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SELF-MASKING, SPECIFICITY OF MASKING, AND EVIDENCE FOR REGULATION BY THE INTRACELLULAR LEVEL OF Leu3p

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Recent work suggests that the masking of the activation domain (AD) of yeast transactivator Leu3p, observed in the absence of the metabolic signal o-isopropylmalate, is an intramolecular event. Much of the evidence came from the construction and analysis of a mutant form of Leu3p (Leu3-dd) whose AD is permanently masked (Wang, D., Hu, Y., Zheng, F., Zhou, K., and Kohlhaw, G. B. (1997) J. Biol. Chem. 272, 19383–19392). In a modified two-hybrid experiment, the ADs of both wild type Leu3p and Leu3-dd were shown to interact with the remainder of the Leu3p protein, in an o-isopropylmalate-dependent manner. The finding that masking and unmasking proceed apparently normally when full-length Leu3p is expressed in mammalian cells is also consistent with the notion of intramolecular masking. Here we report on the identification of nine missense mutations (all of them suppressors of the Leu3-dd phenotype) that cause permanent unmasking of Leu3p. The nine mutations map to three short segments located within a 140-residue-long region of the C-terminal part of the middle region of Leu3p. These segments may be part of a spatial trap for the AD. We also performed “domain swaps” between Leu3p and Cha4p, a serine/threonine-responsive activator that, like Leu3p, belongs to the family of Zn(II)2Cys6 proteins. We show that AD masking and response to the appropriate metabolic signal only occur when a given AD remains attached to its own middle region; middle region swapping results in constitutively active proteins. Finally, we show that the extent to which Leu3p regulates reporter gene expression depends on the intracellular concentration of Leu3p. The possible physiological significance of this observation is discussed in light of the known regulation of Leu3p by Gen4p.

Transcriptional regulation of polymerase II-transcribed genes often depends on the intracellular concentration of signal molecules such as hormones or metabolites. Accordingly, transactivators serving as signal transducers need to be equipped not only with DNA binding and transcriptional activation domains but need to also have sites that interact with ligands (either directly or through auxiliary proteins) and structural features that allow appropriate conformational changes to take place in response to ligand binding and ligand dissociation. A prominent example is the nuclear receptor superfamily whose members regulate gene expression in response to steroid and thyroid hormones, retinoids, and vitamin D (1, 2). Although Saccharomyces cerevisiae does not produce such receptors, many of its transactivators also display the modular construction (domains arranged along the primary structure) seen with the nuclear receptors. Leu3p is a case in point. It belongs to a family of proteins characterized by an N-terminal DNA binding domain (DB)1 of the Zn(II)2Cys6 binuclear cluster type. This family encompasses at least 79 fungal proteins, including at least a dozen transactivators (3). The open reading frame encoding Leu3p indicates a monomer length of 886 residues (4, 5). Leu3p exists as a dimer, both in the presence and absence of DNA (6, 7). It recognizes the sequence 5'-CCGCG-3' (8). Binding of full-length Leu3p causes the target DNA to bend, with an apparent flexure angle of 46° (2). The Zn(II)2Cys6 cluster extends from residue 37 to 67. Heptad repeats, thought to be essential for dimerization, are located between residues 85 and 102. The AD had early on been found to be located within the C-terminal 30 residues and to function when fused directly to a DNA binding domain (9). A recent study showed that as few as 9 of the 30 C-terminal residues suffice to allow substantial transcriptional activation (10). This core AD contains three acidic and four hydrophobic residues. In agreement with the functional mapping that placed the DB close to the N terminus and the AD close to the C terminus of Leu3p, large portions of the middle region could be deleted with only minor effects on DNA binding or transcriptional activation (11, 12). Such deletions did, however, create constitutive molecules, i.e. eliminated the response of Leu3p to alpha-IPM, thereby suggesting that the middle region was essential for the modulation process. The finding that Leu3p exhibited perfectly normal regulatory behavior when expressed in mammalian cells (13) was consistent with the notion that masking of the activation domain in the absence of alpha-IPM and its unmasking in the presence of alpha-IPM did not require extraneous factors but were intramolecular events. This idea was supported by a recent analysis of the mechanism of masking (10). Permutation of the AD revealed the presence of two types of residues as follows: those that, when mutated, appear to loosen the masking interactions (e.g. W864A, S866P, V869F, F882Y, and P884A), and those that

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1 The abbreviations used are: DB, DNA binding domain; AD, activation domain; MR, middle region; IPM, isopropylmalate; UASL, upstream activating sequence in the promoters of Leu3p-regulated genes; WT, wild type; PCR, polymerase chain reaction; bp, base pair; HRMs, heme regulatory domains.

2 H. Guo and G. B. Kohlhaw, unpublished results.

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tighten the interactions when mutated (e.g. D872N and D874N). When the latter two aspartates were simultaneously changed to asparagines, the resulting mutant (designated Leu3-dd) was permanently masked (10). Leu3-dd, like all Leu3p mutants with an impaired AD, caused repression of LEU2 expression and severely reduced cell growth in the absence of leucine. A selection for intragenic suppressors of the Leu3-dd phenotype yielded a number of mutations, all of which mapped to the C-terminal half of the middle region of Leu3p. Transfer of the suppressor mutations to wild type Leu3p caused constitutivity, i.e. such mutants were active irrespective of the presence or absence of α-IPM, a result consistent with an involvement of the affected residues in masking (10). In the present work, nine individual constitutivity-causing residues are identified. Remarkably, they map to three very short regions between positions 604 and 741 and may form the spatial backbone for AD masking. These results leave a large segment of Leu3p (encompassing residues 174–603) unaccounted for as far as function is concerned. It is not clear at this point whether this segment is involved in α-IPM binding or signal transmission or represents a “spacer” that improves stability and/or efficiency of the protein, as has been suggested for Gal4p where a similarly large deletion was shown to have little effect on the regulatory properties of the protein (14). The “dispensable” segment of Gal4p and the corresponding segment of Leu3p contain a region of weak homology that is present in most Zn(II)Cys6 cluster proteins (3). There has been no unambiguous assignment of function to this region, either.

