Yeast regulatory protein *LEU3*: a structure – function analysis

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ABSTRACT

Eleven mutations resulting in partially deleted or truncated *LEU3* protein were generated by linker insertion or other modifications at restriction sites, deletion of restriction fragments, or oligonucleotide-directed mutagenesis. Functional studies of these mutants showed the following: (i) A specific DNA binding region is contained within the 173 N-terminal residues, but other regions of the protein are required for optimal binding. (ii) Activation of *LEU2* expression depends on the C-terminal 113 residues of the *LEU3* protein. (iii) Deletion of part or all of a central section of *LEU3* eliminates the ability of the *LEU3* protein to respond to the co-activator α -isopropylmalate, i.e. creates an unmodulated activator. (iv) Overproduction of unmodulated activator slows down cell growth. (v) Specific deletion of two short acidic regions, including one with net charge -19 , has only minor effects on activation and modulation.

INTRODUCTION

In 1983, a yeast gene was identified which, when mutated, led to a much reduced expression of *LEU1* and *LEU2*, structural genes coding for isopropylmalate (IPM) dehydratase and β -IPM dehydrogenase (1). The gene was designated *LEU3*. At the same time, α -IPM, the product of the first leucine pathway-specific reaction catalyzed by α -IPM synthase, was recognized as a second obligatory component for the activation of *LEU1* and *LEU2* (1). Subsequently, the *LEU3* gene was cloned, sequenced, and shown to encode a protein of 886 amino acid residues in length (2–4). It was shown that *LEU3* protein was able to interact specifically with a dyad-symmetrical, upstream activating sequence (UAS_L) present in the 5'-flanking regions of *LEU1*, *LEU2*, *LEU4*, and *ILV2* (5, and P. R. G. Brisco and G. B. Kohlhaw, submitted for publication). The UAS_L was first discovered upstream of the *LEU2* gene where it has the sequence (–197) GCCGGAACCGGC (–186) (6). Recent experiments have shown that α -IPM, while needed for activation of *LEU2* expression, is *not* required for the binding of *LEU3* to its target DNA (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication; also, Yeast Genetics and Molecular Biology, Abstracts of 1989 Meeting, p. 140B).

Based on our present knowledge, the *LEU3* protein can be predicted to have the following functions: (i) a specific DNA binding function; (ii) an activation function, broadly defined as

a region of the protein capable of interacting with the RNA polymerase II-dependent transcription machinery; (iii) a modulation function, allowing direct or indirect recognition of the presence of α -IPM and possibly initiating an allosteric transition within the *LEU3* protein; (iv) a nuclear entry function; and (v) region(s) involved in oligomerization (dimerization ?) of the *LEU3* protein. The present analysis makes use of assays developed for the first three of the above functions, delimits regions involved in specific DNA binding and activation of *LEU2* expression, and shows that shortened *LEU3* proteins can be generated which, while being good activators, have lost the ability to be modulated by changes in the intracellular α -IPM production.

MATERIALS AND METHODS

Plasmids, Strains, and Special Materials

Most of the *LEU3* deletions described here were expressed behind the inducible yeast *PHO5* promoter. Construction of the parent plasmids was as follows. First, a multi-purpose plasmid, pKZ1, was constructed by opening plasmid pYE7, a 6.55 kb vector containing the *PHO5* promoter, 2 μ m DNA, the yeast *TRP1* marker, a yeast origin of replication, and bacterial sequences including the Ap^r marker (7), at the unique *EcoRI* site and inserting a polylinker region containing the T7 promoter from plasmid pIBI76. Plasmid pYE7 was kindly provided by R. A. Kramer, Hoffmann-LaRoche, Nutley, NJ; pIBI76 was purchased from International Biotechnologies, Inc. Plasmid pKZ1 was digested with restriction endonucleases *SacI* and *XhoI*, and the resulting long fragment was ligated to a fragment derived from pGB4 that contained the *LEU3* region between a *BspMI* site at position –45 and a *SnaBI* site at position +3060 (3). The resulting construct was designated pKZ3. A related plasmid, designated pKZ5, was generated from pKZ3 by removing two undesired unique restriction sites, *BamHI* and *SphI*, through cutting, blunt-ending, and religation. A map of pKZ5 is shown in Fig. 1A. The structure of pKZ5 was confirmed by restriction enzyme analysis and DNA sequencing at the polylinker-*LEU3* junction. Both pKZ3 and pKZ5 were able to complement yeast strain XK157-3C to leucine prototrophy. Strain XK157-3C (kindly provided by P. R. G. Brisco of this laboratory) contains a total deletion of *LEU3* as well as *trp1* and *ura3* mutations (genotype: *MAT α leu3- Δ 2::HIS3 trp1-289 ura3-52*).

