superose 12 column (total volume, 23.6 ml; Pharmacia-LKB) that had been equilibrated with buffer A. Fractions of 0.5 ml were collected, and aliquots of 24 µl were used in electrophoretic mobility shift assays with UASl-α as DNA probe. Far left lane, control (material prior to chromatography); other lanes, fractions 15-26, corresponding to 75-13 ml of eluate collected after application of the Leu3 preparation. Designations α, β, and γ refer to shifted bands α and β and free DNA probe, respectively. The arrows indicate the peak positions of molecular weight standards and represent (from left to right) apoferritin (443,000), β-amylace (206,000), alcohol dehydrogenase (156,000), phosphorylase b (97,000), and bovine serum albumin (66,000).
scriptional regulation, and response to the metabolite α-IPM, and that its *in vitro* behavior faithfully mimics that observed *in vivo*. The stage is therefore set for an *in vitro* study of interactions between components of the transcription machinery and Leu3 under both activating and repressing conditions, which may have important repercussions for our understanding of transcriptional regulation in general.

Highly purified Leu3 protein displayed a doublet of bands when subjected to polyacrylamide gel electrophoresis under denaturing conditions. The apparent molecular weight of the two protein bands was slightly above that predicted by the sequence of the *LEU3* gene, making it highly unlikely that the two species were the result of some proteolytic event. Both protein species co-eluted from a UAS-leucine affinity column, and both reacted with antibodies prepared against a peptide containing the 28 COOH-terminal residues of Leu3 as well as with antibodies prepared against a peptide containing the DNA binding region of Leu3. Thus, they were both Leu3 proteins. It is interesting in this context that the size of the smaller species was very close to that of the largest protein observed in earlier experiments in which full-length LEU3 mRNA was used to direct Leu3 protein synthesis in a rabbit reticulocyte lysate (41). That protein appeared as a singlet with an apparent molecular weight of 106,000, suggesting that the larger species seen in the present work (the 110,000 species) was generated by a yeast-specific process.

Since alkaline phosphatase treatment cleanly converted the slower moving band into the faster moving one, we believe that the two Leu3 bands represent phosphorylated and nonphosphorylated forms of Leu3. It is well known that the electrophoretic mobility of a protein can be reduced upon phosphorylation (33-35). Phosphorylated residues of Leu3 can be obtained and studied as separate, nonphosphorylated entities. To obtain nondenatured Leu3 protein of different states of phosphorylation may require identification of the phosphorylation site(s) and the use of site-directed mutagenesis to eliminate them.

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