In addition to identifying residues potentially involved in AD masking, we show here that the modulation function of Leu3p is activator specific and that the degree of activation by Leu3p is Leu3p concentration-dependent. The latter observation is of special interest since the intracellular level of Leu3p is, at least in part, controlled by Gcn4p, a major regulator of amino acid metabolism in S. cerevisiae (4).

**MATERIALS AND METHODS**

**Strains and Growth Conditions**—The yeast strains used in this work were YW2-3 (MATa leu2-3::HIS3 ura3-52 trp1-289 rbi1-55) (15), and 17 (601) (MATA leu4 ura3-52 trp1-289 his3Δ1, YK82-49 (MATA leu4 ura3-52 trp1-289), DK1 (MATA leu3-Δ2:LEU2 leu2-3 leu2-112 ura3-52 trp1-289 his3Δ1), TG494 (MATA leu3-Δ2: HIS3 ura3-52 trp1-289), and XK41-7 (MATA leu3-Δ2: HIS3 ura3-52 trp1-289). Unless stated otherwise, yeast cells were grown on SD medium supplemented with required nutrients. Cells were grown at 30 °C and harvested at an A600 of about 1. Escherichia coli strains used for DNA manipulations were XL-1-Blue and XL-2-Blue from Stratagene and DH5α and DH5α-F' IQ from Life Technologies, Inc. E. coli cells were grown at 37 °C in LB medium with the addition of 100 μg/ml penicillin.

**Principal Plasmids Used**—pYB1 is a centromere-containing plasmid carrying a LEU2-laZ fusion (15); pPC62-H/86T is a centromere-containing vector carrying an ADC1 promoter, kindly provided by W. A. Laskey, Purdue University; pRS423 is a multicopy (2-μm) plasmid, kindly provided by P. Hieter, The Johns Hopkins University; pBTM117 is a multicopy plasmid containing the full-length leaX gene, kindly provided by J. Colicelli, UCLA; p1155 is a multicopy plasmid containing a single consensus leaX operator (16), kindly provided by M. Johnston, Washington University; pTK327 is a centromere-containing vector carrying the full-length CHA1 gene (21); pTK120 is a centromere-containing vector carrying the CHA1 promoter (1–1 to 699, where +1 refers to the point of transcription start) fused to the lacZ reporter gene (17).

**Site-directed Mutagenesis of the Leu3p Middle Region**—A previous effort to identify suppressors of the Leu3-dd phenotype had yielded multiple site suppressors that mapped to a region bordered by residues 472 and 765 (10). To find out which, if any, particular residue was responsible for activation, a PCR mutagenesis approach was used, that generates all of the single mutations that made up a given multiple mutation suppressor. Two sets of three universal primers were used, plus one specific primer for each individual mutation. The universal primers were 5'-AACGTTGCGTCTGCATGTTGCTC-3' or 5'-GACGTTTTAATGCCTGCTTGATGTCTGTAG-3' (for the 5' ends of two subregions); 5'-TCGGAGCCCTCAGGATGATTCG-3' or 5'-CAGTTGCGTCTGCTTGATGTCTGTAG-3' (for the 3' ends of two subregions); and 5'-TCGGAGCCCTCAGGATGATTCG-3' or 5'-TCGGAGCTCGCTGCTTGATGTCTGTAG-3' (as the mismatched primers; mismatched sequence is underlined). The PCR products containing all single mutations were purified using the QiAquick PCR purification kit (Qiagen). They were then digested with appropriate restriction enzymes and inserted into pPC62-H/86T-LEU3dd (10) that had been cut with either SpeI and NotI or NotI and AvaI. The ligation solutions were used to transform XL1-blue cells. After overnight incubation at 37 °C, single colonies were picked, and DNA was isolated using QiAprep spin columns (Qiagen), and sequences were determined using a DNA sequencing kit (Amersham Pharmacia Biotech). Plasmids containing the desired mutations were used to transform XK157-3C cells that already contained the pYB1 plasmid. The transformed cells were grown on SD medium. Cell-free extracts were prepared and β-galactosidase activities measured (10). Single mutations that caused phenotypes similar to the corresponding multiple mutations were transferred to wild type LEU3 by performing cassette exchanges from pPC62-H/86T-LEU3dd to pPC62-H/86T-LEU3, as described (10).

**Construction of the LEU3-CHA1 Chimera**—To transfer the CHA1 gene from pTK327 to pPC62-H/86T (see above for plasmid details), two oligonucleotides, 5'-TCCGAGCTCCTCGAGAATTCGAGCATGATG-3' (5' end) and 5'-GATTATGTCGCGGTTAGAATTGCGG-3' (3' end) (PstI and SacII restriction sites underlined), were used as primers to amplify the full-length CHA1 gene with desired restriction sites on each end by PCR methodology. In all the experiments where PCR products were digested with restriction enzymes, the respective enzymes were either not present in the DNA fragments to be amplified by PCR or were introduced by the primers. The PCR-synthesized CHA1 gene was digested with PstI and SacII and then inserted into pPC62-H/86T-LEU3 that had been cut with the same enzymes. In the resulting plasmid, pPC62-H/86T-CHA1, the CHA1 gene was expressed from an ADC1 promoter.