A different parent plasmid was constructed for the purpose of creating site-directed mutations (deletions Δ 10, Δ 13, and Δ 14, see Fig. 2B). A map of this plasmid, designated pYB5, is shown

Figure 1. Maps of plasmids pKZ5 (A) and pYB5 (B). Blocks represent yeast DNA sequences, with the stippled part showing the *LEU3* coding region; thin lines represent bacterial sequences. The plasmids are not drawn to scale.

in Fig. 1B. The particular elements of this plasmid were derived from plasmids pGB9 (yielding the *LEU3* region between positions -561 and approximately +4700, ref. 3), pSEYC102 (kindly supplied by S. Emr, CalTech, and yielding *CEN4*, *ARS1* as well as bacterial sequences), YRp7 (kindly supplied by H. Zalkin of this department and yielding the *TRP1* marker, ref. 8), and pUC18 (kindly provided by H. Zalkin and yielding the polylinker region) by cutting with appropriate restriction enzymes and religating. The structure of pYB5 was confirmed by restriction enzyme analysis; this plasmid also complemented strain XK157-3C. Plasmid pYB6 is identical to pYB5 except that the *LEU3* region is present in the opposite orientation.

The tester plasmid used to measure the degree of activation of the *LEU2* gene was pYB1. This plasmid contained a *LEU2-lacZ* fusion (at the *EcoRI* site of *LEU2*), *CEN4* *ARS1* sequences, the *URA3* marker, and bacterial sequences containing the *Ap^r* gene and a bacterial origin of replication. The construction and detailed features of pYB1 have been described elsewhere (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication).

The *E. coli* strains used were MC1000 (9), and JM101 and 103 (10). Cloning vectors M13mp18 and M13mp19 and universal single-stranded M13 primer were obtained from P-L Biochemicals. Oligonucleotides were synthesized, purified, and end-labeled as described elsewhere (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication). All other biochemicals were purchased from various national suppliers. [γ - 32 P]ATP (5000 Ci/mmol) and [α - 35 S]dATP (1000 Ci/mmol) were from Amersham Corp.

Growth Conditions

Cells were grown aerobically either at 30°C (yeast) or at 37°C (*E. coli*). Yeast were routinely grown in SD minimal medium (11) and were harvested in late log phase at an OD_{600} of about 1.0. When the *PHO5* promoter was to be activated, cells were pregrown in SD minimal medium (supplemented with 2 mM leucine and 1 mM isoleucine when the *LEU3* protein was activation-deficient), harvested, resuspended in YEPD medium (11) that had been depleted of inorganic phosphate by precipitating the phosphate as $MgNH_4PO_4$ (12), and grown for 2–3 additional generations until the OD_{600} was 1.4–1.6. *E. coli*

cells were grown in L-broth and Vogel-Bonner minimal medium (13). Ampicillin was added to a final concentration of 50 μ g/ml.

Nucleotide Sequencing

Sequencing was performed by the dideoxy chain termination method (14) both by shuttling DNA into M13 vectors (10) and by directly using alkali-denatured, double-stranded DNA (Promega Catalog and Applications Guide 1988/89).

Site-directed Mutagenesis

The two alternative methods used were the thionucleotide-restriction site protection method offered as 'Oligonucleotide-directed *in vitro* mutagenesis system version 2' by Amersham Corp. and the gapped duplex method offered as 'Site-directed mutagenesis Kit' by Boehringer-Mannheim. To delete positions 298–351 of *LEU3* (corresponding to amino acid residues 100–117), the following 63-mer was used: 5'-CCTGAG-TTGTTTTACTTTTTCTTTTGTGAAATTACTGTTTAAATTTGTCAAAGTTCTGGTGAG-3'. To delete positions 2032–2091 (corresponding to amino acids 678–697), the following 63-mer was used: 5'-GCTATCCATATTTTCTGGA ACTTTACTACTTAGAACATTGTAAGCTGT-AGAAACCTTGGTCTC-3'.

Electrophoretic Mobility Shift Assays

With the exception of strains containing the pYB5 and $\Delta 10$ plasmids, cells were grown in low phosphate medium as described above; pYB5 and $\Delta 10$ cells were grown in minimal medium as described above. Cells were harvested at the end of log phase, washed, frozen, and disrupted by French pressure cell treatment in 25 mM HEPES buffer, pH 7.5, containing 5 mM $MgCl_2$, 200 mM KCl, 0.05 mM $ZnCl_2$, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 1 mM phenylmethanesulfonyl-fluoride, and 2 μ M pepstatin A. Two to four μ l cellfree extract were incubated for 15 min at 30°C with 0.5 to 1.0 ng of 32 P 5'-end-labeled UAS_{L2}-30 (5'-AGGT-GAGAGGCCGGAACCGGCTTTTCATAT-3') in HEPES buffer, pH 7.5, containing 80 mM KCl, 1mM EDTA, 5% (v/v) glycerol, 4 mM dithiothreitol, and 0.1 mg of poly(dI-dC) per ml. When desired, the binding reaction was supplemented with cold UAS_{L2}-30 ('competitive UAS_{L2}-30') or cold UAS_{L2}-24 (5'-AGGTGAGAGGCCGCGCTTTTCATAT-3', 'noncompetitive UAS_{L2}-30'). Gel electrophoresis was performed as described elsewhere (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication).

