Evidence That Intramolecular Interactions Are Involved in Masking the Activation Domain of Transcriptional Activator Leu3p*  

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The Leu3 protein of Saccharomyces cerevisiae regulates the expression of genes involved in branched chain amino acid biosynthesis and in ammonia assimilation. It is modulated by α-isopropylmalate, an intermediate in leucine biosynthesis. In the presence of α-isopropylmalate, Leu3p is a transcriptional activator. In the absence of the signal molecule, the activation domain is masked, and Leu3p acts as a repressor. The recent discovery that Leu3p retains its regulatory properties in mouse cells (Guo, H., and Kohlhaw, G. B. (1996) FEBS Lett. 390, 191–195) suggests that masking and unmasking of the activation domain occur without the participation of auxiliary proteins. Here we present experimental support for this notion and address the mechanism of masking. We show that modulation of Leu3p is exceedingly sensitive to mutations in the activation domain. An activation domain double mutant (D872N/D874N; designated Leu3-dd) was constructed that has the characteristics of a permanently masked activator. Using separately expressed segments containing either the DNA binding domain-middle region or the activation domain of wild type Leu3p (or Leu3-dd) in a modified yeast two-hybrid system, we provide direct evidence for α-isopropylmalate-dependent interaction between these segments. Finally, we use the phenotype of Leu3-dd-containing cells (slow growth in the absence of added leucine) to select for suppressor mutations that map to the middle region of Leu3-dd. The properties of nine such suppressors further support the idea that masking is an intramolecular process and suggest a means for mapping the surface involved in masking.

The Leu3 protein of yeast belongs to a class of transcriptional regulators whose members are characterized by a Zn(II)₂-Cys₆ binuclear cluster in their DNA binding domains (1, 2). Many of them also have the ability to be modulated, i.e. to respond to metabolic signals. Well studied examples of modulation include Gal4p, which is activated ("unmasked") by galactose in a process that involves changes in the interaction between Gal4p and an auxiliary protein known as Gal80p (3, 4), and Hap1p, the activation of which by heme is thought to be brought about by dissociation of cellular factors that allow the DNA binding domain to become functional and its activation domain (AD) to become exposed (5, 6). Leu3p is modulated by α-IPM, an intermediate in leucine biosynthesis (7). Leu3p binds to UAS₉ elements in the promoters of genes involved in the biosynthesis of branched chain amino acids (8, 9) and, surprisingly, in ammonia assimilation in yeast (10). The finding that the GDH1 gene is regulated by Leu3p and α-IPM (10) led to the hypothesis that α-IPM serves as a signal molecule that communicates the status of amino acid biosynthetic activity (as represented by the leucine pathway) to more central points of nitrogen metabolism. It is of interest in this context that the LEU3 gene itself is controlled by Gcn4p (11).

Leu3p binds to target DNA irrespective of the presence or absence of α-IPM (9). In the absence of α-IPM, Leu3p represses gene expression below the basal level seen in leu3Δ null cells (9, 12). In the presence of α-IPM, it causes strong activation. The zinc cluster part of Leu3p’s DNA binding domain is located between amino acids 37 and 67 of the 886-residue protein (11, 13, 14), and a putative dimerization domain is found between amino acids 85 and 99 (14). A region responsible for transcriptional activation has been identified within the C-terminal 30 amino acids of Leu3p (14, 15). Truncated Leu3p molecules lacking the AD still bind to DNA. They are totally devoid of activation potential and act as repressors (9, 15, 16). Deleting as many as 600 amino acids from the middle of Leu3p leaves DNA binding and transcriptional activation functions intact but eliminates modulation (16, 17). Such molecules have a constitutively high activation potential that usually exceeds that of modulated forms of Leu3p. The implication of these results is that Leu3p’s middle region contains information that is essential for modulation. In dealing with the question of modulation, it may be helpful to break the modulation process down into discrete steps, e.g. the binding of the modulator α-IPM, conformational changes caused by the binding of α-IPM, and the eventual exposure of the AD that allows it to interact with components of the transcription apparatus. Our current view is that the unmasking of the AD as well as its masking in the absence of α-IPM occur without the participation of auxiliary proteins. The strongest support for this notion comes from recent observations made when full-length yeast Leu3p was expressed in mammalian cells (18). It was found that the behavior of Leu3p in mouse cells was almost indistinguishable from that seen in its native environment; Leu3p was stable but inactive when α-IPM was absent and caused an apparent severalfold repression of reporter gene expression. When α-IPM was present in the cell culture medium, Leu3p was converted into a strong activator. Since mammalian cells do not synthesize branched chain amino acids, it appeared

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1 The abbreviations used are: AD, activation domain; IPM, isopropylmalate; kbp, kilobase pair; UAS₉, upstream activating sequence in the promoters of Leu3p-regulated genes; WT, wild type; PCR, polymerase chain reaction; MR, middle region; DB, DNA binding domain.
highly unlikely that they would elaborate Leu3p-specific factor(s) for masking, unmasking, or α-IPM interaction.