To make L-L, a fragment encoding the AD of Leu3p was synthesized using oligonucleotides 5'-CTCGAGGCTCCTCGAGAATTCGAGCATGATG-3' (PstI site underlined) and 5'-CGCGTTGCGGTTAGAATTGCGG-3' (SacII site underlined). The PCR product encoding the DB of Leu3p was digested with SacI and SpeI and inserted into pPC62-H/86T-LEU3 that had been digested with the same enzymes. The resulting plasmid, pPC62-H/86T-L-L, encoded a truncated Leu3p molecule containing residues 1–173 (the DB) fused to residues 524–926 of CHA1. To construct pPC62-H/86T-CLC had earlier been constructed in a similar way and was used to make L-L, a fragment encoding the AD of Leu3p was synthesized using oligonucleotides 5'-CTCGAGGCTCCTCGAGAATTCGAGCATGATG-3' (PstI site underlined) and 5'-ACAGGTTGCGGTTAGAATTGCGG-3' (SacII site underlined) and 5'-TGGACTGCGGTCGTAGCAGCGG-3' (SacII site underlined). The PCR product encoding the DB of Leu3p was digested with PstI and EcoRI and that encoding the AD of Cha4p was digested with EcoRI and SacII. The two digested fragments were simultaneously inserted into plasmid pPC62-H/86T-LEU3 that had been digested with PstI and SacII, resulting in pPC62-H/86T-L-C. The DNA manipulations caused the introduction of a foreign serine residue (Ser524) in both Leu3p and Cha4p molecules. To make pPC62-H/86T-LLC, two DNA fragments encoding the MR of Cha4p and the AD of Leu3p, respectively, were synthesized by PCR with the following two pairs of oligonucleotides: 5'-ACAGGTTGCGGTTAGAATTGCGG-3' (SacII site underlined) and 5'-CTCGAGGCTCCTCGAGAATTCGAGCATGATG-3' (PstI site underlined) and 5'-ACAGGTTGCGGTTAGAATTGCGG-3' (SacII site underlined). The resulting plasmid, named pPC62-H/86T-LLC, had been cut with the same enzymes. The resulting plasmid, pPC62-H/86T-LLC, which contained the DNA encoding the DB of Leu3p, was digested with EcoRI and SacII and that coding for the AD of Cha4p was digested with EcoRI and SacII and that coding for the AD of Leu3p was digested with EcoRI and SpeI. The digested fragments were then simultaneously ligated with SacII and SpeI-digested pPC62-H/86T-LEU3 which contained the DNA encoding the DB of Leu3p. The resulting plasmid was named pPC62-H/86T-LCL. Plasmid pPC62-H/86T-LCL had earlier been constructed in a similar way and was used to make pPC62-H/86T-LLC. The latter was constructed by insertion of the SacII/SacII fragment of pPC62-H/86T-CLC that coded for the MR of Leu3p and the AD of Cha4p (the “LC” portion), into pPC62-H/86T-LEU3 which had been digested with the same enzymes and provided the DNA encoding the DB of Leu3p. Construct LCC was made by first synthesizing, by PCR, a DNA fragment encoding the MR of Cha4p (5'-CTCGAGGCTCCTCGAGAATTCGAGCATGATG-3' (SacII site underlined) and 5'-GATTATGTCGCGGTTAGAATTGCGG-3' (SacII site underlined)), PCR product was digested with SacII and then inserted into pPC62-H/86T-LEU3 that had been digested with the same enzymes. The resulting plasmid was named pPC62-H/86T-LCC. To make plasmid pPC62-H/86T-CLL, a DNA fragment encoding the DB of Leu3p.
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Cha4p was synthesized by PCR using the following oligonucleotides: 5'-TCCTGAAAGCTTCCAGAATCCCGGTAGAATG-3' (SacI site underlined) and 5'-AGGAAAATCTGAGCTGCTCGCAAGCTTAC-3' (SfiI site underlined). The PCR product was digested with SfiI and Sall and inserted into pPC62-H/86T-LEU3 that had been digested with the same enzymes.

All junction regions of the LEU3-CHA4 chimeric constructs were verified by restriction digestion analysis and those of constructs L1, L-2C, LCC, and CLL were further confirmed by DNA sequence analysis using the Sequenase (version 2.0) sequencing kit from Amersham Pharmacia Biotech. Unless otherwise stated, the above Leu3-Cha4 fusion proteins did not contain any foreign amino acid residues.