Other Methods

Yeast and bacterial transformations, plasmid DNA isolation, restriction enzyme digestions, Klenow enzyme treatment, DNA ligations, and β -galactosidase assays were performed as described previously (2,3).

RESULTS AND DISCUSSION

Outline of Experiments

Mutations in *LEU3* resulting in partially deleted or truncated *LEU3* protein were generated by linker insertion or other modifications at restriction sites, deletion of restriction fragments, or oligonucleotide-directed mutagenesis. Eleven such mutations are listed in Fig. 2. Extracts from strains carrying these mutations were analyzed for three functions of the *LEU3* protein: DNA binding capability; ability to activate *LEU2* expression ('activation

Figure 2. Deletion analysis of *LEU3* protein. *Part A:* Computer-generated hydropobicity plot using the scale of Kyte and Doolittle (25) with a window size of 11 residues. *Part B:* List of partially deleted or truncated *LEU3* proteins and qualitative description of their behavior. The top (open) bar shows the location of a cysteine-rich region ('finger motif') and of four acidic regions, 16–20 residues in length, that carry net charges of –4 to –19. Also shown are the points where, on the *LEU3* DNA, the sites for *SalI*, *EcoRV*, *NdeI*, and *AvrII* restriction endonucleases would be located. With the exception of mutations designated Δ10, Δ13, and Δ14, all other mutations were constructed by making use of these four restriction sites. To allow greater flexibility, *BamHI* linkers were introduced at *SalI*, *EcoRV*, and *NdeI*. Individual mutations were obtained as follows: Δ3 was generated from Δ9 (see below); it is a frameshift mutation, with the Δ3 protein containing 172 N-terminal amino acid residues of *LEU3* plus 22 additional residues (RDPDPKIFITSGQGSLINLLH). Δ9 (a pKZ5 derivative) was constructed as a frameshift mutation by introducing a *BamHI* linker (10-mer) at the *EcoRV* site of *LEU3*. Δ9 protein contains 463 N-terminal residues of *LEU3* plus the same 22 additional residues as those found in Δ3. (The Δ3 deletion was generated from Δ9 by replacing the *XhoI-BamHI* fragment of Δ9 [10-mer *BamHI* linker insertion at *EcoRV* of *LEU3*] with another *XhoI-BamHI* fragment [8-mer *BamHI* linker insertion at *SalI* of *LEU3*].) V¹1 (a pKZ5 derivative) was constructed as a frameshift mutation by introducing *BamHI* linkers (10-mers) at the *NdeI* site of *LEU3* after treatment with Klenow enzyme. Sequencing showed that two linkers were present in tandem. Δ11 protein contains 608 N-terminal residues of *LEU3* plus 12 additional residues (RDPAGSRMEFIC). Δ8 (a pKZ3 derivative) was constructed as a frameshift mutation by cutting at the *AvrII* site of *LEU3*, treatment with Klenow enzyme, and religation. Δ8 protein contains 773 N-terminal residues of *LEU3* plus one additional residue (S). Δ4 was constructed from Δ11 by replacing the *XhoI-BamHI* fragment of Δ11 (*BamHI* linker insertion at *NdeI* of *LEU3*) with another *XhoI-BamHI* fragment (*BamHI* linker insertion at *SalI* of *LEU3*). Δ4 protein contains 172 N-terminal residues of *LEU3* followed by four additional residues (RDPV), and continues in-frame with W-609 of *LEU3*. Δ5 was constructed exactly like Δ4 except that the replacement *XhoI-BamHI* fragment was from a *BamHI* linker insertion at *EcoRV* of *LEU3*. Δ5 protein contains 463 N-terminal residues of *LEU3* followed by RDPV, and continues in-frame with W-609 of *LEU3*. Δ7 was constructed by cutting Δ11 at *BamHI* and *AvrII*, treatment with Klenow enzyme to generate flush ends, and religation. Δ7 protein contains 608 N-terminal residues of *LEU3* followed by three additional residues (RDP), and continues in-frame with R-774 of *LEU3*. Δ12 was constructed by cutting pKZ5 at *SalI* and *AvrII*, treatment with Klenow enzyme, and religation. Δ12 protein contains 173 N-terminal residues of *LEU3* attached directly to the C-terminal section at R-774. Δ10, Δ13, and Δ14 were constructed by site-directed mutagenesis from pYB5 or pYB6 plasmids (see MATERIALS AND METHODS). The Δ10 protein lacks amino acid residues 678 through 697, Δ13 lacks residues 100 through 117, and Δ14 contains both deletions. pKZ6 was constructed by replacing the short *SalI-KpnI* fragment of pKZ5 with a corresponding piece from pGB11 in which residue D-463 of *LEU3* is connected to Δ-9 of *lacZ* via a 3-residue bridge of GDP (3). With the exception of pKZ6 whose *LEU3-lacZ* junction had been sequenced earlier (3), all constructs were verified by DNA sequencing at the new junctions. The statements regarding expression of *LEU2-lacZ*, regulation of *LEU2*, and binding to UAS_L are qualitative. See Table 1 and Fig. 3 for further information. N.D. = not determined.

