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Detection of Leucine-Independent DNA Site Occupancy of the Yeast Leu3p Transcriptional Activator In Vivo

CATHERINE REYNOLDS KIRKPATRICK† AND PAUL SCHIMMEL*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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The product of the *Saccharomyces cerevisiae LEU3* gene, Leu3p, is a transcriptional activator which regulates leucine biosynthesis in response to intracellular levels of leucine through the biosynthetic intermediate α-isopropylmalate. We devised a novel assay to examine the DNA site occupancy of Leu3p under different growth conditions, using a reporter gene with internal Leu3p-binding sites. Expression of the reporter is inhibited by binding of nuclear Leu3p to these sites; inhibition is dependent on the presence of the sites in the reporter, on the integrity of the Leu3p DNA-binding domain, and, surprisingly, on the presence of a transcriptional activation domain in the inhibiting protein. By this assay, Leu3p was found to occupy its binding site under all conditions tested, including high and low levels of leucine and in the presence and absence of α-isopropylmalate. The localization of Leu3p to the nucleus was confirmed by immunofluorescence staining of cells expressing epitope-tagged Leu3p derivatives. We conclude that Leu3p regulates transcription in vivo without changing its intracellular localization and DNA site occupancy.

In the yeast *Saccharomyces cerevisiae*, the biosynthesis of many amino acids is coordinately regulated by the general control system through the transcriptional activator encoded by *GCN4* (24). However, leucine biosynthesis is regulated, largely independently, by the *LEU3* transcriptional activator (Leu3p) in response to the levels of the metabolic intermediate α-isopropylmalate (α-IPM), which serves as a sensor of leucine availability (19, 28). The first enzyme of the leucine biosynthetic pathway is α-IPM synthase; it is feedback inhibited by leucine, so that high levels of leucine lead to low levels of intracellular α-IPM and reduced transcriptional activation by Leu3p. In contrast, leucine starvation leads to increased levels of α-IPM, which serves as an inducer of Leu3p activity. Leu3p activates the transcription of *LEU1* and *LEU2*, which encode the subsequent enzymes of the pathway, as well as *LEU4*, one of the genes for α-IPM synthase; it may also activate *ILV2* and *ILV5*, which encode enzymes of isoleucine and valine biosynthesis (20). Leu3p activates transcription by binding to palindromic sites upstream of the regulated genes; these sites function as leucine-dependent upstream activation sequences (20, 39).

The domains of Leu3p responsible for DNA binding and transcriptional activation have been localized by deletion analysis and by construction of functional fusion proteins between Leu3p and other transcriptional activators (18, 48, 53, 55). The DNA-binding domain is located near the N terminus and resembles the DNA-binding domains of several other yeast transcriptional activators (5, 37), including that of Gal4p, for which the structure is known. These domains are organized around six conserved cysteine residues which bind two zinc ions in a binuclear cluster which forms the structural core of the DNA-binding domain; an alpha-helix C terminal to the cluster appears to be involved in activator dimerization (7, 30, 33). The domain of Leu3p responsible for activating transcription is located near the C terminus of the protein (53, 55). The minimal functional activation domain has a net negative charge (48); it therefore may be considered an acidic activation domain, although it is possible that other features of the region are also important for activation, as has been suggested for other activation domains (13, 31, 52).

Deletion analysis has also demonstrated that the middle region of Leu3p is required for regulating Leu3p activity in response α-IPM: proteins consisting of the C-terminal activation domain fused directly to the N-terminal DNA-binding domain activate transcription constitutively (18, 53). The mechanism of this regulation by α-IPM is not known. Some transcriptional activators, such as the yeast activator Hap1p, cannot bind to DNA in the absence of inducer (36); however, it appears that α-IPM does not regulate DNA binding by Leu3p, because extracts from cells which cannot synthesize α-IPM give the same Leu3p gel shift as wild-type extracts do (18). Another possibility is that α-IPM binds to the middle region of Leu3p, causing a conformational change which allows transcriptional activation to take place (for instance, by exposing a surface which can interact with some component of the transcriptional machinery). Alternatively, α-IPM may influence the level or activity of a hypothetical Leu3p regulatory protein, which might interact with the middle region of Leu3p; α-IPM could also affect the binding of such a protein to Leu3p.

We wished to explore the possibility that the middle region of Leu3p is required for proper intracellular localization of the protein. The diameter of the nuclear pore channel limits the size of globular proteins which can enter the nucleus by diffusion to about 40 to 60 kDa (15). Wild-type Leu3p is too large to enter the nucleus by simple diffusion, whereas the constitutively active deletion proteins are small enough to diffuse freely into the nucleus. Wild-type Leu3p could therefore be prevented from activating transcription by sequestering the protein in the cytoplasm when α-IPM levels are low. Higher levels of α-IPM could trigger import of Leu3p to the nucleus by causing a conformational change in the protein to reveal a masked nuclear localization signal or by affecting the interaction of Leu3p with a binding protein required to retain it in the cytoplasm. Analogous mechanisms have been observed in other systems: the steroid hormone receptors, such as the glu-

* Corresponding author. Mailing address: Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Rm. 68-230, Cambridge, MA 02139. Phone: (617) 253-6739. Fax: (617) 253-6636.

† Present address: Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280.