Preparation of LexA-containing Chimeric Constructs—Plasmid pBTM117, which contained the full-length LexA gene, was used to make the LexA-containing constructs. First, a working plasmid called pPC62-H/86T-LexA was constructed as follows. Plasmid pPC62-H/86T was partially digested with HindIII and then digested to completion with PstI. A 6.7-kilobase pair HindIII/PstI fragment was isolated by electrophoresis and saved. Then, pBTM117 was digested to completion with HindIII and PstI. A 655-bp fragment (which contained the LexA gene) was isolated by electrophoresis. Insertion of this fragment into the HindIII/PstI-digested vector pPC62-H/86T resulted in pPC62-H/86T-LexA. Since this version of the LexA gene did not contain a stop codon at its end, one such codon was incorporated by replacing the 3' end portion with a PCR-synthesized copy of the same element containing a TAA codon. The oligonucleotides used were 5'-CCGCTGATCAGCCGAGGTCGTCGACGAGCTTA-3' and 5'-TGCGAATTCAAACGAGATTTCAGAAGAA-3' (EcoRI site and stop codon underlined). The PCR product and pPC62-H/86T-LexA were both digested with MluI and EcoRI, and the replacement of the LexA sequence was performed by cassette exchange. The resulting plasmid, named pPC62-H/86T-A, encoded a full-length LexA protein. Plasmid pPC62-H/86T-AL172–886 was constructed by inserting a Sall/SoiI fragment from pPC62-H/86T-LEU3 into plasmid pPC62-H/86T-LexA digested with the same enzymes. Plasmids pPC62-H/86T-AL1-886, pPC62-H/86T-AL70-886, and pPC62-H/86T-A-L100-886 were made in a similar way. Three DNA fragments were synthesized by PCR, one for each construct. The oligonucleotide pairs for synthesizing the DNA fragments were T-TTGGATCGATCGAGGATCAGCGAGGATCC-3' and 5'-ACAGGGTCGGAATTCGTCGACGAGCTTA-3' (EcoRI site and stop codon underlined). The PCR products and pPC62-H/86T-LexA were both digested with MluI and EcoRI, and the replacement of the LexA sequence was performed by cassette exchange. The resulting plasmid, named pPC62-H/86T-LEU3 that had been digested with the same enzymes, was inserted into pPC62-H/86T-LexA digested with the same enzymes.

RESULTS AND DISCUSSION

Genetic Selection Reveals Three Clusters of Single Residue Mutations That Abolish Masking and Are Located within a Short Stretch of the Middle Region (MR) of Leu3p—In a previous paper, we initiated an analysis aimed at identifying residues of Leu3p that were important for AD masking (10). Since available evidence indicated that neither the extended DNA binding domain (DB, residues 1–173) nor a region adjacent to the AD (residues 774–854) were required for modulation of Leu3p, whereas deletion of residues 174–733 abolished modulation (9, 11, 20, and this paper), the analysis focused on the “middle region” (MR, residues 174–733). Starting with a slow growing strain that contained a mutant Leu3p whose AD was permanently masked (Leu3p (D872V/D874N), designated Leu3-dd), faster growing suppressors were isolated following mutagenic PCR of the entire MR. The suppressor mutations were designed in a strain, restoring to varying degrees activation potential and modulation to Leu3-dd. When transferred to wild type LEU3 by cassette exchange, the same mutations caused the Leu3 protein to become constitutively active. As it turned out, however, all but one of the suppressor mutants contained multiple (double to quadruple) mutations in the MR, allowing only a general delimitation of an area potentially involved in masking (10). We therefore undertook to find out whether any one of the multiple mutations of a given suppressor might be responsible for the phenotype of that suppressor. By using PCR methodology, we created Leu3-dd proteins containing separately each of the multiple mutations found in a given suppressor. For example, one of the suppressors carried mutations that altered amino acid residues in four places as follows: A472V, E505K, 11536Q, and 1604K. We determined that the activation-modulation behavior of Leu3-dd(A472V), Leu3-dd(E505K), and Leu3-dd(11536Q) was very similar to that of Leu3-dd itself, whereas Leu3-dd(1604K) behaved very much like the original four-mutation suppressor molecule. The 1604K mutation was therefore considered to be the actual suppressor. With this approach, we identified eight separate single suppressor mutations. Each mutation was subsequently transferred to wild type LEU3 by cassette exchange. As shown in Table I, each of these mutations will turn an otherwise wild type Leu3p molecule into an essentially 8-IP3-independent “constitutive” activator. Moreover, all eight mutant proteins display significantly stronger activation capacities than wild type Leu3p, suggesting that they are stable molecules that approach their maximal innate activation potential. Remarkably, all eight mutations map to three small areas of the MR that cover residues 604–611, 643–664, and 738–741 (Fig. 1).
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Table I

| Leu3 protein | Reporter gene activity | Modulation ratio |
|--------------|------------------------|------------------|
| None         | High α-IPM             | Low α-IPM        |
|              | 8.1                     | 8.2              |
| Wild type    | 156                     | 18               |
| Leu3p(604K)  | 281                     | 218              |
| Leu3p(S607G) | 205                     | 169              |
| Leu3p(L6111) | 351                     | 269              |
| Leu3p(R643G) | 308                     | 261              |
| Leu3p(Y664D) | 372                     | 276              |
| Leu3p(K664E) | 342                     | 295              |
| Leu3p(N738D)| 415                     | 318              |
| Leu3p(N741Y)| 467                     | 183              |

* The host strain, XR157–3C, contained a total deletion of the LEU3 gene. It was first transformed with plasmid pYB1 that contained the reporter gene (see Footnote *) and then with plasmid pC62/H86T containing either no LEU3 gene, or wild type LEU3, or a mutant LEU3 encoding a protein with the indicated single mutation. All LEU3 genes were under the control of the ADC1 promoter.

* Specific activity of β-galactosidase in Miller units. The reporter gene was LEU2-lacZ (containing positions –1 to –645 of the LEU2 promoter, relative to the transcription start). High or low α-IPM levels were generated by supplementing the media with either 0.2 mM leucine or 2 mM leucine plus 1 mM each of isoleucine and valine. Assays were performed in duplicate and averaged. The error was <15% except for Leu3p(N741Y) where it was 23%.