Figure 3. Electrophoretic mobility shift assays. The assays were performed as described in MATERIALS AND METHODS. Note that, with the exception of pYB5 and $\Delta 10$, *LEU3* was under the control of the *PHO5* promoter. The different panels show separate experiments. Designation of mutations is as in Fig. 2. **A**, lane 1: free UAS_{L2}-30; lane 2: pKZ5 (wild type *LEU3*)—both shifted bands and band at origin represent specific complexes, see panel **B**; lane 3: $\Delta 9$ —band remaining at origin represents specific complex (competition experiment not shown); lane 4: $\Delta 11$ —band remaining at origin represents specific complex (competition experiment not shown); lane 5: $\Delta 8$ —pattern similar to wild type, but shifted to different positions, possibly reflecting smaller protein size; lane 6: $\Delta 5$ —band remaining at origin represents specific complex (competition experiment not shown); lane 7: $\Delta 7$ —only one shifted complex visible in addition to material remaining at origin; lane 8: $\Delta 12$ —pattern similar to wild type, but shifted to different positions, possibly reflecting much smaller protein size. **B**, lanes 1, 2, and 3: pKZ5 (wild type *LEU3*)—no addition, addition of noncompetitive UAS_{L2}-24 (see MATERIALS AND METHODS), and addition of competitive UAS_{L2}-30, respectively; lanes 4, 5, and 6: $\Delta 4$ —no addition, addition of noncompetitive UAS_{L2}-24, and addition of competitive UAS_{L2}-30, respectively; lane 7: pYB5 (wild type *LEU3*)—pattern is similar, but relatively weaker, than that seen with pKZ5; lane 8: $\Delta 10$. **C**, lane 1: pKZ5 (wild type *LEU3*); lane 2: $\Delta 3$. The band with the fastest mobility represents nonspecific binding since it also appears when extract from a strain with a total *LEU3* deletion is used (not shown). **D**, lane 1: free UAS_{L2}-30; lane 2: UAS_{L2}-30 plus purified β -galactosidase; lanes 3, 4, and 5: pKZ6—no addition, addition of noncompetitive UAS_{L2}-24, and addition of competitive UAS_{L2}-30, respectively; lane 6: pKZ5 (wild type *LEU3*).

function'); and ability to respond to changes in the intracellular concentration of α -IPM ('modulation function'). The DNA binding function was monitored by an electrophoretic mobility shift assay. The DNA fragment serving as a target for the *LEU3* protein consisted of 30 bp of the 5'-flanking region of *LEU2* (positions -206 to -177). This fragment, designated UAS_{L2}-30, contains the sequence (-197) GCCGGAACCGGC (-186) which is known to interact with *LEU3* protein (5, and P. R. G. Brisco and G. B. Kohlhaw, submitted for publication) and to be required for high-level expression and regulation of *LEU2* (6). A control fragment, designated UAS_{L2}-24, covers the same region except for 6 bp previously recognized as essential for *LEU2* expression (6) and *LEU3* protein binding (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication). The activation function was examined by measuring the expression of a *LEU2-lacZ* translational fusion present on a centromere-containing yeast-*E. coli* shuttle vector (pYB1). In order to achieve greater flexibility in expressing weakly activating or unstable *leu3* mutants, most of the mutants described here (all except $\Delta 10$, $\Delta 13$, and $\Delta 14$, see Fig. 2) were placed behind the *PHO5* promoter which can be stimulated by lowering the inorganic phosphate concentration (15). The modulation function was measured in the same *LEU2-lacZ* system by comparing β -galactosidase activities in extracts from yeast cells grown in the absence or in the presence of excess leucine plus isoleucine. It has recently been demonstrated that *LEU2* expression is severely

repressed when cells are grown in the presence of 2 mM leucine plus 1 mM isoleucine and that this 'repression' is likely to be the consequence of diminished α -IPM production (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication).

