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A Large Internal Deletion Converts Yeast LEU3 to a Constitutive Transcriptional Activator

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***LEU3* of *Saccharomyces cerevisiae* encodes an 886-amino-acid polypeptide that activates transcription of at least five genes by binding to an upstream decanucleotide sequence. This activation is dependent on the inducer α-isopropylmalate, the synthesis of which is repressed by leucine. We created a 285-amino-acid LEU3 derivative by removing a large block of internal sequences, including a dense cluster of acidic residues. This deletion protein bound to the decanucleotide sequence in vitro and activated gene expression in vivo. In contrast to wild-type LEU3, the truncated LEU3 protein was an effective transcriptional activator when α-isopropylmalate synthesis was repressed by leucine.**

The genes required for leucine biosynthesis in *Saccharomyces cerevisiae* (*LEU1*, *LEU2*, and *LEU4*) are regulated by leucine at the transcriptional level (1, 6, 11). This control is mediated by α-isopropylmalate (α-IPM), an intermediate in the leucine biosynthetic pathway. α-IPM synthesis, in turn, is controlled by leucine through feedback inhibition of α-IPM synthase, the first enzyme in the pathway (20, 21). The involvement of α-IPM in the regulation of leucine biosynthesis has been determined on the basis of the following results: (i) the levels of the *LEU1* and *LEU2* gene products are sharply decreased in a strain that lacks a functional α-IPM synthase; (ii) *LEU1* and *LEU2* mutants, which are expected to accumulate intermediates in the pathway, exhibit increased levels of the products of *LEU2* and *LEU1*, respectively; (iii) a strain that contains a feedback-resistant α-IPM synthase, which produces elevated amounts of α-IPM, also has increased amounts of the *LEU1* and *LEU2* gene products (2).

Another component of this regulatory process is *LEU3*. This gene was identified by mutations that result in a leaky Leu$^-$ phenotype as a result of decreased expression of *LEU1* and *LEU2* (2, 14). LEU3, an 886-amino-acid regulatory protein, has been shown to bind in vitro to a conserved decanucleotide sequence (CCGGNNCCGG) that is present upstream of *LEU1*, *LEU2*, and *LEU4* (6, 7). This sequence was sufficient to confer *LEU3*-dependent and leucine-sensitive regulation on a *CYC1-lacZ* fusion gene when placed upstream (7). In addition, LEU3-binding sites have been identified upstream of *ILV2* and *ILV5* (7), suggesting a role for LEU3 in the regulation of all three branched-chain amino acids. A model for transcriptional control of leucine biosynthesis is that α-IPM acts as an inducer of LEU3. According to this model, elevated levels of leucine result in a decrease in the synthesis of α-IPM, which leads to a decrease in LEU3 activity. When cells are starved for leucine, α-IPM synthesis increases, resulting in the activation of LEU3.

LEU3 contains a repeated cysteine motif located between amino acids 37 and 67, which is similar to the zinc finger originally identified in the *Xenopus laevis* transcription factor IIIA (6, 17). Similar sequences have been identified in other regulatory proteins found in yeast and mammalian cells (5). A number of reports indicate that these sequences are involved in sequence-specific DNA binding (9, 12, 18). LEU3 also contains a cluster of acidic amino acids (19 out of 20) located between amino acids 678 and 697. The importance of negatively charged amino acids in gene activation has been documented for a number of transcriptional regulatory proteins (10, 15).

In this paper, we describe experiments which provide evidence that the DNA-binding domain of LEU3 resides in the amino terminus of the protein, a region which also contains the zinc finger-like sequence. In addition, we show that up to 68% of the internal coding region of LEU3 (including the cluster of acidic amino acids) can be deleted without seriously impairing the activity of the protein. This small LEU3 derivative is an effective transcriptional activator, which suggests that the deleted region contains sequences that, in the absence of α-IPM, inhibit this function.