In this study, we have used biochemical and molecular genetic approaches to advance our understanding of the modulation process. We show that modulation is exquisitely sensitive to mutational changes in the AD of Leu3p. Using wild type Leu3p and a mutant form with drastically intensified masking, we show that the AD and the remainder of Leu3p interact and that this interaction depends on α-IPM. Isolation of intragenic suppressors of the slow growth phenotype of the masking mutant shows the usefulness of this approach for identifying regions or individual residues of Leu3p that are involved in masking.

MATERIALS AND METHODS

*Strains and Growth Conditions*—The *Saccharomyces cerevisiae* strains used were DBY746 (MATa leu2-3 leu2-112 trp1-289 ura3-52 his3-A1; YGSC), DK1 (MATa leu2-3:LEU2 leu2-3 leu2-112 trp1-289 ura3-52 his3-A1; see below), and XK157-3C (MATa leu2-3::HIS3 trp1-289 ura3-52 his3-A1; 16). The latter two contain total leu3 deletions. DK1 was constructed as follows. Yeast shuttle vector pRS305 (19) was amplified by PCR using appropriate primers. A 1-kbp fragment of DNA containing new regions or individual residues of Leu3p that are involved in masking.

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by digesting pPC62H/86T-LEU3-dd with Sall and AvrII, followed by Klenow enzyme treatment and re-ligation. It was designated pPC62H/86T-LEU3dd12.

**Modified Yeast Two-hybrid System**—The DNA binding part of the two-hybrid system was designed to contain the extended DNA binding region (residues 1–173 or SubRII) and a short subregion (MR, residues 174–773). It was expressed behind the ADCI promoter. An appropriate centromere-containing plasmid was constructed by digesting pPC62H/86T-LEU3 (see above) with AvrII, filling in the overhangs with T4 DNA polymerase, and re-cloning the plasmid with T4 DNA ligase. This created an in-frame stop codon at amino acid position 773 of Leu3p. The gene was replaced with the arginine at position 774 with a serine. The resulting plasmid was designated pPC62H/86T-DB-MR.

The activation domain constructs for use in the two-hybrid system were also expressed behind the ADCI promoter. Plasmid pNLVP16 (a gift from E. Taparowsky, Purdue University) served as starting material for pVP-LEU3-WT-AD. A pair of primers was used to clone, by PCR, a fragment encoding the AD of VP16. The primers 5′-CTGAGCTTATTCGACATTGAGGAG3′ (5′ end primer) and 5′TCGACGGGTCGACGTAGGCGCCGGGAA-3′ (3′ end primer) were designed to contain a PstI site and an AvrII site, respectively (underlined). The PCR product was digested with PstI and AvrII and then ligated into plasmid pPC62H/86T-LEU3 that had also been cut with PstI and AvrII, thus fusing the VP16 AD sequence to the N terminus of and in-frame with the first subregion (subregion I) of the AD sequence (residues 774–886). This yielded pPC62H/86T-VP-LEU3-WT-AD. To transfer the VP-LEU3-WT-AD sequence to plasmid pRS423 (multi-copy, different marker; ref. 25), pPC62H/86T-VP-LEU3-WT-AD was digested with Apal and PovI. A 3-kbp Apal-PovI fragment containing the VP-LEU3-WT-AD sequence was ligated into pRS423 that had been cut with Apal and SmaI. The resulting plasmid was designated pVP-LEU3-WT-AD. Plasmid pLEU3-dd-GACCTAGGACCCGGGGAA was constructed in the same way except that pPC62H/86T-LEU3dd was used instead of pPC62H/86T-LEU3. To ligate into plasmid pRS423 that had been cut with either NdeI or SpeI, the resulting plasmid, pPC62H/86T-VP, containing the AD of Leu3p as being contained within the C-terminal 30 residues (14, 15). This region is sufficient to cause transcriptional activation not only in yeast but also in mammalian cells. Leu3p molecules lacking the AD are inactive. Full-length Leu3p is subject to metabolic modification, requiring the presence of α-IPM to be transcriptionally active (9, 12). To gain a better understanding of the contribution of individual amino acids of the AD to activation and modulation, we extensively mutagenized the AD, both by random and site-directed mutagenesis methods. The results are shown in Table I. In this table, the mutated sequence of the AD is followed by two columns that show the activity of a LEU2-lacZ reporter gene at high and low intracellular α-IPM concentrations; the third column shows the ratio of these numbers (modulation ratio). Since yeast does not take up α-IPM from the medium, “low” and “high” levels of α-IPM were established by supplementing the growth medium either with an excess of branched chain amino acids (conditions that severely diminish the production of α-IPM (9)) or with a limiting amount of leucine. Under both conditions, the specific activity of β-galactosidase was close to 10 when Leu3p was absent. This value therefore represents a basal level of expression of the reporter gene. The long upper part of Table I shows the effect of single amino acid mutations on the activation and modulation functions of Leu3p. Twenty