The DNA Binding Domain of the *LEU3* Protein is Located within the N-terminal 173 Amino Acid Residues, but Normal, Wild Type-like Interaction with Target DNA Requires Additional Region(s) of the Protein

Electrophoretic mobility shift assays showed that interaction of wild type *LEU3* protein with target DNA (UAS_{L2}-30) resulted in the formation of two *LEU3*-specific shifted bands and, in most experiments, an additional band that remained at the origin (Fig. 3). There was competition against all three bands when excess of unlabeled target DNA (UAS_{L2}-30) was present in the reaction mixture, but not when a control fragment (UAS_{L2}-24) was present (Fig. 3B, lanes 1, 3, and 2). This suggests that the immobile band consists of larger complexes which, while their composition is unknown, still reflect specific *LEU3* protein-target DNA interaction. Of those *LEU3* mutants whose DNA binding capability was determined, only $\Delta 8$, $\Delta 12$, and $\Delta 10$ showed a pattern similar to that of wild type, with respect to the presence of two clearly identifiable shifted bands. All three of these mutant proteins contained the N-terminal 173 residues of the native protein. In addition, they contained either an intact middle part, defined as the region between residue 173 and acidic region II

Table 1. Expression of a *LEU2-lacZ* fusion in *LEU3* mutants)

<i>LEU3</i> -carrying plasmid ^{b)}	Specific activity of β -galactosidase in extracts of cells grown in:		
	Minimal medium 'nonrepressing' ^{c)}	Minimal medium 'repressing' ^{c)}	Low phosphate medium ^{d)}
pKZ5-1 (WT)	566	68	443
Δ 3-27	18	16	13
Δ 9-3	12	12	16
Δ 11-3	15	16	18
Δ 8-1	2	4	5
Δ 4-3	55	65	900
Δ 5-2	27	25	56
Δ 7-3	202	214	603
Δ 12-3	700	701	2429
None	14	15	23
pYB5 (WT)	242	29	
Δ 10-1	187	24	
Δ 13-1	152	36	
Δ 14-1	387	81	
None	9	10	

a) The *LEU3* mutants are the ones listed and described in Fig. 2. β -Galactosidase activity was determined in cell-free extracts prepared by suspending 1 g of cells (wet weight) in 1.5 ml of breaking buffer and twice passing the suspension through a French pressure cell, followed by centrifugation at 35,000 $\times$ g for 20 min. Breaking buffer was Z-buffer (24) supplemented with 1mM phenylmethanesulfonyl-fluoride, 6 mg/ml benzamidine HCl and 0.1% Triton X-100. The values listed are averages of at least duplicate assays, with the experimental error not exceeding 15%.

b) All plasmids were introduced into recipient strain XK157-3C (carrying a total deletion of *LEU3*, see MATERIALS AND METHODS) which already contained the *LEU2-lacZ* tester plasmid pYB1. Plasmids listed in the upper part of the Table (pKZ5 derivatives) are multicopy, i. e. contain 2 μ m sequences, and contain the inducible *PHO5* promoter in front of *LEU3*. Numbers following the hyphen refer to the particular isolate studied. Plasmids listed in the lower part of the Table (pYB5 derivatives) contain *CEN4-ARS1* sequences, i. e. are, on the average, single-copy, and express the *LEU3* gene behind its own promoter.

c) 'Nonrepressing' minimal medium contains 0.2 mM leucine (upper part) or no leucine (lower part); 'repressing' minimal medium contains 2 mM leucine plus 1 mM isoleucine.

d) 'Low phosphate medium' (see MATERIALS AND METHODS) serves to induce the *PHO5* promoter.

(see Fig. 2), or the C-terminal 113 residues (and no middle part at all), or both.

The only amino acid residues which the Δ 8 and Δ 12 proteins have in common are 1–173. They therefore must contain the DNA binding function. This conclusion is supported by the fact that the N-terminal region of the *LEU3* protein contains a cysteine-rich stretch of amino acids, extending from residues 37 to 67, that shows strong similarities with at least five other regulatory proteins of lower eukaryotes (14 identities and 3 conservative changes within a total of 31 residues, ref. 3). The cysteine-rich stretch of one of these proteins, *GAL4* of yeast, has been shown to form a complex with zinc ions and to be involved in the binding of *GAL4* protein to its DNA targets (16).

Mutant proteins Δ 11, Δ 9, and Δ 3 lacked more and more of the middle part of the *LEU3* protein when compared with Δ 8; internal deletion proteins Δ 4, Δ 5, and Δ 7 lacked different sections of the middle part, leaving other sections of it intact. All of these mutants showed aberrant DNA binding behavior, defined as the presence of only one shifted band, a predominance of the immobile band, the near absence of bands, or a combination of these. We conclude that proper DNA binding is interfered with whenever the integrity of the middle part of the *LEU3* protein is perturbed. (In further support of this conclusion, an in-frame linker insertion mutation, named pKZ5NKBL-9/3, which adds four residues (RDPV) to a position in the protein corresponding to the *NdeI* site in the *LEU3* DNA also showed aberrant DNA binding behavior). In addition, DNA binding ability is greatly diminished when both the middle part and the C-terminal 113 residues are absent, as is the case in the Δ 3 mutant. In exactly which way a damaged middle part interferes with proper DNA binding is not clear. Possible reasons include instability of the

mutant proteins, misfolding, and inability to assume the correct oligomeric structure for binding.