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

Table II

| Plasmid-encoded Leu3 protein | Reporter gene activity |
|-----------------------------|------------------------|
| None                        | 2.6                    |
| Wild type                   | 20                     |
| Leu3p(604K)                 | 489                    |
| Leu3p(S607G)                | 255                    |
| Leu3p(L6111)                | 285                    |
| Leu3p(R643G)                | 414                    |
| Leu3p(Y654D)                | 393                    |
| Leu3p(K664E)                | 382                    |
| Leu3p(N738D)                | 374                    |
| Leu3p(N741Y)                | 145                    |

* The host strain, XR62–49, was deficient in LEU4 and LEU5 and therefore unable to produce α-IPM. It was transformed with a plasmid carrying the reporter gene (see legend to Table I), followed by pC62/H86T that contained either no insert, or the wild type LEU3 gene, or a mutant LEU3 encoding a protein with the indicated single mutation.

* Specific activity of β-galactosidase in Miller units. See legend to Table I for further details. Assays were performed in quadruplicate and averaged (error <20%). The numbers are the average of at least two independent experiments (error was <17% except for Leu3p(L6111) where it was 31%).

The host strain contained one genomic copy of LEU3 expressed under its own promoter. The Leu3 protein produced under these conditions and in the absence of α-IPM represses target gene expression (15) (see Table VII).

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**Fig. 1. Location of important functional domains and of mutations that suppress the Leu3-dd phenotype along the linear structure of Leu3p.** See text for a more exact description of the domain pattern. *The* W609R mutation was identified by default. The original suppressor carried four mutations (S486T, N495I, G603R, and W609R). When each of these was individually transferred to wild type Leu3p, it was found that the first three mutations had essentially no effect, i.e. the resulting proteins behaved like wild type, with modulation ratios between 8.3 and 10.0. On the basis of these results, the protein carrying the W609R mutation was expected to behave like the suppressor, *i.e.* to display strong, constitutive activation potential. However, Leu3p(W609R) was unstable, with reporter gene activities being identical to those observed in the total absence of Leu3p.

We wished to rule out the possibility that the loss of response to changes of the α-IPM concentration and the resulting constitutivity seen with the mutant proteins was caused by an altered, i.e. increased, affinity for α-IPM. We therefore repeated the experiment shown in Table I with host cells that were deficient in LEU4 and LEU5 and were thus unable to produce measurable amounts of α-IPM synthase and, consequently, α-IPM (21, 22). Table II shows that all eight Leu3p mutants were able to activate the reporter gene in the “no α-IPM” cells very much as they had in cells capable of producing α-IPM.

To bolster further the idea that the constitutive behavior caused by the single residue mutations was due to a quasi-permanent unmasking of the AD of Leu3p, we performed a series of modified two-hybrid experiments patterned after similar experiments done previously (10). In these experiments, the “bait” consisted of the DB-MR moiety of Leu3p where the MR was either wild type or one of two mutant forms identified above (L6111 and N738D). The “prey” consisted of the ADs of wild type Leu3p, Leu3-dd, or Cha4p (another Zn[II]2Cys6 cluster-type activator that regulates the utilization of serine and threonine, see below) fused to the AD of herpes simplex virus protein VP16. The latter was included to make interactions between an AD and the DB-MR moiety of Leu3p visible. If interaction occurred, it would be expected to occur at low levels but not at high levels of α-IPM (which would disrupt the interaction). An interacting AD would be masked but would at the same time recruit the VP16 AD to the promoter of the reporter gene LEU2-lacZ and cause it to be activated. The results are shown in Table III. The upper part of the table shows one negative (line 1) and two positive controls (lines 2 and 3). The simultaneous expression of the wild type DB-MR moiety and the wild type AD of Leu3p led to a weak but statistically significant interaction of the components at low, but not at high, α-IPM levels. (That interaction between these components would be weak was to be expected since their interaction was now diffusion controlled and no longer directed by the structure of the intact Leu3p molecule.) The interaction between wild type DB-MR and the AD of Leu3-dd (again seen only at low α-IPM levels, as predicted) was considerably stronger, consistent with the known properties of Leu3-dd (10). By contrast, the reporter gene activities seen when either DB-MR(L6111) (line 4) or DB-MR(N738D) (line 5) were co-expressed with wild type AD were indistinguishable from those seen in the negative control experiment, indicating that there was no measurable interaction. The same was true for the experiment in which wild type DB-MR was co-expressed with the AD of Cha4p (line 6; see also below).

It was shown previously that the DB-MR components used in the two-hybrid experiment are stably expressed (10). That these components, including the mutant ones, are present and functional in vivo is also evident from the fact that they repress reporter gene expression (see below for a functional definition of repression). The earlier paper also documented that the Leu3 wild type and Leu3-dd ADs were present in cell extracts (10).
Under identical conditions, the presence of the Cha4 AD was now likewise established (Fig. 2).

The above results are consistent with the idea that the single amino acid mutations shown in Table I are true "masking minus" mutations, i.e. mutations that make it virtually impossible for Leu3p to assume a conformation that silences the AD. In the absence of structural information, it is not possible to decide whether the corresponding wild type residues (Fig. 1) participate in the masking process in a direct or indirect way. We nevertheless favor the notion that at least some of those residues have a direct role in the masking process. Masking of the AD of Leu3p is known to be extremely sensitive to mutations within the AD itself (10), suggesting that there are structural elements within the AD that are important for masking. We think it is feasible that such elements are in close contact with a complementary structure in the MR of Leu3p and that this contact might be severely perturbed even by minor spatial changes such as the substitution of leucine for valine in position 611. An attractive possibility is that Leu-611, Trp-609, and Ser-607 are part of a short \( \beta \)-pleated sheet segment with an essential role in the interactions that lead to AD masking.

Attempts to find out what, if any, function could be assigned to the region between residues 173 and 604 of Leu3p were unsuccessful since proteins containing deletions of that region were unstable.