**RESULTS**

**The Modulation Function of Leu3p Is Very Sensitive to Mutational Alterations of the AD**—Earlier experiments had defined the AD of Leu3p as being contained within the C-terminal 30 residues (14, 15). This region is sufficient to cause transcriptional activity not only in yeast but also in mammalian cells. Leu3p molecules lacking the AD are inactive. Full-length Leu3p is subject to metabolic modification, requiring the presence of α-IPM to be transcriptionally active (9, 12). To gain a better understanding of the contribution of individual amino acids of the AD to activation and modulation, we extensively mutagenized the AD, both by random and site-directed mutagenesis methods. The results are shown in Table I. In this table, the mutated sequence of the AD is followed by two columns that show the activity of a LEU2-lacZ reporter gene at high and low intracellular α-IPM concentrations; the third column shows the ratio of these numbers (modulation ratio). Since yeast does not take up α-IPM from the medium, “low” and “high” levels of α-IPM were established by supplementing the growth medium either with an excess of branched chain amino acids (conditions that severely diminish the production of α-IPM (9)) or with a limiting amount of leucine. Under both conditions, the specific activity of β-galactosidase was close to 10 when Leu3p was absent. This value therefore represents a basal level of expression of the reporter gene. The long upper part of Table I shows the effect of single amino acid mutations on the activation and modulation functions of Leu3p. Twenty
Effect of mutations in the activation domain of Leu3p on activation and modulation

| Number | Mutation | Activity H | Activity L | Mod. ratio |
|--------|----------|------------|------------|------------|
| Wild type | GWDN WSDMVWRVDILMANEFAPNKV | 209 | 19 | 11.1 |
| 861 | GWDN WSDMVWRVDI LMANEFAPNKV | 159 | 45 | 3.5 |
| 862 | GWDN WSDMVWRVDI LMANEFAPNKV | 270 | 63 | 4.3 |
| 862 | GWDN WSDMVWRVDI LMANEFAPNKV | 270 | 63 | 4.3 |
| 863 | GWDN WSDMVWRVDI LMANEFAPNKV | 217 | 57 | 3.8 |
| 863 | GWDN WSDMVWRVDI LMANEFAPNKV | 288 | 90 | 3.2 |
| 864 | GWDN WSDMVWRVDI LMANEFAPNKV | 397 | 120 | 3.2 |
| 864 | GWDN WSDMVWRVDI LMANEFAPNKV | 241 | 44 | 5.5 |
| 865 | GWDN WSDMVWRVDI LMANEFAPNKV | 430 | 253 | 1.7 |
| 865 | GWDN WSDMVWRVDI LMANEFAPNKV | 184 | 13 | 14.2 |
| 865 | GWDN WSDMVWRVDI LMANEFAPNKV | 132 | 10 | 13.2 |
| 866 | GWDN WSDMVWRVDI LMANEFAPNKV | 477 | 274 | 1.7 |
| 866 | GWDN WSDMVWRVDI LMANEFAPNKV | 516 | 285 | 1.8 |
| 867 | GWDN WSECDMVWRVDI LMANEFAPNKV | 204 | 18 | 11.3 |
| 867 | GWDN WSECDMVWRVDI LMANEFAPNKV | 328 | 89 | 3.8 |
| 867 | GWDN WSECDMVWRVDI LMANEFAPNKV | 283 | 83 | 3.4 |
| 868 | GWDN WSECDMVWRVDI LMANEFAPNKV | 218 | 29 | 7.4 |
| 868 | GWDN WSECDMVWRVDI LMANEFAPNKV | 245 | 23 | 10.7 |
| 869 | GWDN WSECDMVWRVDI LMANEFAPNKV | 586 | 424 | 1.4 |
| 869 | GWDN WSECDMVWRVDI LMANEFAPNKV | 654 | 425 | 1.5 |
| 870 | GWDN WSECDMVWRVDI LMANEFAPNKV | 194 | 16 | 12.5 |
| 870 | GWDN WSECDMVWRVDI LMANEFAPNKV | 151 | 9 | 16.8 |
| 872 | GWDN WSECDMVWRVDI LMANEFAPNKV | 45 | 2.6 | 17.3 |
| 872 | GWDN WSECDMVWRVDI LMANEFAPNKV | 51 | 2.0 | 25.5 |
| 874 | GWDN WSECDMVWRVDI LMANEFAPNKV | 42 | 1.7 | 24.7 |
| 874 | GWDN WSECDMVWRVDI LMANEFAPNKV | 78 | 3.6 | 21.7 |
| 874 | GWDN WSECDMVWRVDI LMANEFAPNKV | 63 | 3.3 | 19.1 |
| 875 | GWDN WSECDMVWRVDI LMANEFAPNKV | 239 | 51 | 4.7 |
| 875 | GWDN WSECDMVWRVDI LMANEFAPNKV | 305 | 39 | 7.8 |
| 876 | GWDN WSECDMVWRVDI LMANEFAPNKV | 162 | 13 | 12.5 |
| 879 | GWDN WSECDMVWRVDI LMANEFAPNKV | 124 | 4.3 | 29.2 |
| 879 | GWDN WSECDMVWRVDI LMANEFAPNKV | 272 | 85 | 3.2 |
| 880 | GWDN WSECDMVWRVDI LMANEFAPNKV | 264 | 49 | 5.4 |
| 882 | GWDN WSECDMVWRVDI LMANEFAPNKV | 599 | 466 | 1.3 |
| 883 | GWDN WSECDMVWRVDI LMANEFAPNKV | 283 | 94 | 3.0 |
| 883 | GWDN WSECDMVWRVDI LMANEFAPNKV | 124 | 6.3 | 19.7 |
| 884 | GWDN WSECDMVWRVDI LMANEFAPNKV | 533 | 311 | 1.7 |
| 884 | GWDN WSECDMVWRVDI LMANEFAPNKV | 320 | 140 | 2.3 |
| 884 | GWDN WSECDMVWRVDI LMANEFAPNKV | 472 | 283 | 1.7 |
| 884 | GWDN WSECDMVWRVDI LMANEFAPNKV | 645 | 441 | 1.5 |
| 885 | GWDN WSECDMVWRVDI LMANEFAPNKV | 184 | 8 | 23.0 |