Taken together, these results show that the DNA binding domain as presently defined is not a stable entity by itself, but can be stabilized by the middle part of the protein (when present in its entirety) or by the C-terminal 113 residues. In this sense, the remainder of the molecule is not irrelevant for DNA binding.

The Activation Function of the *LEU3* Protein Resides Within the C-terminal 113 Amino Acid Residues

All of our deletion mutants that retained the C-terminal 113 amino acid residues also maintained at least some ability to activate *LEU2* gene expression (Fig. 2 and Table 1). The strongest activator among these mutant proteins was the Δ 12 protein which lacks 602 internal residues, or about 70% of the total structure. The Δ 4, Δ 5, and Δ 7 proteins were weaker activators. As demonstrated in the previous section, internal deletions that remove only a portion of the middle part of the *LEU3* protein affect DNA binding; they would therefore also be expected to influence the activation function.

No deletions lacking the C-terminal 113 residues (proteins Δ 3, Δ 9, Δ 11, and Δ 8) displayed any activation function for *LEU2* even under high level expression conditions, i. e. with the *PHO5* promoter switched on (Table 1). However, three of these mutant proteins (Δ 3, Δ 9, and Δ 11) showed aberrant DNA binding behavior, as defined above, and might have failed to activate transcription of *LEU2* for that reason. This is not true for Δ 8 which showed 'normal' interaction with UAS_{L2}-30 DNA. It could, of course, be argued that the Δ 8 protein might be unable to activate not because it lacks the activation region but for some other reason, e.g. because it lacks a nuclear entry signal, a

function not studied in this analysis. However, there is indirect evidence that $\Delta 8$ does indeed enter the nucleus. As shown in Table 1, in the presence of $\Delta 8$ protein expression of *LEU2* falls significantly below the basal level observed when no *LEU3*-derived protein is present. The same phenomenon has been seen and analyzed in a recent study of *LEU2* regulation (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication) where it was concluded that mutant *LEU3* protein which is inactive but still able to bind to the *LEU2* promoter competes with another activator responsible for basal level expression. To do this, the mutant protein would have to enter the nucleus.

The C-terminal region suspected to carry the activation function is acidic in nature, thus conforming to observations made with other eukaryotic activators (summarized in 16, 17). There are two sub-regions (labeled 'acidic III' and 'acidic IV' in Fig. 2B and located between residues 783–798 and 860–879, respectively) that carry a net charge of -4 and -5 , respectively. The latter of these sub-regions also contains three tryptophan residues and a motif, GWDNWE, that appears with only slight variations in several other proteins, including DNA polymerase of human adenovirus (18), yellow fever virus genome polypeptide (19), and the *MEL1* gene product of yeast (20,21). Its significance, if any, with respect to transcription activation remains to be established. Very recent results indicate that the C-terminal 30 residues of the *LEU3* protein, which contain both the tryptophan motif and acidic region IV, are necessary for activation (Zhou and Kohlhaw, unpublished observations).

Internal Deletions Convert the *LEU3* Protein from a Modulated Activator to an Unmodulated Activator

An increase in the intracellular concentration of α -IPM serves as a signal to activate several genes, including *LEU2* (1). The presence of signal is communicated to the target genes by *LEU3*. We have chosen the term 'modulation' to describe the effect of α -IPM on the *LEU3* protein. It has already been established in this laboratory that no measurable activation of *LEU2-lacZ* takes place in a strain incapable of producing α -IPM, and that α -IPM is *not* required for binding of the *LEU3* protein to *UAS_{L2}-30* DNA (P. R. G. Brisco and G. B. Kohlhaw, submitted for publication; also, Yeast Genetics and Molecular Biology, Abstracts of 1989 Meeting, p. 140B). We therefore believe that α -IPM's main and maybe only effect in terms of regulation is to cause the *LEU3* protein to assume an active configuration. α -IPM could do so in a number of ways, e. g. (i) by direct interaction with the activation domain; (ii) by direct interaction with a region other than the activation domain, followed by an allosteric transition; (iii) by interaction with an auxiliary protein covering the activation domain, followed by dissociation of that protein, in a manner similar to that postulated for the *GAL4-GAL80* system; or (iv) by interaction with an auxiliary protein binding outside of the activation domain and causing a conformation change in response to the reaction with α -IPM. (While there is at present no evidence for an auxiliary protein, its existence has not been ruled out either.)