The ADs of Leu3p and Cha4p Are Interchangeable but Cannot be Cross-masked—Evidence provided in this paper and in a previous report (10) shows that the modulation function of Leu3p, i.e. its response to \( \alpha \)-IPM, is very sensitive to point mutations located either in the AD or within a subregion of the MR (residues 604–741) of Leu3p. The MR mutations (all of them suppressors of the Leu3-dd phenotype) identify amino acids that appear to be involved, directly or indirectly, in AD masking interactions (see above). The question arose as to whether the subregion defined by these amino acids was specific for the AD of Leu3p or whether it might be capable of masking other ADs of the same class as well. To answer this question, we performed domain swap experiments between Leu3p and Cha4p. The latter is a yeast regulatory protein that activates genes involved in the utilization of serine and threonine as nitrogen sources (23). Leu3p and Cha4p show a similar pattern of organization along their primary structures. Their N-terminal regions conform to the Zn(II)2Cys6 binuclear cluster that is typical for this group of lower eukaryotic transactivators and constitutes the core of the DNA binding domain (DB). The core DB of Leu3p is located between residues 36 and 68 and that of Cha4p between residues 43 and 71. A peptide consisting of residues 17–147 of Leu3p has strong affinity for US15 (7). Similarly, a Cha4 peptide containing residues 1–174 binds specifically to US15 (23). The ADs of both activators are located near the C terminus (9). Inspection of the C-terminal 30 residues shows that both ADs are acidic with a net charge of \(-4\) (Leu3p) or \(-5\) (Cha4p). Hydrophobic amino acids constitute \(57\%\) of the Leu3p AD and \(40\%\) of the Cha4 AD. There is no sequence homology within these regions except for a 6-residue stretch close to the C termini (872–879 of Leu3p) versus 633–639 of Cha4p). The significance of this homology is not known. It should be remembered, however, that mutating Asp-872 and Asp-874 of Leu3p causes its AD to be permanently masked (10).

Table IV shows the activation and modulation properties of several Leu3p-Cha4p chimeras. In these experiments, the reporter gene was LEU2-lacZ controlled by the natural LEU2 promoter when the Leu3p DB was present; when the Cha4 DB was present, the reporter gene was CHA1-lacZ controlled by the natural CHA1 promoter. Full-length Leu3p (designated L-L) showed a 7- to 8-fold increase in activation potential when the intracellular concentration of \( \alpha \)-IPM was raised from "low" to "high." There was no significant response to increasing the serine level. Full-lengthCha4p (designated CCC) responded only to changes in the serine level. The larger modulation ratio obtained with CCC is at least in part due to the fact that the CHA1 promoter is tighter than the LEU2 promoter, i.e. is essentially silent when serine and threonine are absent from the growth medium. When the AD of Leu3p was fused directly to the DB of Leu3p, the resulting construct (L-L) acted as a strong, constitutive activator. Fusing the AD of Cha4p to the DB of Leu3p (L-C) also caused constitutivity. The apparent difference in activation potential between L-L and L-C is likely due to protein stability differences; the observation that both LLL and LCL show similarly strong activation potential indicates that the ADs of Leu3p and Cha4p are functionally equivalent. Both LLL and LCL are also constitutive activators, i.e. they are permanently unmasked, and their response to changes of the \( \alpha \)-IPM or serine levels is negligible. Specific
modulation returns when both the AD and the MR are from the same protein. Thus, LCC is modulated by serine, but not by α-IPM, and CLL is modulated by α-IPM, but not by serine. That the modulation ratios seen with LCC and CLL differ from those of the parent molecules CCC and LLL probably has to do with differences between the CHA1 and LEU2 promoters and also with overall structural differences caused by the fusion of “foreign” domains.

To avoid the complications caused by two different promoters and DBs, we constructed additional chimeras that uniformly contained the full-length LexA protein as the DNA binding moiety. The reporter gene for these chimeras consisted of a LexA operator fused to the lacZ gene. Table V shows the modulation behavior of constructs that contain the LexA sequence followed either by full-length Leu3p or by Leu3p molecules with N-terminal truncations. All constructs exhibit a strong modulation response. The variation in reporter gene activity, especially at high α-IPM levels, is probably due to differing degrees of instability of the constructs. Although all constructs were active in vivo, they could not be detected in electrophoretic mobility shift assays (data not shown).

Table V further shows that modulation by α-IPM essentially disappears or becomes very weak when the AD of Leu3p (residues 857–886) is replaced with the AD of Cha4p (residues 619–648). Maximal expression of the reporter gene by these fusions was very similar to that seen with the wild-type full-length Leu3p AD.

The results of Tables IV and V show that the DNA binding domains of Leu3p or Cha4p are not required for modulation.

In yet another approach to the specificity question, we utilized the AD-expressing plasmids that were originally constructed for the modified two-hybrid experiments (see Table III) to ask whether a given AD would compete with the masking function of full-length Leu3p. To this end, we overexpressed different ADs in a strain that contained one copy of the LEU3 gene. We expected those ADs that interact with Leu3p to compete with the internal masking process of Leu3p and therefore to diminish the modulation by α-IPM; the stronger the interaction between Leu3p and an AD, the more constitutive Leu3p should become. This was indeed found to be the case. Table VI shows that the presence of excess VP16 AD or Cha4p AD is without effect on the modulation of full-length wild type Leu3p. The presence of excess Leu3p AD has a small but statistically significant effect on modulation. The presence of excess Leu3-dd AD causes full-length Leu3p to be almost permanently inactive. The presence of excess VP16 AD or Cha4 AD shows that the presence of excess VP16 AD or Cha4 AD has no effect on the modulation of full-length wild type Leu3p. The presence of excess Leu3-dd AD causes full-length Leu3p to be almost permanently active. All of the AD constructs were stably expressed (10) (Fig. 2). Apparently, the intracellular concentration of the ADs was not high enough to cause squelching.