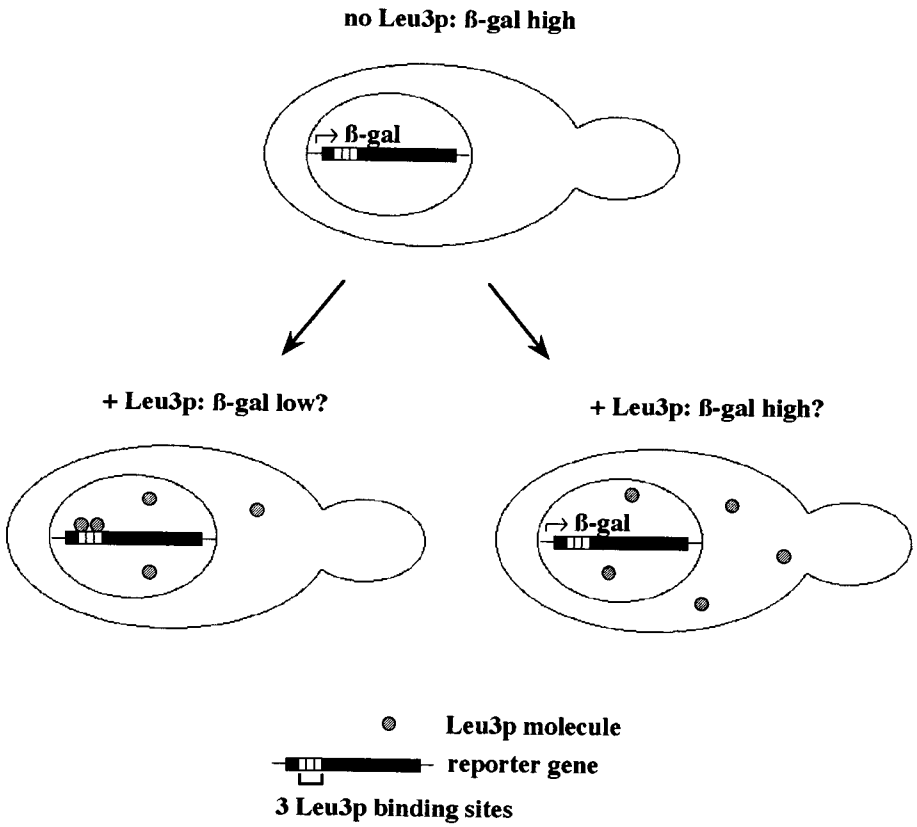

FIG. 1. Overview of the assay for nuclear localization and DNA site occupancy. The *RPL16-lacZ* reporter gene produces β-galactosidase in *leu3* cells, irrespective of the growth medium. When a plasmid encoding Leu3p is introduced into these cells, Leu3p is expected to inhibit production of β-galactosidase from *RPL16-lacZ* when the cells are grown in medium containing a low level of leucine, because under these conditions Leu3p is known to activate transcription and is presumably located in the nucleus; this inhibition is expected to occur because of Leu3p binding to the Leu3p-binding sites introduced within the *RPL16-lacZ* gene. If Leu3p does not enter the nucleus or bind to DNA when the cells are grown in medium containing a high level of leucine, this inhibition would not be observed. In contrast, inhibition of *RPL16-lacZ* even when the cells are grown in medium containing a high level of leucine would indicate that Leu3p is in the nucleus and binds to its DNA site under all conditions.

cocorticoid receptor, localize to the nucleus upon binding of the inducer (hormone) (38); NF-κB is retained in the cytoplasm by its interaction with I-κB and enters the nucleus only when it is released by an appropriate signal (4). Such regulation has not been observed in *S. cerevisiae*, although the *SWI5* and *ACE2* transcriptional activators do appear to be regulated by localization to the nucleus at only the G_1 phase of the cell cycle (16, 34).

To examine this question, we devised a novel assay for the presence of Leu3p in the nucleus and binding to its DNA sites. This indirect assay is based on the principle that Leu3p might inhibit the expression of a reporter gene by binding to copies of the Leu3p-binding site introduced within the reporter, downstream of the start of transcription (Fig. 1). If Leu3p is able to enter the nucleus and occupy its binding site even when α-IPM levels are low, the expression of the reporter gene would be expected to be lower than in a strain lacking Leu3p under all growth conditions. If Leu3p is restricted to the cytoplasm or does not bind to DNA in vivo when α-IPM levels are low, there would be no inhibition of reporter gene expression when the cells are grown in high-leucine medium but only when they are grown in low-leucine medium. An *RPL16-lacZ* fusion gene (40) was chosen as a reporter because the transcription of this ribosomal protein gene does not vary according to whether leucine is available (27a), so that this fusion gene could serve as a constitutively transcribed gene whose product is readily assayable.

We found that expression of the *RPL16-lacZ* fusion was inhibited by Leu3p when cells were grown in both high and low levels of leucine, indicating that α-IPM does not regulate the intracellular localization or DNA site occupancy of Leu3p. This inhibition of expression required the DNA-binding domain of Leu3p and, unexpectedly, the activation domain as well. Heterologous activation domains fused to the Leu3p DNA-binding domain were as effective as the Leu3p activation domain in mediating the repression. We found that α-IPM was not required for inhibition.

To confirm some of the results obtained with the indirect assay for nuclear localization, the intracellular distribution of epitope tagged Leu3p was examined by indirect immunofluorescence. This analysis also revealed that Leu3p is concentrated in the nucleus irrespective of the level of leucine in the growth medium, consistent with the results obtained with the reporter gene.

MATERIALS AND METHODS

Strains and genetic methods. *Escherichia coli* HB101, JM109, and TG1 (Amersham) were used to maintain plasmids and M13-derived vectors. Other methods for working with bacteria and yeasts were as described previously (41, 43).

The yeast strain CRY101-4A (α *ura3-52 his3Δ1 trp1-289*::*TRP1*::*LEU2-lacZ leu2-3,112 leu3Δ*::*HIS3*) is a segregant from a cross between PFY423 (α *ura3-52 his3Δ1 trp1-289*::*TRP1*::*LEU2-lacZ leu2-3,112 leu3Δ*::*HIS3 LEU4*^{r}*-103*) and DBY747 (**a** *ura3-52 his3Δ1 trp1-289 leu2-3,112*) (both from Phil Friden, Massachusetts Institute of Technology). Resistance to trifluoroleucine (conferred by

the *LEU4*^{r}*-103* allele) was assessed by mating candidates with strains F9 (**a** *ade6*) and F11 (α *ade6*) in patches on YPD and replica plating to SD (43) containing 0.6 mM trifluoroleucine (purchased from Fairfield Chemical Co., Inc.).

The yeast strain CRY102-2B (α *ura3-52 his3Δ1 trp1-289 leu2-3,112 leu3Δ::HIS3*) is a segregant from a cross between PFY421 (α *ura3-52 his3Δ1 trp1-289 leu2-3,112 leu3Δ::HIS3 LEU4*^{r}*-103*) and DBY747 (**a** *ura3-52 his3Δ1 trp1-289 leu2-3,112*) (both from Phil Friden). Resistance to trifluoroleucine was assayed as above.

The yeast strain CRY103 (**a** *ura3-52 his3*? *trp1Δ::hisG leu3Δ::HIS3 leu4-190 leu5-190*) was constructed from PFY425-2C (**a** *ura3-52 his3*? *leu3Δ::HIS3 leu4-190 leu5-190*), which is a segregant from a cross between PDY111 (α *ura3-52 leu4-190 leu5-190*) and PFY420 (**a** *ura3-52 his3Δ1 leu3Δ::HIS3 LEU4*^{r}*-103*). The *TRP1* gene was disrupted by the method of Alani et al. (1). PFY425-2C (from Phil Friden) was transformed with plasmid pNKY1009 that had been digested with *Eco*RI and *Bgl*II. Ura$^+$ transformants were screened for tryptophan auxotrophy and then streaked on plates containing 5-fluoroorotic acid to select for loss of the *URA3* gene by recombination between the flanking *hisG* sequences. The resulting cells should retain a single copy of *hisG* disrupting the *TRP1* gene. Candidates were transformed with a *TRP1* plasmid to confirm that the Trp$^-$ phenotype was due to a mutation in the *TRP1* gene.