Among the deletions analyzed in this work, there were four ($\Delta 4$, $\Delta 5$, $\Delta 7$, and $\Delta 12$) which resulted in unmodulated, 'simple' regulators, i.e. regulators possessing a permanently active configuration unresponsive to changes in the α -IPM concentration (Table 1). (In this terminology, native *LEU3* protein would be a modulated, 'composite' regulator.) Three of the unmodulated regulators ($\Delta 4$, $\Delta 7$, and $\Delta 12$) were efficient activators, compared with native *LEU3* protein. These results show that activation and

Figure 4. Growth behavior of strain XK157-3C/pYB1 carrying either wild type *LEU3* (WT) or *LEU3* mutations. The *LEU3* alleles are the ones described in Fig. 2 and Table 1. Cells were pre-grown as described in the METHODS section. At time zero, they were transferred to YEPD medium that had been depleted of inorganic phosphate (12).

modulation functions can be cleanly separated. We therefore conclude that modulation mechanisms that entail the direct interaction of α -IPM or an auxiliary protein with the activation region (mechanisms (i) and (iii) above) are unlikely with *LEU3* since such mechanisms should work, at least to some extent, as long as the activation region is intact.

We cannot distinguish between modulation mutants defective in *LEU3*- α -IPM interaction and those that may be defective in an allosteric transition-type process. It is therefore not possible at this point to identify a 'modulation domain', even if this term were to be used in a narrow sense, i.e. to describe the site on the *LEU3* protein where direct or indirect interaction with α -IPM takes place. However, it is clear from our results, that the middle part of the *LEU3* protein, as defined above, is essential for the modulation process, whatever the mechanism.

When produced in full medium minus inorganic phosphate, conditions that turn on the *PHO5* promoter, three of the deletion proteins ($\Delta 4$, $\Delta 7$, $\Delta 12$) became much more potent activators, surpassing the activation seen with native *LEU3* grown under the same conditions. This behavior is consistent with a loss of the modulation function. Since full medium contains branched-chain amino acids, wild type *LEU3* action will be limited by the availability of α -IPM, even when the *LEU3* protein is overproduced. By contrast, an increase in the concentration of unmodulated *LEU3* molecules will result in increased expression of the target genes until the targets are saturated with activator.

Overproduction of Unmodulated Activator Slows Down Cell Growth

Growth in phosphate-depleted medium causes the *PHO5* promoter to be switched on (15). Since this medium is not entirely

phosphate-free, it will support normal growth for several generations. During this time, the expression of a *PHO5*-controlled *LEU3* gene can increase several hundred-fold; for example, we found that for a strain carrying a *pPHO5-LEU3-lacZ* fusion (on the pKZ6 plasmid, see legend to Fig. 2) the specific activity of β -galactosidase increased from 0.7 to 300 after the *PHO5* promoter had been switched on. When growth of six of our *LEU3* mutants and of cells carrying wild type *LEU3* was followed after shifting to low-phosphate medium (Fig. 4), mutants producing the *LEU3*- $\Delta 3$, $\Delta 5$, or $\Delta 8$ proteins behaved very much like cells producing the wild type activator, and grew logarithmically for several generations. By contrast, mutants producing the *LEU3*- $\Delta 4$, $\Delta 7$, or $\Delta 12$ activators exhibited a significantly diminished growth rate during the second half of the observation period. The $\Delta 4$, $\Delta 7$, and $\Delta 12$ activators have three things in common: they contain the activation region of the *LEU3* protein, they are incapable of being modulated by changes in the intracellular α -IPM concentration, and they strongly activate *LEU2* expression when overproduced. The *LEU3*- $\Delta 5$ protein has the first two features, but does not strongly activate *LEU2* expression when overproduced. Wild type *LEU3* protein does activate *LEU2* expression when overproduced, but remains subject to α -IPM modulation. These results suggest that it is the overproduction of unmodulated, permanently active *LEU3* protein that is responsible for the slowdown in growth. While there may be trivial reasons for why unmodulated *LEU3* protein should have this effect, such as stress imposed by excessive production of *LEU2-lacZ* fusion protein or the products of other activated genes, it is also possible that excess *LEU3* activator may titrate away a component of the transcription machinery, such as a TATA box binding factor or other transcription factor(s), by interacting with the factor(s) free in solution. The latter mechanism has been proposed to account for the effects of overproduction of *GAL4* in yeast, using a different approach (22).