| Mutation | Activity H | Activity L | Mod. ratio |
|----------|------------|------------|------------|
| Δ789–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 209 | 195 | 1.1 |
| Δ777–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 300 | 252 | 1.2 |
| Δ756–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 289 | 300 | 1.0 |
| Δ735–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 52 | 81 | 0.6 |
| Δ684–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 10 | 12 | 0.8 |
| Δ681–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 4.0 | 4.7 | 0.9 |
| Δ1–886 | GWDN WSDMVWRVDI LMANEFAPNKV | 12 | 8 | 1.5 |

The numbers in the upper part of the table indicate the position of the mutated amino acid. The numbers in the lower part indicate deleted residues. In all instances, a stop codon follows the last amino acid shown. Single residue mutations are shown in bold and underlined type.

Specific activity of β-galactosidase. The reporter gene was LEU2-lacZ, under the control of the natural LEU2 promoter. H and L, activity at high and low α-IPM levels, respectively. See “Materials and Methods” for construction and expression of the LEU3 mutants and for growth conditions. The data are the average of at least two independent experiments. The error was typically <20%.

Ratio of activity at high over that at low α-IPM levels.

The sequence shown is that of the AD of Leu3p (residues 860–886).

The 26 C-terminal residues were found to have been mutated at least once. Remarkably, mutations at 16 of the 20 positions had a significant effect on modulation. Mutations causing lower modulation ratios were in the majority and mapped to both the N-terminal side (positions 861–864, 866, 867, 869) and the C-terminal side (positions 875, 879, 880, 882–884) of the AD. In all of these cases, the main reason for lower positions ratios was a sharp increase in reporter gene activity at low α-IPM levels. Given that this effect was produced by mutation in so many different places, it is very unlikely that it was brought about by improved, i.e. tighter, binding of α-IPM to Leu3p; more likely, the effect was due to impaired masking. Most of these mutations also showed a substantial rise in activation potential at high α-IPM levels (with a disproportionate increase at low α-IPM levels). The strongest increase in activation potential was seen with mutant proteins that also had the lowest modulation ratios (W864A, S866F, S867Y, V867F, V869A, F882Y, P884A, P884R, and P884A), suggesting that loss of modulation allowed these molecules to approach their maximal inherent activation capacity.

A significant increase in the modulation ratio was caused by mutations at positions 872, 874, 883, and 885. Of these, the D872X and D874X mutations (including a seemingly conservative D872E change) caused a marked 3–5-fold decline of the activation potential at low α-IPM levels. The activation potential at high α-IPM levels also declined but to a lesser extent (3–5-fold). This decline was not due to instability of the mutant proteins, as judged by the results from electrophoretic mobility shift assays (Fig. 1A; see Ref. 23). These results are consistent with the idea that the mutations at
position 872 and 874 prevent Leu3p from assuming an active configuration, by strengthening the masking interactions. (Another possibility, namely that the mutations at positions 872 and 874 reduce the affinity for α-IPM is considered unlikely (see “Discussion”).) We wondered whether mutating Asp-872 and Asp-874 simultaneously might amplify the effect caused by the individual mutations and therefore constructed a D872N/D874N double mutant by site-directed mutagenesis. Properties of a strain expressing the D872N/D874N mutant protein (designated Leu3-dd) are shown in Table II. The Leu3-dd protein had lost essentially all of its activation potential. Again, the loss of the activation potential was not caused by protein instability (Fig. 1B). To learn more about the intrinsic activation potential of the AD of Leu3-dd, we modified Leu3-dd by deleting the 600 amino acid residues between positions 173 and 774. The same deletion, when performed on wild type Leu3p (designated Leu3-Δ12), had resulted in a highly active, non-modulatable Leu-3 molecule (16). Table II shows that the Leu3-dd-Δ12 protein also regained substantial activation potential which in this case amounted to about one-third of that of wild type Leu3p. This value must be considered minimal since Leu3-dd-Δ12 appeared to be somewhat unstable. Like Leu3-Δ12, Leu3-dd-Δ12 had lost essentially all modulatability. These results strongly suggest that the inability of full-length Leu3-dd to activate was not caused by an elimination of the activation function; they are consistent with the idea that the D872N/D874N mutation caused a severe defect in modulation that keeps the Leu3-dd AD in a masked configuration (e.g. through stronger interactions with a complementary surface) and thus prevents it from interacting with the transcription machinery.