A lack of interaction between the AD of Cha4p and the

### Table IV

| Regulator | Reporter gene activity | Modulation ratio |
|-----------|------------------------|------------------|
|           | α-IPM level | Serine level | α-IPM level | Serine level |
| LLL       | 193        | 26         | 32        | 26         | 7.4 | 1.2 |
| CCC       | 0.6        | 0.4        | 81        | 0.4        | 1.5 | 202 |
| L-L       | 237        | 278        | 178       | 278        | 0.9 | 0.6 |
| L-C       | 45         | 53         | 47        | 53         | 0.9 | 0.9 |
| LLC       | 478        | 388        | 327       | 388        | 1.2 | 0.8 |
| LCL       | 450        | 493        | 405       | 493        | 0.9 | 0.8 |
| LCC       | 6.2        | 5.0        | 349       | 5.0        | 1.2 | 70  |
| CLL       | 61         | 0.5        | 0.7       | 0.5        | 121 | 1.4 |

* The host strain (TG494) contained total deletions of both LEU3 and CHA4. Cells were transformed first with either pYB1 or pTK120 carrying the desired reporter genes (see below) and subsequently with pPC62-H/86T carrying the indicated regulator constructs. The composition of the regulators is given in form of a tripartite code. LLL and CCC, wild type Leu3p and Cha4p, respectively, where the 1st letter symbolizes the extended DB of the activators (residues 1–173 and 1–174, respectively), the 2nd letter symbolizes their MR (residues 174–854 and 176–618, respectively), and the 3rd letter symbolizes their AD (residues 855–886 and 619–648, respectively); L-L, DB of Leu3p fused to AD of Cha4p; L-C, DB of Leu3p fused to AD of Cha4p; LCL, DB of Leu3p fused to AD of Cha4p; LCC, DB of Leu3p fused to MR plus AD of Cha4p; LCL, DB of Leu3p fused to MR plus AD of Cha4p.

### Table V

| Construct | Reporter gene activity | Modulation ratio |
|-----------|------------------------|------------------|
|           | α-IPM level | Serine level | α-IPM level | Serine level |
| Control   | 0.3         | 0.6         | 0.5         |             |
| A (LexA)  | 1.1         | 2.2         | 0.5         |             |
| AL1–886   | 267         | 7.6         | 35.1        |             |
| AL100–886 | 1280        | 7.5         | 165         |             |
| AL172–886 | 785         | 5.2         | 151         |             |
| AL172–886 | 1467        | 9.1         | 161         |             |
| AL172–886 | 1302        | 441         | 2.9         |             |
| AL172–886 | 887         | 695         | 1.3         |             |
| AL172–886 | 1098        | 776         | 1.4         |             |
| AL172–886 | 1131        | 834         | 1.4         |             |

* The host strain (TG494) contained total deletions of LEU3 and CHA4. Cells were first transformed with reporter plasmid p1155 and then with pPC62-H/86T carrying either no insert (control) or DNA encoding the indicated constructs. All constructs contain the full-length LexA protein, designated by the letter A. The letter L designates the portion of this construct that is derived from Leu3p (subscript numbers indicate amino acid residues; note that full-length Leu3p has 886 residues). The letter C designates the transcriptional activation domain of Cha4p (residues 619–648).
formed successively with plasmid pYB1 carrying the reporter gene and isoleucine.

We wanted to ascertain that these differences were not limited to the "no-promoter) had a reporter gene activity of 20. We have on several occasions observed that overex-pression of the gene also causes stronger expression of the target gene expression in response to heme (26). A very similar example is Met4p, a principal regulator of the sulfur network in S. cerevisiae. It contains an “inhibitory region,” about 45 residues in length, that not only inhibits its own AD (in response to high intracellular concentrations of S-adenosylmethionine) but also that of Gal4p when the latter replaces the native AD in a fusion protein (24, 25). The inhibitory region is also required for the formation of a complex between Met4p and Met30p, a pleiotropic repressor. Although the mechanism of inhibition is not understood, it is possible that Met30p acts by shielding the Met4 AD rather than by specifically interacting with it. Another example for cross-masking is Hap1p, an activator of yeast genes encoding cytochromes. Hap1p contains seven heme reg-ulatory domains (HRMs), one of which (HRM7) regulates the AD of Hap1p itself in a way that results in the stimulation of target gene expression in response to heme (26). A very similar response is seen when the AD of Hap1p is replaced by the major AD of Gal4p, implying that there are no specific interactions between the AD of Hap1p and the region containing HRM7. A mechanism was proposed according to which an as yet unidentified repressor would bind to the HRM7 region and would nonspecifically shield a nearby AD until dislodged by heme.