Surgical Deletion of Two Acidic Regions of the *LEU3* Protein, Including One With Net Charge -19, Has Only Minor Effects on Activation and Modulation

Amphipathic acidic regions have been identified as one important class of activation domains in *trans*-activating proteins. There are four noticeable acidic regions in the *LEU3* protein (labeled Acidic I-IV in Fig. 2). The first two of these could be deleted, singly or in combination, with only minor effects on activation or modulation. They were located between residues 100-117 ('acidic I' in Fig. 2B, net charge -5) and residues 678-697 ('acidic II' in Fig. 2B, net charge -19), the latter constituting a remarkable stretch of 20 residues, 19 of which are glutamate or aspartate. Removal of these 20 residues caused a decrease of only about 30% in activation efficiency; α -IPM-dependent modulation remained normal (Table 1). Removal of acidic region I caused a 37% loss of activation efficiency. This was accompanied by a change in the modulation ratio (induced activity/uninduced activity) from about 8 to about 4. Surprisingly, a construct containing deletions of both acidic regions I and II ($\Delta 14$) was a better activator than native *LEU3* protein. However, it retained the lower modulation ratio seen with the $\Delta 13$ protein.

The two remaining acidic regions (labeled III and IV in Fig. 2) are located in the C-terminal part of the protein now recognized to contain the activation function. A more detailed analysis of these elements is underway.

Apparent Correlation Between the Hydropathy and Some Major Functions of the *LEU3* Protein

Inspection of the hydropathy plot of the *LEU3* protein (Fig. 2A) shows that, on a large scale, three regions can be distinguished: a broad amphiphilic middle section extending roughly from residue 165 to residue 675, flanked by N-terminal and C-terminal regions rich in hydrophilic residues. It is obvious that the three major functions studied here follow the same gross spatial distribution. While this could be coincidental, it could also reflect a 'linear' arrangement of individual domains. Consistent with this notion is the observation that the 'borderlines' between the major structural sections are rather insensitive to perturbation: an in-frame linker insertion adding 12 amino acid residues to a position corresponding to the *SalI* restriction site had almost no effect on the properties of the *LEU3* protein (data not shown), and deletion of residues 678-697 (see above) had only minor effects. It is possible that these regions constitute 'hinges' separating the major functional parts of the protein.

While this paper was readied for publication, we learned that Friden *et al.* (23) have also created and analyzed deletions in *LEU3* of *S. cerevisiae*. Their deletions closely resemble the ones designated $\Delta 3$, $\Delta 8$, and $\Delta 12$ in this work.

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REFERENCES

- Baichwal, V. R., Cunningham, T. S., Gatzek, P. R., and Kohlhaw, G. B. (1983) *Curr. Genet.* **7**, 369-377.
- Brisco, P. R. G., Cunningham, T. S., and Kohlhaw, G. B. (1987) *Genetics* **115**, 91-99.
- Zhou, K., Brisco, P. R. G., Hinkkanen, A. E., and Kohlhaw, G. B. (1987) *Nucl. Acids Res.* **15**, 5261-5273.
- Friden, P., and Schimmel, P. (1987) *Mol. Cell Biol.* **7**, 2708-2717.
- Friden, P., and Schimmel, P. (1988) *Mol. Cell Biol.* **8**, 2690-2697.
- Martinez-Arias, A., Yost, H. J., and Casadaban, M. J. (1984) *Nature* **307**, 740-742.
- Schaber, M. D., DeChiara, T. M., and Kramer, R. A. (1986) *Methods Enzymol.* **119**, 416-423.
- Parent, S. A., Fenimore, C. M., and Bostian, K. A. (1985) *Yeast* **1**, 83-138.
- Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., and Chou, J. (1983) *Methods Enzymol.* **100**, 293-308.
- Messing, J. (1983) *Methods Enzymol.* **101**, 20-78.
- Fink, G. R. (1970) *Methods Enzymol.* **17A**, 59-78.
- Rubin, G. M. (1974) *Europ. J. Biochem.* **41**, 197-202.
- Davis, R., Botstein, D., and Roth, J. R. (1980) In: *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
- Oshima, Y. (1982) In: *The Molecular Biology of the Yeast Saccharomyces* (Strathern, J. N., Jones, E. W., and Broach, J. R., eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 159-180.
- Johnston, M. (1987) *Microbiol. Revs.* **51**, 458-476.
- Hinnebusch, A. (1988) *Microbiol. Revs.* **52**, 248-273.
- Gingeras, T. A., Sciaky, D., Gelin, R. E., Bing-Dong, J., Yen, C. E., Kelly, M. M., Bullock, P. A., Parsons, B. L., O'Neill, K. E., and Roberts, R. J. (1982) *J. Biol. Chem.* **257**, 13475-13491.
- Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H. (1985) *Science* **229**, 726-733.
- Sumner-Smith, M., Bozzato, R. P., Skipper, N., Davies, R. W., and Hopper, J. E. (1985) *Gene* **36**, 333-340.
- Liljeström, P. L. (1985) *Nucl. Acids Res.* **13**, 7257-7268.

22. Gill, G., and Ptashne, M. (1988) *Nature* **334**, 721–724.
23. Friden, P., Reynolds, C., and Schimmel, P. (1989) *Mol. Cell. Biol.* **9**, 4056–4060.
24. Miller, J.-H. (1972) In: *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.