β-Galactosidase assays. β-Galactosidase assays were performed essentially as described previously (22). Cultures were grown in synthetic complete medium selective for the appropriate plasmid(s), containing 36 mg of leucine per ml (approximately 0.3 mM leucine [low leucine]) or 2 mM leucine (high leucine). Unless indicated otherwise, all β-galactosidase assays were performed on cells grown in high levels of leucine. Values (in Miller units [22]) are generally given as the mean of multiple determinations with an error equal to the standard deviation.

Plasmid constructions. Standard protocols (41) were followed for the construction of all plasmids. All enzymes were used as specified by the manufacturer. Site-directed mutagenesis was carried out with the Amersham site-directed mutagenesis kit as specified by manufacturer, except that multiple oligonucleotides were sometimes used together in the initial annealing step. When necessary, three oligonucleotides were 5′ phosphorylated together in a single reaction (each at one-third of the usual concentration of 1.67 pmol/μl) and the usual volume (2.5 μl) was added to the annealing reaction mixture; this allowed as many as nine different oligonucleotides to be annealed to the single-stranded template simultaneously.

(i) Introduction of new silent restriction sites into *LEU3*. A unique *Bam*HI site was introduced into plasmid pPF763 (18) upstream of the *LEU3* sequence by inserting a *Bam*HI linker at the *Tth*111I site in the plasmid backbone. Large fragments of *LEU3* were then subcloned into either M13mp19 or pBluescript KS+ for site-directed mutagenesis with the oligonucleotides listed in Table 1, which were designed to create new restriction sites without altering the protein product of the gene. As many as seven different restriction sites were introduced simultaneously by using mixtures of oligonucleotides in the mutagenesis reactions; all changes were confirmed by sequencing. The mutagenized fragments were reintroduced into the parental plasmid backbone to generate a *LEU3* gene containing all 11 new silent restriction sites. This gene gave rise to the same level of Leu3p activity as the unmutagenized gene, as judged by the amount of β-galactosidase activity measured from the *LEU2-lacZ* fusion in the yeast strain CRY101-4A (data not shown). For most of the experiments described, Leu3p constructs were carried in the vector pRS426, a 2μm *URA3* vector (provided by Neal Silverman, Massachusetts Institute of Technology) similar to pRS316 (44). For low-level Leu3p expression, constructs were introduced into pRS316 (as a *Bam*HI-*Nhe*I fragment).

(ii) Leu3p deletion constructs. Constructs with deletions internal to Leu3p are named Leu3pΔX–Y, where residues X to Y inclusive are deleted from the protein. Truncated proteins are named Leu3p:1–X, where X is the last residue of Leu3p present.

Leu3pΔ173–773 (called Leu3Δ601 in reference 18) was created by removal of sequences between the unique *Sal*I and *Avr*II sites of *LEU3* followed by treatment with the Klenow fragment of DNA polymerase I and religation of the blunt ends with T4 DNA ligase. This protein is a constitutive transcriptional activator (18). Leu3pΔ143–828 was created by digesting Leu3p at the unique introduced *Sfi*I site, treating with mung bean nuclease, adding *Sac*II linkers, digesting with *Sac*II (which also cleaves at the unique *Sac*II site introduced by site-directed mutagenesis), and religating. Leu3pΔ143–828 was the smallest of several constitutively active Leu3p deletion proteins which could activate transcription of the *LEU2-lacZ* fusion as well as full-length Leu3p (27). Leu3p:1–774 was created by linearizing Leu3p at the unique *Avr*II site, treating with the Klenow fragment of DNA polymerase I, and religating. Leu3p:1–142 was created by removing sequences between two *Not*I sites introduced by site-directed mutagenesis (see below) and religating; this protein has three alanine residues at the C terminus following residue 142 of Leu3p. These last two proteins are stably expressed in vivo (Leu3p:1–774 is slightly less abundant and Leu3p:1–142 is more abundant than full-length Leu3p); they bind to DNA but do not activate transcription (18, 27). All constructions were confirmed by sequencing of the altered region.

(iii) Epitope tagging of Leu3p. Two oligonucleotides were synthesized to encode the 9-amino-acid epitope from influenza virus hemagglutinin recognized by the monoclonal antibody 12CA5 (29) plus two flanking amino acids (sequences, GTCCTACCCATACGACGTCCCAGACTACGCTCG and GACCGAGC GTAGTCTGGGACGTCGTATGGGTAG). These were annealed to one another and ligated into the unique introduced *Rsr*II site near the 5′ end of *LEU3*; because the two ends generated by *Rsr*II are complementary but not identical, all insertions were in the desired orientation. Plasmids containing one, two, and three copies of the hemagglutinin tag were recovered, and Leu3p produced from them was analyzed in a Western immunoblot with the 12CA5 monoclonal antibody. The triple tag gave a much stronger signal than either the single or double tag (data not shown) and was used for all subsequent work. The tagged protein activated transcription of *LEU2-lacZ* in the yeast strain CRY101-4A almost as well as the untagged protein did (e.g., 88.3 U of β-galactosidase activity compared with 100 U for untagged Leu3p) but was not repressed to quite the same extent when the cells were grown in high-leucine medium (33.0 U compared with 11.6 U). In all repression assays with the yeast strain CRY102-2B, however, the tagged and untagged forms of Leu3p and its derivatives behaved similarly. The epitope tag was moved to other Leu3p constructs within a *Bam*HI-*Sph*I fragment, which was used to replace the corresponding wild-type sequence.