Our random mutagenesis of the AD of Leu3p also yielded a number of nonsense mutations, creating stop codons at various points along the AD. As shown in the lower part of Table I, deleting the eight C-terminal residues of Leu3p (mutant Δ879–886) totally eliminated the modulation response while the activation potential was fully retained. This result is consistent with the effect of point mutations in this region, exemplified by F882Y, P884D, P884R, and P884A. Shortening the AD further by deleting two or three additional residues actually stimulated the activation potential. A decrease of the activation potential to about 25% wild type was noted when the 17 C-terminal residues were deleted (Leu3p-Δ870–886). Deleting 23 C-terminal residues (Δ864–886) resulted in an apparently complete loss of the activation potential. It is likely, however, that Leu3p-Δ864–886 retained just enough potential to overcome the repression of reporter gene expression that is typically seen with Leu3p molecules that are totally devoid of activation potential. Finally, deleting 26 residues from the C terminus (Leu3p-Δ861–886) created a protein that did cause repression of reporter gene expression, indicating that the activation function had been lost. Again, electrophoretic mobility shift assays showed that low activation potentials were not due to loss of mutant protein (Fig. 1C). For example, the level of Leu3-Δ864–886 protein (not an activator) was at least as high as that of Leu3p-Δ876–886, Δ877–886, or Δ879–886 (all good activators).

**TABLE II**

| Leu3 protein | Reporter gene activitya | Ratiof |
|--------------|-------------------------|--------|
| Wild type    | 618                     | 65     | 9.5   |
| None         | 10                      | 13     | 0.8   |
| Leu3-dd      | 11                      | 5      | 2.2   |
| Leu3-dd-Δ12  | 176                     | 150    | 1.2   |

a Specific activities of β-galactosidase. The reporter gene was LEU2-lacZ under the control of its own promoter. See “Materials and Methods” for construction of mutants and growth conditions. The data are the average of three independent experiments. The experimental error was <25%.

b Host cells contained a total deletion of the LEU3 gene (leu3-Δ2, Ref. 9). The Leu3 protein was expressed from a single copy plasmid behind the ADC1 promoter. Leu3-dd, full-length Leu3 protein with D872N/D874N mutations. Leu3-dd-Δ12, Leu3 protein with D872N/D874N mutations and lacking amino acid residues 174–773.

c Ratio of activity at high over that at low α-IPM levels.
not require extraneous factors, we wondered whether there was direct interaction between the Leu3 AD and the remainder of the molecule. To answer this question, we turned to the yeast two-hybrid system in which the interaction of two proteins, separately fused to a DNA binding domain and a transcriptional activation domain, results in the formation of a functional transactivator and subsequent activation of a reporter gene (28). Since Leu3 was a DNA binding protein itself, we chose the following experimental setup. The two segments of Leu3 to be tested for interaction, expressed from separate plasmids, consisted of residues 1–773 and 775–886, respectively. The former contained the DNA binding domain (DB) plus the middle region (MR) of Leu3 and was used directly as the DNA binding part of the two-hybrid system; the latter contained the AD of Leu3 and an apparently functionless connecting peptide (15). Interaction between the two segments, expected to occur at low α-IPM levels, would by definition create a silent activator since the AD would be masked. To be able to recognize interaction, we therefore fused the AD of the herpes simplex virus protein VP16 (designated VP) to the N terminus of the 775–886 segment. We expected that interaction between the Leu3 AD and the remainder of Leu3 at low α-IPM levels would recruit VP to the UASL-containing promoter and activate the reporter gene. At high α-IPM levels, the two segments would not interact, VP would not be recruited, and the reporter gene would not be activated. An important additional consideration was that interaction between the two separated segments of Leu3 would likely be considerably weaker than interaction between the same regions in the intact molecule since the contact between the regions would be diffusional and no longer directed by the shape of the intact protein. For this reason, we also included the 775–886 segment of the Leu3-dd mutant protein (containing the Leu3-dd AD) in the analysis. If the interactions leading to AD masking were indeed stronger for Leu3-dd than for wild type Leu3, we would expect interaction of the severed Leu3-dd AD with the remainder of Leu3 also to be stronger. The experimental design is illustrated in Fig. 2.

All of the above expectations were fulfilled by experimental results. Fig. 3 shows that a low basal level of reporter gene expression occurred when the DB-MR segment of Leu3 was expressed by itself; changing the intracellular α-IPM concentration had no effect (column pair 1). The same result was obtained when the DB-MR segment of Leu3 was co-expressed with the VP16 AD (column pair 2). When the DB-MR segment was co-expressed with the VP-Leu3-WT AD segment, a weak but statistically significant (2-fold) activation of the reporter gene was observed at low but not at high α-IPM concentrations (column pair 3). Finally, when the DB-MR segment was co-expressed with the VP-Leu3-dd AD segment, a strong, approximately 11-fold activation occurred at low (but not at high) α-IPM levels (column pair 4). The effects were specific for the Leu3 ADs since no activation was seen with VP alone. Specificity was also implied by the fact that reporter gene activation depended on the α-IPM concentration. That is, the DB-MR segment showed no sign of interacting with the Leu3 ADs when the α-IPM concentration was high; the very same DB-MR segment did interact with the Leu-3 ADs when the α-IPM concentration was low. The α-IPM dependence was as predicted, i.e., activation was seen when the α-IPM level was low, a condition which should promote AD-MR interaction. The results are consistent with the idea that the Leu-3 AD interacts with the remainder of the Leu3 molecule and that this interaction is intensified when aspartate residues 872 and 874 are mutated to asparagines (see also Fig. 7). Importantly, the results also demonstrate that Leu3-dd is capable of interacting with α-IPM.