**Overproduction of Leu3p Results in Stronger Activation Potential**—We have on several occasions observed that overexpression of the LEU3 gene also causes stronger expression of Leu3p-controlled reporter genes. Since the strains in question usually had different genetic backgrounds in addition to the difference in LEU3 expression, interpretation of the results was difficult. One exception was the experiment shown in Table II. The 1st two lines of Table II represented reporter gene activity in cells that differed only with respect to the amount of Leu3p synthesized. Cells that possessed only their single genomic copy of LEU3 (under its own promoter) had a reporter gene activity of 2.6; cells that contained in addition a LEU3 gene on a single copy plasmid (under the control of the ADC1 promoter) had a reporter gene activity of 20. We wanted to ascertain that these differences were not limited to the “no-α-

### Table VI

**TABLE VI**

| Competing AD* | Reporter gene activityb | Modulation ratiob | Relative competitionb |
|---------------|------------------------|-------------------|-----------------------|
| None          | 170                    | 2.5               | 68                    | 1.0                   |
| VP16          | 229                    | 3.6               | 64                    | 1.1                   |
| Cha4p         | 227                    | 2.9               | 78                    | 0.9                   |
| Leu3p         | 212                    | 7.1               | 30                    | 2.3                   |
| Leu3-dd       | 377                    | 279               | 1.4                   | 49                    |

* Host strain XK122–6B (wild type with regard to LEU3) was transformed successively with plasmid pYB1 carrying the reporter gene and with plasmid pPC62-H/86T carrying either no insert or an insert encoding the indicated AD constructs (see “Materials and Methods”). High and low α-IPM levels were generated by either not supplementing the growth media or adding 2 mM leucine and 1 mM each of valine and isoleucine.

a Shown as specific activity of β-galactosidase in Miller units. See legend of Table I for details. Assays were performed in quadruplicate (error <9%). The data shown are the average of three independent experiments (error <29%).

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

c Reciprocal of the modulation ratio, normalized with respect to the no-AD experiment.

### Table VII

**TABLE VII**

| Leu3p level* | Reporter gene activityb |
|---------------|------------------------|
| High α-IPM    | 240                    |
| Low α-IPM     | 7.8                    |
| Overexpressed | 416                    |

* Strain XK41–7 (wild type with regard to LEU3) was used for these experiments. Cells were first transformed with reporter plasmid pYB1. For “normal” levels of Leu3p, cells were further transformed with pPC62-H/86T without insert, so that the only source for Leu3p was the genomic copy of LEU3 under the control of its own promoter. For “overexpressed” levels, cells were transformed with pPC62-H/86T containing an additional LEU3 gene under the control of the ADC1 promoter.

### Figure 3

**Electrophoretic mobility shift assays with extract from cells producing Leu3p at different levels.** Electrophoretic mobility shift assays were performed as described (10) using a UAS5-containing 30-mer. The protein concentration in the extracts was measured using a Coomassie Blue-based assay kit from Bio-Rad. The amount of extract used in each reaction was then adjusted to ensure that equal amounts of total protein were present. Control, no extract; normal level Leu3p, extract from cells expressing Leu3p from the genomic LEU3 gene; overexpressed Leu3p, extract from cells expressing Leu3p from both genomic and plasmid-borne LEU3 genes; L, low α-IPM level; H, high α-IPM level. See legend to Table I for conditions to generate different levels of α-IPM.

IV” condition (cells deficient in LEU4 and LEU5) of the experiment described in Table II. We therefore performed additional experiments with two isogenic strains that were wild type with respect to the branched chain amino acid-related genes and again contained either just the native genomic copy of LEU3 or that copy plus an additional, plasmid-borne copy under the control of the ADC1 promoter (Table VII). We again observed the significant differences in Leu3p activation potential at the two Leu3p levels, reflected by a 3.5-fold difference of reporter gene activity under “low α-IPM” conditions and a 1.7-fold difference under “high α-IPM” conditions. Expression of the reporter gene at low α-IPM is repressed but only when the Leu3p levels are “normal” (low).

Before discussing how increased Leu3p production can lead to increased reporter gene expression, it might be instructive to review briefly what is known about reporter gene expression in
cells containing one genomic copy of LEU3 (or mutants thereof). With LEU2-lacZ as the standard reporter gene, four levels of expression can be distinguished (see Ref. 15 and this paper) as follows. (i) A basal level, typically around 12 Miller units, is seen in the absence of Leu3p, e.g. in cells with a total deletion of LEU3. (ii) A repressed level, 2–5-fold lower than the basal level, is seen either with truncated Leu3 molecules that can still bind to UASL, but don’t have an AD or in cells elaborating normal Leu3p but producing no α-IPM or very low levels of α-IPM. Note that repression, as defined here, is an event separate from masking. “Masking-unmasking” describes an equilibrium between inactive and active conformations of Leu3p that is influenced by α-IPM. “Repression” refers to a negative effect by Leu3p on transcription that requires neither the MR nor the AD of Leu3p (20). It may involve a separate negative factor (27). (iii) An active level, 15–20 times above basal level, is observed with fully functional Leu3p in the presence of increased (high) levels of α-IPM. (iv) A hyperactive level, about twice the active level, is observed when cells generate and accumulate large quantities of α-IPM.

Overproduction of Leu3p at low α-IPM concentrations leads to yet another level of reporter gene expression that we call slightly active (about twice the basal level or 4–10 times the repressed level), whereas at high α-IPM concentrations overproduction of Leu3p changes reporter gene expression from active to hyperactive. The first of these observations in particular would appear to rule out the possibility that increased LEU2-lacZ expression is simply due to increased saturation of the reporter gene promoter with Leu3p. If anything, increased saturation of the promoter should lead to continued repression rather than to activation, an expectation reinforced by the observation that a Leu3 protein lacking only the AD will repress reporter gene expression even when present in high levels of α-IPM (32). The emerging picture is one of a large network of metabolic control in which Gcn4p substantially extends its reach by virtue of up-regulating the expression of LEU3. It is also a network that is capable of a high degree of fine-tuning, not only through the general control system but also in response to changes in the levels of α-IPM and Leu3p. That α-IPM is an important link in this network would also explain the unexpectedly complex control of α-IPM synthase (33, 34) and the presence in yeast of at least three forms of α-IPM synthase (35, 36).

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