(iv) Leu3p fusion proteins. Site-directed mutagenesis was carried out with two oligonucleotides (sequences, GAACCGCGGGCGGCCGCCTCTTCTCTA and GTCCTTTTCTGCGGCCGCGTAATGTTTA) to introduce *Not*I sites on either side of the Leu3p activation domain in a plasmid encoding Leu3pΔ143–828. The activation domains of Gal4p (amino acids 769 to 881) and VP16 (amino acids 413 to 490) were amplified by PCR from plasmids pMA242 (32) and 2μGVWT (8), respectively, with the following primers: GAL4N (CCCGGGATCCGCGGCC GCCAATTTTAATCAAAGTGGGAATATTGC), GAL4C (CCCGGGATCC GCGGCCGCTACTCTTTTTTTGGGTTTGGTGG), VP16N (CCCGGGATC CGCGGCCGCGGCCCCCCCGACCGATGTCAGC), and VP16C (CCCGGG ATCCGCGGCCGCTACCCACCGTACTCGTCAATTCC). The *Not*I-digested PCR products (kindly provided by Neal Silverman and Julie Agapite, Massachusetts Institute of Technology) were inserted in place of the Leu3p activation domain. The junctions between Leu3p and the inserted sequences were confirmed by sequencing. Both fusion proteins constitutively activate the *LEU2-lacZ* gene in the yeast strain CRY101-4A as well as or somewhat better than does Leu3pΔ143–828 (data not shown).

(v) Construction of reporters. The *RPL16-lacZ* reporter was constructed in a three-way ligation of the following DNA fragments: a 2.4-kb fragment of pMR10 (40) containing the 5′ end of the *RPL16-lacZ* fusion, obtained by digestion with *Nde*I, treatment with the Klenow fragment of DNA polymerase I, and digestion with *Sac*I; a 1.2-kb *Sac*I-*Pst*I fragment of pMC1871 (Pharmacia) containing the 3′ end of *lacZ*; and plasmid pRS314 (44), which had been digested with *Eag*I, treated with the Klenow fragment of DNA polymerase I, and digested with *Pst*I. Leu3p-binding sites were inserted at the *Bam*HI site at the junction between the *RPL16* and *lacZ* sequences, after amino acid 49 of Rpl16p, using two oligonucleotides (sequences, GATCTAGAGTGAGAGGCCGGAACCGGCTTTTCG and GATCCGAAAAGCCGGTTCCGGCCTCTCACTCTA). These were annealed with one another and ligated with the linearized (partially digested) reporter plasmid. Products were screened by sequencing to identify the number and orientation of any inserted oligonucleotides. A plasmid containing three copies of the Leu3p-binding site was generally used as the reporter plasmid. In preliminary experiments, it gave slightly better expression than did a reporter containing only one Leu3p-binding site but showed similar levels of inhibition by Leu3p (data not shown).

Western blot analysis. Protein samples were prepared for Western blot analysis by the method of Silve et al. (45). Briefly, cells were collected from 3 ml of culture (with an optical density at 600 nm of about 1) and subjected to alkaline lysis followed by trichloroacetic acid precipitation. The pellet was boiled in 100 μl of sodium dodecyl sulfate gel loading buffer, and 7 μl was loaded per lane. Protein (10 to 15 μg per lane) from extracts made for gel shift assays (20) was also used for Western blot analysis. Protein gels (9, 12, or 15% polyacrylamide) were prepared and run as described previously (2). Proteins were transferred to polyvinylide difluoride membranes by using a Millipore dry blot apparatus as suggested by the manufacturer. Blots were stained with amido black to visualize the molecular weight markers and to check the success of the transfer, destained in 90% methanol–7% acetic acid, rinsed twice in Tris-buffered saline, and blocked in a 5% solution of nonfat dry milk in Tris-buffered saline containing 0.1% Triton X-100. Western blot analysis was continued as described previously (23), with 1:5,000 12CA5 monoclonal antibody (Berkeley Antibody Co.) and 1:2,000 horseradish peroxidase-linked anti-mouse immunoglobulin secondary antibody (Amersham). Proteins were detected by the Amersham enhanced chemiluminescence system.

Immunofluorescence. For immunofluorescence, the plasmids of interest were transformed into strain CRY101-4A. Cultures were grown in synthetic complete medium to an optical density at 600 nm of 0.1 to 0.2. The cells were fixed by adding formaldehyde directly to the culture and incubating on ice for 1 h (31a). Immunofluorescence was performed as described previously (46). The primary antibody was 1:1,000 12CA5 monoclonal antibody (Berkeley Antibody Co.); the secondary antibody was 1:1,000 fluorescein-conjugated goat anti-rabbit antibody (Cappel). Cells were examined on a Zeiss Axioplan microscope, and photographs were taken with Kodak Tri-X Pan 400 ASA film.

TABLE 1. Oligonucleotides used for mutagenesis in this study

Restriction site	Oligonucleotide sequence[a]	Position[b]
*Rsr*II	ATGGAAGGACGGTCCGATTTTGTG	10
*Sph*I	GAAATTCGCATGCGTGGAATGT	106
*Sac*I	ATTCAAGGAGCTCACCAGA	271
*Sac*II	AAATAGAACCGCGGTCATACAAAACAC	421
*Mlu*I	CTTAATGACGCGTTTATCAGT	778
*Pfl*MI	TGTTGTATCCCAAACAGTGGCATCATCATT	1101
*Xho*I	CTAAGTCAACTCGAGATTAGTCTTGAAGA	1354
*Eag*I	TTATAATGCGGCCGTAAACTT	1565
*Not*I	GAAGGTGGCGGCCGCCTAAATAATGATTTC	1880
*Bgl*II	AGAAGAAGATCTAAGTAGTAAAGTT	2088
*Sfi*I	CACTACCGGCCACACAGGCCAACT	2478

[a] Bases which differ from the wild-type base at the corresponding position in *LEU3* are underlined in the oligonucleotide sequence, and the new restriction sites are boxed.

[b] The position of the first base of the introduced restriction site is given, with the numbering beginning at the first base of the *LEU3* open reading frame.

RESULTS

Leu3p enters the nucleus under all conditions tested. As described above, the assay for nuclear import of Leu3p was based on the fact that DNA binding by Leu3p caused reduced expression of an *RPL16-lacZ* reporter gene. The assay was generally performed with the yeast strain CRY102-2B, which is a *leu3* deletion strain with the appropriate selectable markers for maintaining both the reporter plasmid and a second plasmid carrying a *LEU3* derivative. The plasmids were transformed together into this strain, and β-galactosidase assays were performed on cultures of several transformants. As shown in Fig. 2, Leu3p is able to inhibit expression of the *RPL16-lacZ* fusion: there is approximately threefold less β-galactosidase activity in cells containing wild-type *LEU3* on a multicopy plasmid than in those which have only the corresponding vector. The minimal Leu3p constitutive activator, Leu3pΔ143–828, can also inhibit expression of the fusion to about the same extent as the full-length protein. Both forms of Leu3p cause this inhibition when the cells are grown in either high- or low-leucine medium. This behavior was expected of the constitutive activator, which is clearly able to reach the nucleus and bind its DNA site under both growth conditions; the results indicate, however, that the full-length protein also occupies its site even when α-IPM levels are low. High levels of α-IPM therefore do not seem to be required for the correct localization of Leu3p.