Proof that the constructs used in this experiment were stably expressed in the cells was obtained from electrophoretic mobility shift assays and Western blots (Fig. 4, A–C).

**Intragenic Suppression of the Leu3-dd Mutation as a Means to Identify Residues in the Middle Region (MR) of Leu3 That Are Potentially Involved in AD Masking—** Cells expressing the Leu3-dd protein grew very slowly on plates lacking leucine (Fig. 5), probably because of a repressive effect by Leu3-dd on the expression of LEU2 and possibly other LEU genes. Similar growth behavior and concurrent in vivo repression of LEU2 expression had earlier been observed with mutants of Leu3 that carried a stop codon at position 772 or 812 (9, 29). The slow growth of Leu3-dd-containing cells provided an opportunity to select for suppressors. We argued that if the phenotype exhib-
Intramolecular Interactions in Leu3p

WT LEU3

LEU3dd

Fig. 5. Growth behavior of yeast cells expressing the Leu3-dd protein. Growth was on SD medium (20) for 3 days at 30°C. The leu3 null strain XK157–3C, transformed with pYB1 (9) was used throughout. It was transformed additionally either with pPC62H/86T-LEU3, a plasmid that generates full-length wild type Leu3p (WT LEU3), or with pPC62H/86T-LEU3dd, a plasmid that generates full-length Leu3-dd (LEU3dd).

Fig. 4. Demonstration that the fragments used in the modified two-hybrid system were stably expressed. A, electrophoretic mobility shift assays using whole-cell extracts as protein source and a UASleu3 30-mer (9) as probe. Lane 1, no extract; lane 2, extract from cells expressing full-length Leu3p from its native promoter; lane 3, extract from cells expressing full-length Leu3p from the ADC1 promoter (which was used in all subsequent experiments); lanes 4 and 5, extract from cells expressing DB-MR only; lane 6, extract from cells expressing DB-MR and the VP AD; lane 7, extract from cells expressing DB-MR and the VP-Leu3 WT AD fusion; lane 8, extract from cells expressing DB-MR and the VP-Leu3-dd AD fusion. B, Western blot (with anti-VP16 antibody) using Hybond C nitrocellulose membrane (Amersham Corp.). Lane 1, extract from cells not expressing a VP AD; lane 2, extract from cells expressing the VP-Leu3 WT AD fusion; lane 3, extract from cells expressing the VP-Leu3-dd AD fusion. The position of carbonyl anhydrase (M, 30,000) is marked. C, Western blot using Immobilon-P membrane (Millipore). Lane 1, extract from cells not expressing a VP AD; lane 2, extract from cells expressing VP AD. The position of lysozyme (M, 14,300) is marked. See “Materials and Methods” for further details. B, bound DNA; F, free probe.

Residues 172–469, 470–607, and 608–772, respectively. The mutated fragments were inserted into a cloned LEU3-dd gene by cassette exchange, and plasmids carrying a mutated LEU3-dd gene were then used to transform leu3 yeast cells that contained a LEU2-lacZ reporter gene (9). Fast-growing transformants were isolated and analyzed for β-galactosidase activity. Mutants of interest, i.e., those that caused reporter gene activation (at high α-IPM levels) corresponding to at least 25% that caused by wild type Leu3p, were collected and subjected to DNA sequence analysis. A batch of nine such mutants is shown in Table III (mutants 1A–9A). Suppression of the phenotype of the Leu3-dd mutant was evident from the activation potential of the mutants, which ranged from 29 to 118% wild type value (Leu3-dd’s activation potential is <2% wild type). The modulation ratios of the suppressors also covered a wide spectrum, ranging from relatively large values (mutants 1A–4A and 6A–8A) to a value that was closer to normal (mutant 5A). The behavior of mutants 1A–8A is readily explained by assuming that, to a varying degree, their Leu3 molecules regained the ability to expose their AD in response to α-IPM. The modulation ratio of one mutant (9A) was close to 1. The phenotype of this mutant (strong activation potential, essentially no response to α-IPM) was identical to that of cells containing constitutive Leu3p molecules with a permanently active (unmasked) conformation. The behavior of all nine mutant proteins is therefore consistent with a relaxation of tight AD masking interactions present in the Leu3-dd parent molecule. If the masking interactions in Leu3-dd proceeded through the same (or very similar) contact regions as in wild type Leu3p, one would expect wild type protein containing the above mutations also to show diminished AD-masking capabilities. This was indeed found to be the case. When the MR mutations present in mutants 1A–9A were introduced into wild type Leu3 (by the same cassette exchange that was used to introduce them into Leu3-dd), it was found that all nine mutant proteins (1B–9B, right half of Table III) had become essentially constitutive (modulation ratios <2). At the same time, their activation potential was either significantly above or close to that seen with wild type Leu3p. These results strongly suggest that the underlying mutations occurred in residue(s) that are important for the masking of the AD. For most of the MR mutants listed in Table III, identification of individual critical residues is not possible at this point since the mutants are the result of multiple (double, triple, or quadruple) residue changes (see...
Intragenic ("second-site") suppressor mutations were generated as described under "Materials and Methods." The mutations of interest were first identified and analyzed in the context of Leu3-dd (left half of table). The mutations were then transferred to wild type Leu3p and re-analyzed (right half of table). The suppressor mutations were as follows: 1A + 1B: K664E; 2A + 2B: Y654D, S708N, T713S, K723T; 3A + 3B: R643G, S645T, P754T; 4A + 4B: N493S, I496V, K578R, S607G; 5A + 5B: F510L, W553R; 6A + 6B: L611H, F612L, S700G; 7A + 7B: V702D, Q709L, N738D, R765S; 8A + 8B: L668V, L611H, Q635R, K624M; 9A + 9B: A472V, E505K, H536Q, I604K.