α-IPM is not required for sequence-specific DNA binding in vitro. Genetic studies with various mutants in the leucine biosynthetic pathway indicate that the leucine precursor α-IPM acts as an inducer of LEU3. There are two known functions of LEU3 which α-IPM could modulate: DNA binding and transcriptional activation. To determine whether DNA binding is α-IPM dependent, extracts for gel mobility shift assays were prepared from an α-IPM-overproducing strain (PDY102-1A; α *LEU4-103 ura3-52*), a strain which lacks α-IPM as a result of point mutations in two genes required for α-IPM synthase activity (PDY111; α *leu4-190 leu5-190 ura3-52*), and a strain with disruptions in both *LEU4* and *LEU5* (PDY242-5B; α *leu4-2::URA3 leu5-1::URA3 trp1 ura3-52*), as described previously (7). Extracts were incubated with a ^{32}P-labeled oligonucleotide (LEU2-26 [7]) containing the LEU3 decanucleotide-binding site. Bound complexes were separated from free DNA on nondenaturing polyacrylamide gels. There were no α-IPM-dependent differences in the DNA-binding activity of LEU3 (Fig. 1). In addition, we have not detected any α-IPM-dependent differences in the base contacts made by LEU3 to its DNA-binding site, as determined by methylation interference footprinting (data not shown). These results suggest that α-IPM affects LEU3 at a step other than DNA binding.

Construction of LEU3 deletion and frameshift derivatives. To understand the functional importance of the zinc finger-like sequence and the cluster of acidic residues, as well as

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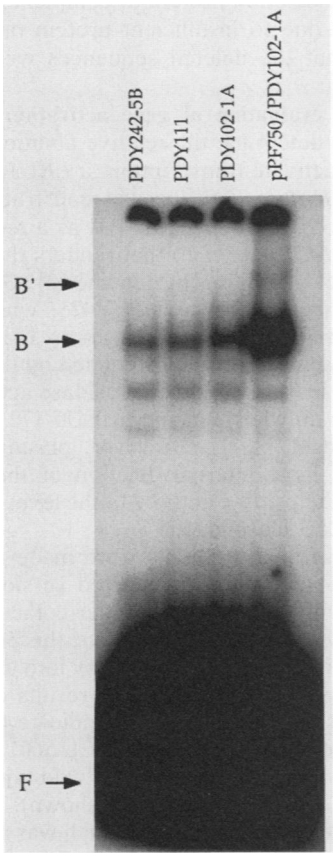

FIG. 1. Effect of α-IPM on LEU3 DNA binding in vitro. Extracts for DNA-binding assays were prepared from PDY242-5B (*leu4-2::URA3 leu5-1::URA3*; α-IPM deficient) (3), PDY111 (*leu4-190 leu5-190*; α-IPM deficient) (3), PDY102-1A (*LEU4-103 LEU5*; α-IPM overproduced) (3), and PDY102-1A (wild type) transformed with the *LEU3* multicopy plasmid pPF750. The DNA substrate in the binding reactions consisted of 1.0 ng of ^{32}P-labeled LEU2-26 oligonucleotide (7). This oligonucleotide encodes the LEU3-binding site of the *LEU2* promoter. The amount of extract per reaction was 30 μg. B and B', Major and minor LEU3-dependent complexes (7), respectively; F, free DNA.

other sequences in the LEU3 protein, a number of frameshift and deletion derivatives of *LEU3* were constructed. The constructs discussed here are shown in Fig. 2. Plasmid pLEU1-172, which encodes a 172-amino-acid N-terminal fragment of LEU3, was constructed by deleting the sequences between the *Sal*I site at base pair (bp) 515 and the *Avr*II site at bp 2320, with the subsequent introduction of a frameshift at the *Sal*I site (which was recreated by the ligation). This truncated LEU3 derivative retains the repeated cysteine motif. Plasmid pLEU1-774 was constructed by creating a frameshift in plasmid pPF750 (6) at the *Avr*II site located at bp 2320 in the *LEU3* sequence. This plasmid encodes a LEU3 derivative that lacks the C-terminal 112 amino acids of the protein, leaving intact the putative DNA-binding domain and the cluster of acidic amino acids. A third plasmid, pLEUΔ601, was constructed by making an in-frame deletion of the sequences between the *Sal*I site and the *Avr*II site. This plasmid encodes a protein that consists of the N terminus of LEU3 (amino acids 1 to 172) fused to the C terminus (amino acids 774 to 886), with a deletion of approximately 68% of the internal coding sequences. The parent plasmid for both pLEU1-172 and pLEUΔ601 is plasmid pPF763. This plasmid was constructed from plasmid pPF750 (6) by deleting sequences between a *Bst*EII site located ~800 bp upstream of the *LEU3* coding region and the *Pvu*II site in the vector sequences so as to remove the *Sal*I site located within the vector sequences.

The initial screen of the LEU3 derivatives was a test of their ability to complement a *LEU3* deletion-disruption strain (PFY420 [7]). Plasmids pLEU1-172 and pLEU1-774 failed to complement this strain (Table 1). However, plasmid pLEUΔ601, which is deleted for approximately two-thirds of the *LEU3* coding region, was able to complement the *LEU3* deletion strain.