To confirm that the inhibition of *RPL16-lacZ* expression seen in this assay was due to Leu3p binding within the reporter gene and not to some other effect of Leu3p on transcription of the gene, the assay was repeated with a reporter lacking the Leu3p-binding sites, in parallel with the original reporter (Fig. 3). While repression was still seen with the reporter containing Leu3p-binding sites, there was little difference in the β-galactosidase activities observed with the reporter which lacked Leu3p sites. In addition, we constructed a Leu3p derivative which could not bind to DNA because of a mutation in one of the conserved cysteine residues in the DNA-binding domain (Leu3p C47S, in which cysteine 47 of Leu3p was changed to serine by site-directed mutagenesis). This protein was stably expressed in vivo to nearly the same level as wild-type Leu3p was, but it was unable to bind to DNA as assayed by gel shift (data not shown) and did not inhibit β-galactosidase production in the nuclear import assay (Fig. 3). These results suggest that Leu3p must be able to bind to DNA in order to inhibit expression of *RPL16-lacZ* and that inhibition is caused by binding of Leu3p within the reporter gene.

To confirm that the inducer of Leu3p, α-IPM, was not required for Leu3p to enter the nucleus and repress the *RPL16-lacZ* fusion, the assay was repeated in the yeast strain CRY103, which cannot synthesize α-IPM because of mutations in the *leu4* and *leu5* genes. The results are shown in Fig. 4, where the β-galactosidase activity observed with the reporter containing Leu3p-binding sites is expressed as a fraction of the activity observed with the control reporter lacking Leu3p-binding sites. These data are very similar to those obtained in CRY102-2B: both full-length Leu3p and the constitutively active deletion protein inhibit β-galactosidase production. Because *leu4 leu5* strains appear to lack α-IPM synthase completely (6), it ap-

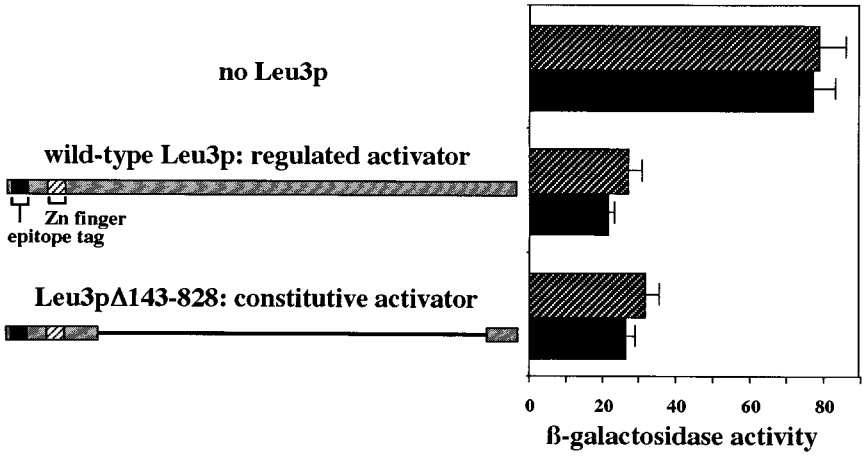

FIG. 2. Leu3p represses reporter gene expression under both high-leucine (■) and low-leucine (▨) growth conditions. Strain CRY102-2B was cotransformed with the reporter plasmid, which carries the *RPL16-lacZ* fusion gene containing three Leu3p-binding sites, and plasmids expressing epitope-tagged wild-type Leu3p, a constitutively active deletion mutant of Leu3p, or no Leu3p. Transformants were assayed for β-galactosidase activity after growth in medium containing a low level or a high level of leucine, as described in Materials and Methods. The values (shown as bar graphs) are means from four transformants assayed in duplicate.

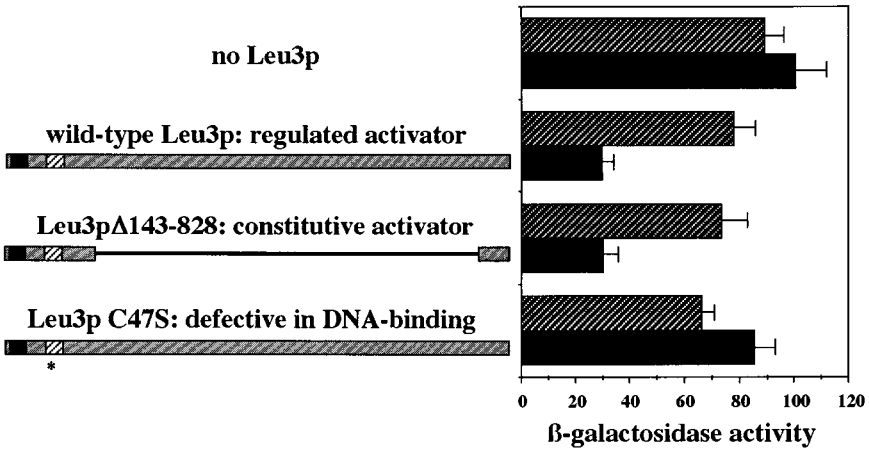

FIG. 3. Inhibition of reporter gene expression is dependent on the Leu3p-binding sites in the reporter and on the ability of Leu3p to bind to DNA. Strain CRY102-2B was cotransformed with plasmids expressing the epitope-tagged Leu3p variants shown on the left of the figure, together with either the reporter plasmid containing *RPL16-lacZ* with three internal Leu3p-binding sites (■) or a reporter plasmid containing *RPL16-lacZ* without the Leu3p-binding sites (▨). Transformants were assayed for β-galactosidase activity after growth in medium containing a high level of leucine. The values (shown as bar graphs) are means from four transformants assayed in duplicate.

pears that even low levels of α-IPM are not required for correct localization and DNA site occupancy of Leu3p.

Repression is still seen with low-level Leu3p expression. Leu3p derivatives on CEN plasmids were also tested in this assay, to see if lower-level Leu3p expression might be sufficient for repressing the reporter. As shown in Fig. 5A, epitope-tagged full-length Leu3p and the constitutive activator Leu3p Δ173–773 each inhibit β-galactosidase production when cells are grown in high levels of leucine, although the levels of epitope-tagged Leu3p from a CEN plasmid are at least 5- to 10-fold lower than are those from the corresponding 2μm plasmid (Fig. 5B). These data suggest that even low levels of Leu3p can inhibit expression of the reporter gene.