For construction of mutant proteins and other details see "Materials and Methods."

**TABLE III**

### Intramolecular Interactions in Leu3p

| Activation domain, dd | Activation domain, wild type |
|-----------------------|-----------------------------|
| No. | Activation potential | Modulation ratio | No. | Activation potential | Modulation ratio |
|-----|---------------------|------------------|-----|---------------------|------------------|
| 1A  | 29  | 30.0 | 1B  | 163 | 1.8 |
| 2A  | 32  | 19.0 | 2B  | 158 | 1.3 |
| 3A  | 34  | 15.5 | 3B  | 168 | 1.5 |
| 4A  | 36  | 18.5 | 4B  | 107 | 1.4 |
| 5A  | 38  | 7.3  | 5B  | 86  | 1.1 |
| 6A  | 43  | 24.2 | 6B  | 162 | 1.6 |
| 7A  | 49  | 22.2 | 7B  | 168 | 1.8 |
| 8A  | 73  | 16.9 | 8B  | 140 | 1.4 |
| 9A  | 118 | 0.8  | 9B  | 68  | 1.3 |

*Reporters gene (LEU2-leu2) activity at high α-IPM, in percent of wild type. The numbers are the average of at least two independent experiments. The error was <11%, except for mutant 7B where it was 24%.

**DISCUSSION**

In this paper, we have taken a first step toward understanding the mechanism by which Leu3p responds to α-IPM. The functional change brought about by α-IPM is quite dramatic. At low concentrations of the metabolite or in its absence, Leu3p acts as a repressor, causing a 4- to 5-fold drop in reporter gene expression below the level observed in cells lacking Leu3p (12, 14). When the intracellular α-IPM concentration rises above a threshold value, which is probably in the 10th-millimolar range (12, 18), Leu3p becomes a strong activator of gene expression. This transition is thought to be accompanied by a conformational change that somehow allows the sole AD of Leu3p, located near the C terminus, to interact with elements of the transcription machinery. The form of Leu3p that represses transcription is a masked mode even at relatively high concentrations, as long as α-IPM is absent (12). Finally, the demonstration in this paper that the Leu3 AD can directly interact with the remainder of the protein provides a very strong argument for the self-contained nature of Leu3p with respect to its modulation.

If masking of the AD of Leu3p is achieved intramolecularly, the next question is which parts of the Leu3p molecule are involved in this process. Recent domain-swap experiments with the serine/threonine-responsive activator Cha4p of yeast have shown that the extended DNA binding domain of Leu3p (encompassing residues 1–173) is not required for modulation by α-IPM. Also, deleting a region adjacent to the AD of Leu3p (residues 774–854) had no effect on modulation (15). In the present work, we therefore focused on the AD and the MR of Leu3p as potentially holding the key to the modulation process.

Our attempt to understand modulation was aided significantly by the construction of the Leu3-dd mutant. This mutant not only proved useful in the two-hybrid experiment but also led to the isolation of modulation-related mutants that map to the MR of Leu3p. It is therefore important to inquire about the mechanism by which the D872N/D874N mutation keeps the regulator in a virtually inactive state. A priori, at least three possibilities to explain the behavior of Leu3-dd might be considered: (i) the D872N/D874N mutation drastically reduces the binding of α-IPM to Leu3p; (ii) the double mutation does not affect α-IPM binding but essentially quells a conformational change that, in wild type Leu3p, is a consequence of α-IPM binding and eventually leads to the exposure of the AD; or (iii) the double mutation allows the AD to interact more strongly with the remainder of the molecule. There are several observations that argue against the first possibility. First and foremost, Leu3-dd still responds to changes in the α-IPM concentration; a strong dependence on α-IPM was clearly evident in the two-hybrid experiment with cleaved Leu3-dd. Second, if the expression of the LEU3 gene was directed by the human cytomegalovirus major intermediate early promoter. No other yeast-specific genes were present. Since the leucine biosynthetic pathway is absent from mammalian cells and such cells do not normally contain a Leu3p-type regulator, they would not be expected to contain a specific Leu3p-masking factor either.

It is important to note in this context that expression of LAC9 of Kluyveromyces lactis (a Gal4p homolog) in mammalian cells produced a protein that was not masked unless GAL80 was co-expressed (31). The argument that Leu3p does not require a separate masking factor is further supported by the observation that, in an in vitro transcription system using yeast whole-cell extract from Leu3p-deficient cells, purified Leu3p was unable to out-titrate a presumptive masking factor and stayed in a masked mode even at relatively high concentrations, as long as α-IPM was absent (12). Finally, the demonstration in this paper that the Leu3 AD can directly interact with the remainder of the protein provides a very strong argument for the self-contained nature of Leu3p with respect to its modulation.