DNA-binding activities of the LEU3 derivatives. The failure of plasmids pLEU1-172 and pLEU1-774 to complement the *LEU3* deletion strain could have resulted from the inability to direct synthesis of either a stable protein or a protein with sufficient DNA-binding activity. Gel mobility shift assays were performed, using whole-cell extracts prepared from the *LEU3* deletion strain containing the various plasmids. All of the plasmids gave rise to proteins with DNA-binding activity (Fig. 3). Competition experiments using wild-type and mutant LEU3-binding sites showed that the indicated bands

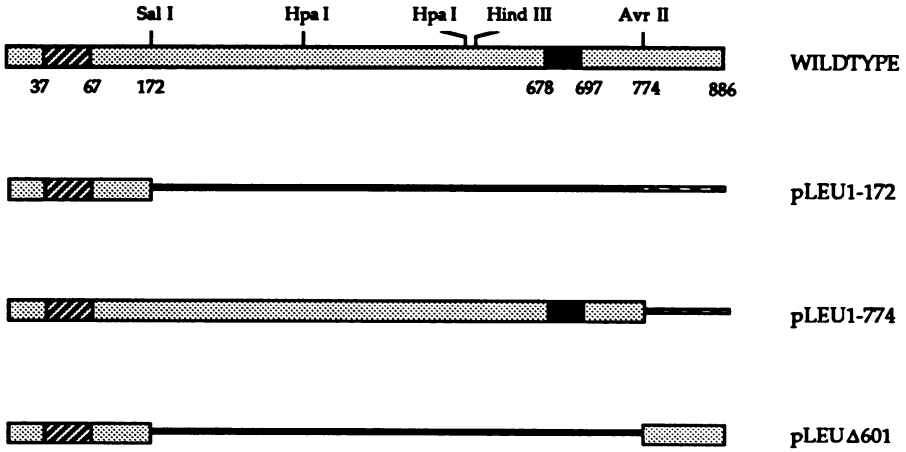

FIG. 2. Diagrams of the *LEU3* coding region and LEU3 derivatives. Symbols: ▭, translated sequence; ▬, deleted sequence; ▬, untranslated sequence; ▨, zinc finger-like sequence; ■, block of acidic amino acids.

TABLE 1. Activities of LEU3 deletion proteins

LEU3 derivative	Complementation in *leu3Δ::HIS3*[a]	DNA binding[b]	β-Galactosidase activity with *LEU2-lacZ* fusion in an α-IPM-overproducing strain[c]
Wild-type (pPF750)	++	++	57
pLEU1-774	−−	++	0.1
Wild-type (pPF763)	++	++	100
pLEU1-172	−−	+/−	0.2
pLEUΔ601	++	+	97
YEp24	−−	−−	0.2

[a] Measured as growth on minimal medium without leucine and, separately, with 0.6 mM trifluoroleucine in strain PFY420 (7).
[b] Summarized from Fig. 3.
[c] Measured in strain PFY423 transformed with the various plasmids as described previously (7). Values are means from at least two experiments, each done in duplicate. Standard errors were less than 25%.

resulted from specific DNA binding (data not shown). The positions of the bound complexes in the gel were consistent with their predicted sizes. The smaller LEU3 derivatives gave rise to proportionally more of the corresponding B′ complex (this complex appeared to contain multiple LEU3 proteins). The amount of DNA-protein complex formed with the various LEU3 derivatives was less than that formed with the wild-type, suggesting decreased synthesis, stability, or DNA affinity. However, the amount of DNA-binding activity associated with plasmid pLEU1-774 was greater than that seen with plasmid pLEUΔ601 or a single chromosomal copy of *LEU3*, both of which complemented a *LEU3* deletion strain. This finding suggested that the inability of the C-terminal LEU3 deletion protein to complement the deletion strain was not due to insufficient protein or DNA-binding activity and that the deleted sequences were involved in gene activation.

Quantitative evaluation of gene activation by the LEU3 derivatives. To determine the relative abilities of the LEU3 derivatives to activate transcription, a *LEU3* deletion strain containing a *LEU2-lacZ* fusion was constructed (PFY423). This strain also overproduced α-IPM as a result of a mutation in *LEU4* (*LEU4-103* Tfl^r) that renders the cells insensitive to feedback inhibition by leucine (2). β-Galactosidase activity was assayed in strain PFY423, which was transformed with plasmids carrying the various LEU3 derivatives as described elsewhere (7). As predicted by the complementation data, no increase in β-galactosidase activity was seen in the tester strain when plasmid pLEU1-172 or pLEU1-774 was introduced (Table 1). However, plasmid pLEUΔ601, which has the large internal deletion of the *LEU3* gene, increased β-galactosidase activity to the levels obtained with a wild-type *LEU3* plasmid.