Results with Leu3p variants: is the activation domain required for repression? Several Leu3p variants produced during deletion analysis of the protein (27) were tested in the localization assay. As expected, all constitutively active variants with less extensive deletions than Leu3pΔ143–828 behaved similarly: all repressed *RPL16-lacZ* expression under all growth conditions tested (data not shown). Surprisingly, however, a truncated protein lacking the C-terminal 113 amino acids (Leu3p:1–774), which appears to be stably expressed to nearly the same level as full-length Leu3p in vivo (27); also see the immunofluorescence data below) and to bind to DNA in vitro (18, 53), did not substantially inhibit β-galactosidase production in this assay (Fig. 6). This result raised the possibility that the C terminus of Leu3p is involved in the repression in some way.

Figure 6 shows the results of some additional assays that have a bearing on this question. As shown above, Leu3pΔ143–828 inhibits expression of *RPL16-lacZ*. Removal of the C-terminal 58 amino acids needed for activation renders the

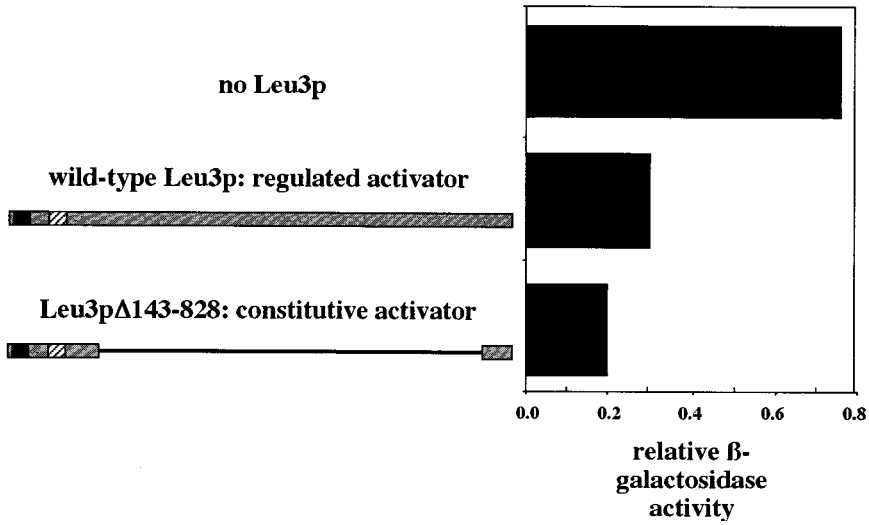

FIG. 4. Leu3p can repress reporter gene expression in the absence of α-IPM. The α-IPM-deficient strain CRY103 was cotransformed with plasmids expressing epitope-tagged wild-type Leu3p, Leu3pΔ143–828, or no Leu3p, together with one of the two reporter plasmids with and without Leu3p-binding sites. β-Galactosidase activities were determined, in duplicate, for four transformants from each combination of plasmids. The mean value obtained for cells containing the reporter with Leu3p-binding sites is graphed as a fraction of the mean activity obtained for cells containing the reporter lacking Leu3p-binding sites. (The latter values ranged from 33 to 48 units.)

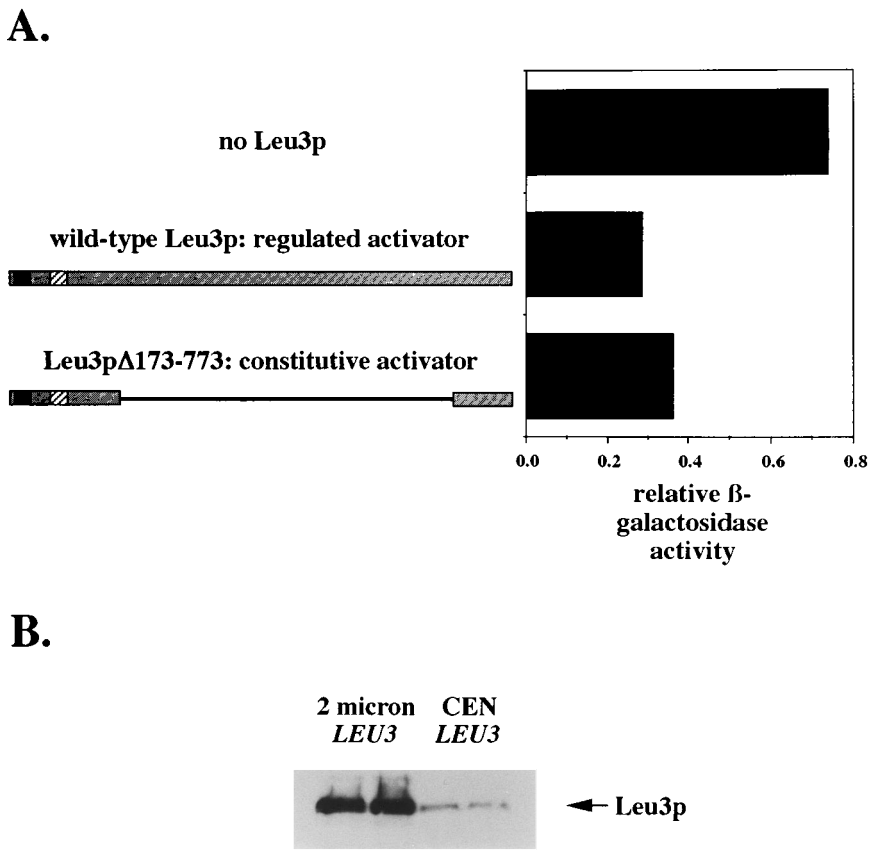

FIG. 5. (A) Reporter gene repression occurs even with low levels of Leu3p. Strain CRY102-2B was cotransformed with CEN plasmids encoding epitope-tagged wild-type Leu3p, Leu3pΔ173–773, or no Leu3p, together with each of the two reporter plasmids (with and without Leu3p-binding sites). β-Galactosidase activities were determined in duplicate for four transformants from each plasmid combination grown in high-leucine medium. The data are graphed as in Fig. 4. (The mean values for cells in which the reporter lacked Leu3p binding sites ranged from 41 to 51 units.) (B) Western blot analysis of Leu3p protein levels. Extracts were made from CRY102-2B cells (grown as for β-galactosidase assays) expressing epitope-tagged Leu3p from high-copy (2μm) and low copy (CEN) plasmids; protein from each extract was electrophoresed on a 12% acrylamide gel and subjected to Western blot analysis as described in Materials and Methods. The duplicate lanes for each protein are from cultures containing the two different reporter plasmids. The position of full-length Leu3p is indicated.

truncated protein nonfunctional in this assay, although the short N-terminal fragment is stably expressed at somewhat higher levels than Leu3pΔ143–828 and binds to DNA in a gel shift assay (27; also see the immunofluorescence data below). Addition of a heterologous activation domain from Gal4p or the herpes simplex virus transcriptional activator VP16 to the DNA-binding domain of Leu3p restores the ability to repress *RPL16-lacZ*, as shown in Fig. 6. These data confirm the role of the activation domain in the inhibition of transcription seen in this assay and indicate that the ability to participate in repression is not a special property of the Leu3p activation domain, because other activation domains can substitute for it.