![Fig. 6. Regions of interest along the primary sequence of Leu3p. Chequered region, DNA binding motif (residues 57–67) and heptad repeats (residues 85–99; dark gray region, region to which the suppressor mutations map (resides 472–765, Table III); light gray region, non-essential sequence (resides 774–854, Ref. 15); black region, activation domain (resides 861–886, see text).](image-url)
D872N/D874N mutation had caused severe impairment of α-IPM binding, it would be quite improbable that a relatively large number of second-site suppressor mutations would be found that would repair a damaged α-IPM binding pocket. Also, if the mutations leading to suppression of the Leu3-dd phenotype did so by repairing the α-IPM binding pocket of Leu3-dd, those same mutations would not be expected to create generally strong and nearly α-IPM-independent activators when introduced into wild type Leu3p. Yet that is what is observed (Table III). Turning to the second possibility, it is also unlikely that the D872N/D874N mutation interferes with a conformational change caused by α-IPM. If this were the case, one would expect the intramolecular masking interactions (which by definition occur before any α-IPM-induced conformational change could take place) to be the same with wild type Leu3p and Leu3-dd. Yet these interactions, as observed in the two-hybrid experiment, are much stronger with Leu3-dd. We therefore conclude, in accordance with the third possibility and based on the evidence from the two-hybrid experiment, that the behavior of Leu3-dd is a consequence of stronger interactions between the AD and the remainder (very likely the MR) of Leu3p. In Leu3-dd, these interactions are so strong that the normal exposure of the AD following α-IPM binding cannot occur. A different behavior results when the AD of Leu3-dd is severed from the rest of the molecule. The now diffusion-controlled interactions are weaker than those in the intact Leu3-dd molecule, yet are still strong enough to resemble those of wild type Leu3p. A schematic representation of interactions proposed to take place is shown in Fig. 7. The observation that the Leu3-dd suppressor mutations studied so far not only reverse the behavior of Leu3-dd but also lead to essentially permanent unmasking of the AD of wild type Leu3p suggests that residues identified in this way are also involved in the normal masking process. The question of whether a given residue participates directly in masking (e.g. by making contact with the AD) or has an indirect effect (e.g. by stabilizing a configuration favorable for masking) will be difficult to answer in the absence of structural information. However, we think that Lys-664 is a good candidate for direct participation because the K664E mutation (Table III) causes a change in side chain size and a drastic change in side chain chemistry, yet the mutated protein is very active, making it seem unlikely that the mutation caused a gross conformational change.

We now turn to the portion of Leu3p that is presumed to interact with the MR, i.e. the AD. This region is remarkably sensitive to mutation with respect to the modulation function, suggesting that important secondary or tertiary structural configurations are present. CD spectroscopy has indicated a propensity for α-helical structure in the 859–886-residue region, and it is possible that the intactness of helical structure is important for efficient masking. However, drastic effects on modulation are seen both when the effect of a mutation on α-helical stability is expected to be strong (e.g. F882Y) and when it is expected to be minor (e.g. S866Y). Strong effects on modulation are also seen when Pro-884 (which is very likely not part of an α-helix) is mutated. This indicates that a need to conserve α-helical structure per se is not sufficient to explain the sensitivity of the modulation function to mutation. Another interesting feature of the AD is the presence of two types of amino acid residues: those that loosen and those that tighten the masking interactions. Mutations that appear to loosen the interactions the most are found at positions Trp-864, Ser-866, Val-869, Phe-882, and Pro-884. It is reasonable to assume that those same residues facilitate masking in wild type Leu3p. Residues Asp-872 and Asp-874, which are located in the center of the AD and which, when mutated, increase masking efficiency, would not be expected to contribute much to masking in wild type Leu3p and might even antagonize masking. This pattern of residues that either favor or oppose (or are neutral toward) masking might be important for achieving a physiologically desirable balance between open and closed forms of Leu3p.

In striking contrast to their effect on the modulation function of Leu3p, mutations in the AD have a much smaller effect on

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3 K. Uma, E. Remboutsika, G. B. Kohlhaw, and S. A. Johnston, unpublished observations.
the activation potential of the protein. This is most evident from the deletion analysis (Table I). A Leu3p molecule lacking 17 of the 26 residues of the AD still has about 25% the activation potential of full-length Leu3p. It should be noted that the remaining short region still contains three of the original six acidic residues, as well as four hydrophobic residues. In view of the well-known fact that even random (acidic) sequences can bestow transcriptional activation potential upon DNA binding regions to which they are fused (32), the apparent promiscuity of the Leu3p AD does not come as a surprise.

Although this work has provided important evidence supporting intramolecular interactions as a mechanism for masking the AD of Leu3p, we are not yet able to determine whether these interactions occur in cis or in trans. Since Leu3p very likely acts as a dimer (33, 34), masking could in theory be achieved either by intra-monomer or by intra-dimer interactions. Experiments addressing this question are underway.

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