Because these measurements were made in strains with either the wild-type gene or a deleted version of *LEU3* on multicopy plasmids, α-IPM or another cofactor required by the system may be limiting. To examine this possibility, both plasmids were integrated in single copy into the *URA3* locus of the *LEU2-lacZ* tester strain. The results of the β-galactosidase assays with the integrants indicate that the LEU3 derivative encoded by plasmid pLEUΔ601 was approximately fivefold less effective than the wild type in activating the *LEU2-lacZ* fusion gene (data not shown). This difference was comparable to the difference (which was at least fivefold

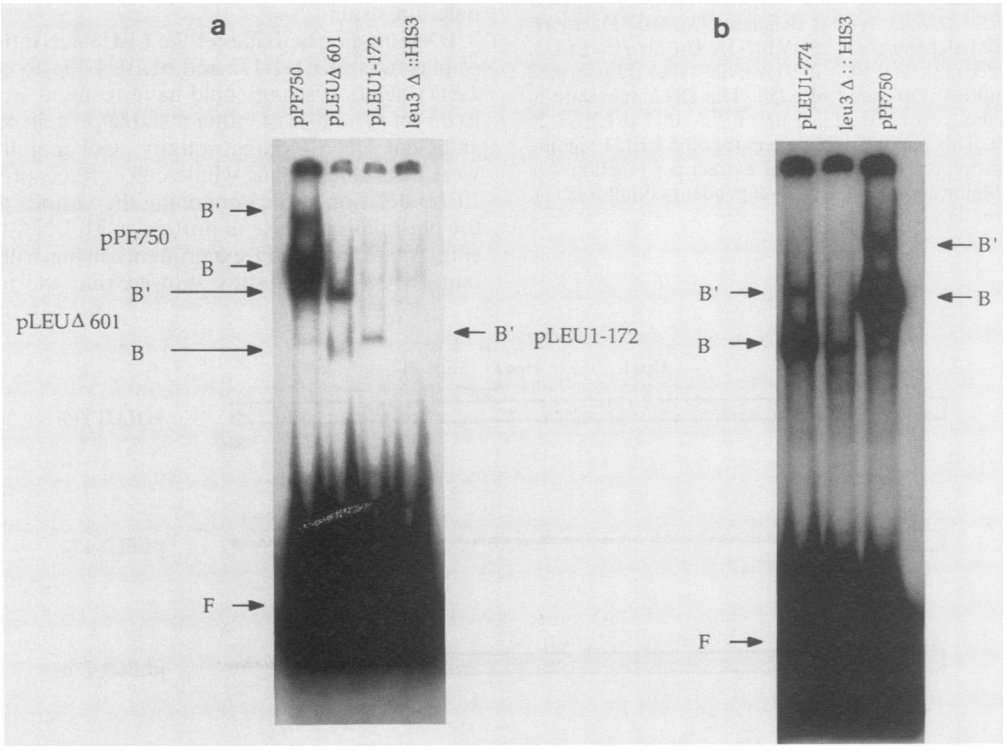

FIG. 3. DNA-binding analysis of LEU3 deletion derivatives. Extracts for DNA-binding assays were prepared from PFY420 (*leu3Δ::HIS3*) with and without the indicated plasmids. The DNA substrate in the reactions was as described for Fig. 1. The amount of extract per lane was ~20 μg (a) or ~50 μg (b). B and B′, Lower and upper *LEU3*-dependent protein-DNA complexes, respectively (in the case of pLEU1-172, only a complex in the expected position for B′ is observed); F, free DNA.

TABLE 2. Effect of leucine on gene activation by wild-type protein and a deletion derivative of LEU3[a]

LEU3 derivative	β-Galactosidase activity with *LEU2-lacZ* fusion in a leucine-sensitive strain	
	+ Leucine	− Leucine
Wild-type (pPF763)	5.3	112
pLEUΔ601	121	303
YEp24	1	1

[a] CRY101-4A (α *ura3-52 his3-1 trp1-289::TRP1 LEU2-lacZ leu2-3,112 leu3Δ::HIS3 LEU4*) was transformed with the indicated plasmids, and β-galactosidase activity was determined as described previously (7) on cultures grown in SD minimal medium or SD medium containing 2.0 mM leucine. Values are means from three separate experiments, each performed in triplicate. Standard errors were less than 35%.