Immunofluorescence confirms that Leu3p and its derivatives are localized to the nucleus. Cells expressing various epitope-tagged *LEU3* constructs were examined by indirect immunofluorescence as another assay for Leu3p localization. Cells expressing tagged Leu3p give rise to nuclear fluorescence (Fig. 7), which is not seen in the absence of the epitope tag. Not all cells in the population showed this fluorescence; the lack of consistency might be due to variability in the level of expression, because the *LEU3* constructs were present on 2μm plasmids whose copy number can vary, or it might be due to incomplete permeabilization of the cells to antibody. Still, the pattern of nuclear localization is reasonably clear. Full-length Leu3p gave this pattern whether the cells were grown in low- or high-leucine medium (the data in Fig. 7 are from cells grown in high-leucine medium), confirming that Leu3p localization is not regulated by α-IPM.

The localization of some derivatives of Leu3p was also examined by immunofluorescence. Epitope-tagged Leu3p:1–774, which lacks the activation domain, appears to be localized to the nucleus (Fig. 7). This result contrasts with the lack of repression of the reporter gene by Leu3p:1–774 in the indirect assay (Fig. 6) and suggests that this protein is not defective in localization but in some other property required for the inhibition of *RPL16-lacZ* expression. The fragment consisting of the N-terminal 142 amino acids of Leu3p also appears to be in the nucleus by immunofluorescence, as is the fusion protein Leu3p-VP16 (Fig. 7).

Immunofluorescence was also attempted with *LEU3* constructs carried on CEN plasmids, without success (data not shown); the level of expression of Leu3p from its own promoter may be too low to permit detection. The indirect assay for localization described above therefore appears to be more sensitive for detection of Leu3p than is immunofluorescence, because Leu3p expressed from a CEN plasmid was able to inhibit expression of the reporter gene (Fig. 5). The consistency between the results of the indirect assay and the immunofluorescence data suggests that overexpression of Leu3p did

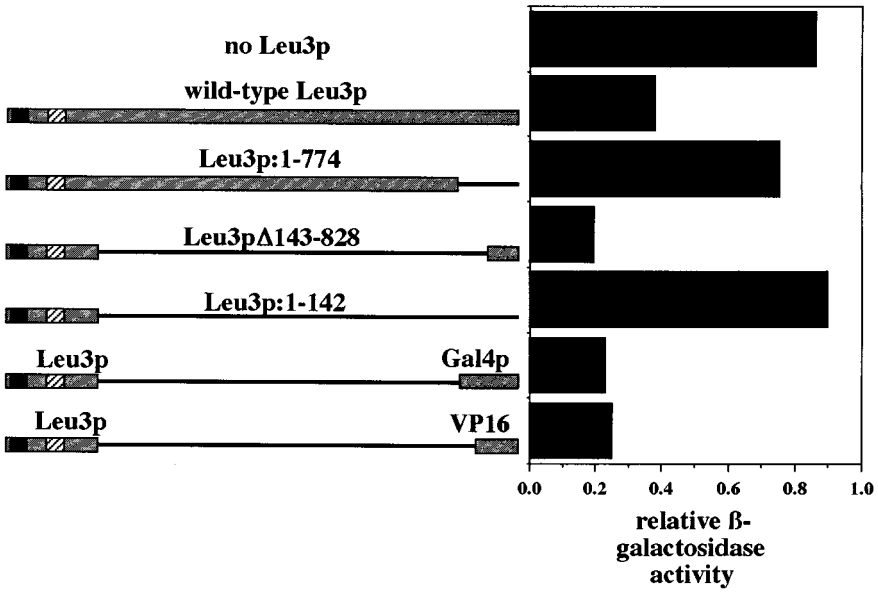

FIG. 6. Inhibition of reporter gene expression requires an activation domain in the repressor protein. Epitope-tagged wild-type Leu3p, Leu3p:1–774, Leu3pΔ143–828, Leu3p:1–142, and fusion proteins Leu3p-Gal4p and Leu3p-VP16 were assayed for their ability to repress expression of *RPL16-lacZ* from the reporter plasmids with and without Leu3p-binding sites. β-Galactosidase activities were determined for four transformants of each, grown in high-leucine medium; the data are graphed as in Fig. 4. (The mean values for cells in which the reporter lacked Leu3p binding sites ranged from 50 to 80 units.)

not lead to artifactual immunofluorescence signals; also, the observed nuclear fluorescence is not surprising, given the function of Leu3p as a transcriptional activator.

DISCUSSION

The results presented here eliminate one model for the regulation of Leu3p activity: neither leucine levels nor α-IPM levels affect the intracellular localization and DNA site occupancy of Leu3p. Previous work demonstrated that α-IPM does not greatly affect the level of Leu3p protein (10, 54) or alter the affinity of Leu3p for DNA in vitro (18). It appears that Leu3p may be bound to its regulatory sites at all times, poised to activate transcription upon receiving the appropriate signal. (The Gal4p, Put3p, Adr1p, and HSTF transcriptional activators may act similarly [3, 25, 42, 47, 50].) This signal may cause a change in Leu3p conformation or a posttranslational modification, which allows activation to take place. The participation of additional proteins in the regulation of Leu3p activity has not been ruled out, although the nature and identity of such proteins are completely unknown. An extensive screen for mutations which affect the regulation of leucine biosynthesis was carried out, but the mutations (in three new genes) isolated in the screen all caused reductions in α-IPM synthase activity rather than reductions in (apparent) Leu3p activity (17). This result is circumstantial evidence against the existence of a protein whose sole function is to regulate Leu3p activity. If such a protein does exist, it presumably interacts with some region in the internal portion of Leu3p which is required for Leu3p regulation. Alternatively, α-IPM may interact directly with the middle region of Leu3p to bring about transcriptional activation. Further work is required to determine if any of these models is correct.