[Fig. 3]) between their in vitro DNA-binding activities when each was expressed from multicopy plasmids. This result suggested that the specific activity of the deletion derivative of LEU3 was equal to or greater than that of the wild-type protein.

Deletion derivative of LEU3 is no longer dependent on α-IPM. Because such a large portion of the protein-coding sequences were removed in making the deletion derivative, we examined whether this protein was still subject to modulation by α-IPM. To do this, PFY423 was crossed with a *LEU4* (Tfl^s) strain (DBY747), and a segregant containing the *leu3Δ::HIS3, trp1-289::TRP1 LEU2-lacZ*, and *LEU4* loci (CRY101-4A) was selected. β-Galactosidase levels were measured in this strain carrying either the wild-type or deletion derivative *LEU3* plasmids in the absence or presence of 2.0 mM leucine as described previously (7).

In contrast to the wild-type protein, the truncated protein encoded by plasmid pLEUΔ601 was a potent transcriptional activator in the presence of leucine (Table 2); the leucine-sensitive regulation was virtually lost (the ~2.5-fold difference in activity observed with plasmid pLEUΔ601 in the presence and absence of leucine may have resulted from the effect of leucine on cellular processes other than α-IPM synthesis). These results indicate that a protein consisting of the N-terminal 172 and the C-terminal 112 amino acids of LEU3 significantly stimulates gene expression in the absence of α-IPM.

It has been proposed that a negatively charged surface on a regulatory protein bound to DNA makes contact with a component of the transcription complex, thus giving rise to activation (10, 15). However, the results obtained with plasmid pLEUΔ601 suggest that the region of highest negative-charge density (the cluster of acidic residues located between amino acids 678 and 697) is not directly involved in transcriptional activation. The C-terminal 112 amino acids of LEU3, which appear to be critical and sufficient for activation, are somewhat acidic, with an overall charge of −11. Other deletion and frameshift derivatives of *LEU3* have been made and analyzed, but all lack detectable DNA-binding activity. Because the presence of that activity is currently our only means of determining whether a stable protein is synthesized, we are unable to draw any conclusions regarding those additional constructions and therefore cannot rule out the possibility that additional regions of the protein, which might become unmasked under particular circumstances, are involved in gene activation.

As mentioned above, it appears that amino acids 173 to 773 of LEU3, which include the block of acidic amino acids located in the C-terminal half of the protein (amino acids 678 to 697), are not required for DNA binding or transcriptional activation. Perhaps the α-IPM-dependent positioning of the acidic domain relative to the C-terminal activation domain influences the ability of the latter to activate gene expression. Another possibility is that α-IPM and the acidic residues somehow alter the interaction of another protein with LEU3, similar to the interaction of GAL4 with GAL80 (13, 16). However there is no genetic evidence implicating additional proteins in the regulation of leucine biosynthesis.

In summary, it appears that most if not all of the amino acid sequences which define the DNA-binding and transcriptional activation domains of LEU3 lie in the amino and carboxyl termini of the protein, respectively. The results presented here suggest that much of the remainder of the protein is dispensable in terms of in vivo gene activation. In the yeast regulatory protein GAL4, fusion of the amino terminus (DNA-binding domain) to the carboxyl terminus (activation domain) gives rise to a protein with activity comparable to that of the wild type, similar to what is seen with LEU3 (15). However, unlike LEU3, the GAL4 deletion derivative is still subject to galactose-dependent regulation. The glucocorticoid receptor is also similar to LEU3 in some respects. This protein has been found to have modular domains encoding the various functions of the protein (4). In addition, deletion of the hormone-binding domain of the receptor gives rise to a constitutive activator (8). Picard et al. (19) have shown that multiple receptor functions are repressed when the hormone-binding region of the protein is unoccupied and that this repression is relieved by ligand binding. A similar situation may exist for LEU3, with α-IPM acting as the ligand.

It is unclear why many transcriptional regulatory proteins have large blocks of apparently dispensable sequences. In the case of LEU3, at least some of the functionally dispensable sequences are required for proper regulation. In addition, perhaps size itself has a functional role, possibly by influencing the presentation of the activating domain to the transcriptional machinery or by limiting access to the nucleus.

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