The striking observation in this work is the requirement for an activation domain, joined to the Leu3p DNA-binding domain, to achieve transcriptional repression. The indirect assay described here requires Leu3p, which normally activates transcription, to serve instead as a repressor. This change of function is not unprecedented, but the mechanism of the repression seen here may be unusual. Three different repression mechanisms have been described previously.

The first type of repression is caused by proteins which bind within a promoter but are incapable of activating transcription. Both the bacterial *lexA* protein and certain Gal4p derivatives inhibit expression of a reporter gene in yeast cells when the appropriate binding site is introduced between the upstream activation sequence and the start of transcription (9, 26). This repression may occur because *lexA* or Gal4p binding interferes with the proper assembly of a transcription initiation complex at the promoter. Mutant forms of Leu3p which bind to DNA but cannot activate transcription inhibit Gcn4p-dependent basal transcription of *LEU2* in vivo (11), perhaps by a similar mechanism. Leu3p has also been reported to act as a repressor of in vitro transcription in the absence of α-IPM (49). The N-terminal region of Leu3p is sufficient for this repression, suggesting that only DNA binding is required of the repressor protein (48). The inhibition seen in the assay described here must differ from these other types of repression because it is dependent on the presence of the activation domain. Also, the Leu3p-binding sites are located downstream of the start of transcription in the localization assay but upstream in the other cases.

The finding that α-IPM must be added to an in vitro transcription reaction for Leu3p to activate transcription in vitro (49) suggests that α-IPM directly or indirectly causes a change in Leu3p which allows it to function as an activator. Whatever this change might be, it is not required for the repression of *RPL16-lacZ* expression, because repression occurs even in the strain CRY103, which cannot synthesize α-IPM. Thus, the role played by the activation domain in inhibiting *RPL16-lacZ* is distinct in some way from the role it plays in activating transcription. However, the ability of other activation domains to substitute for the Leu3p activation domain suggests that inhibition of the reporter may involve some general property

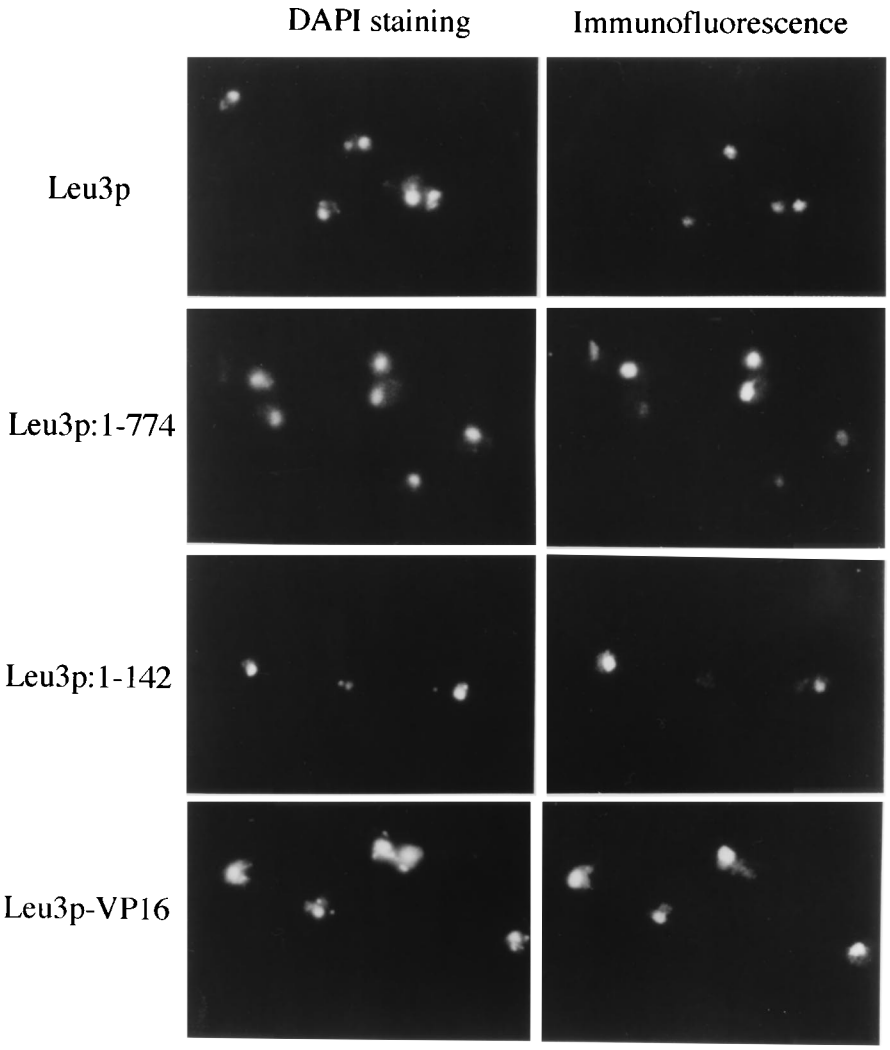

FIG. 7. Immunofluorescence of epitope-tagged Leu3p. Immunofluorescence was carried out as described in Materials and Methods on cells which expressed epitope-tagged Leu3p, Leu3p:1–774, Leu3p:1–142, or Leu3p-VP16 fusion protein, grown in medium containing a high level of leucine. Two panels are shown for each cell type; on the left is fluorescence due to staining with DAPI (4′,6-diamidino-2-phenylindole), which stains DNA, and on the right is immunofluorescence with the 12CA5 monoclonal antibody (from the same cells).

shared by activation domains, such as the ability to interact with some other protein(s).

Squelching is a second form of transcriptional repression which occurs when proteins carrying activation domains are highly overexpressed, to the point that they interfere with the normal transcription of many genes (21, 51); the inhibition is presumably due to titration of a general transcription initiation factor and is not dependent on the presence of a DNA-binding domain in the overexpressed protein. Repression of *RPL16-lacZ* expression by Leu3p does not appear to be a form of squelching, because it occurs even when Leu3p is expressed from a low-copy-number plasmid, because it requires the Leu3p-binding sites within the gene, and because it requires a form of Leu3p which can bind to DNA.

A third form of transcriptional repression, which is most similar to the repression by Leu3p in our assay, is the blockage of *E. coli* RNA polymerase elongation observed in vitro by Pavco and Steege (35) and in vivo by Deuschle et al. (14), which is caused by the binding of a protein to a site within the transcribed gene. Leu3p may similarly inhibit elongation by *S. cerevisiae* RNA polymerase; an activation domain could be required for this process because it permits the assembly of a larger or more stable complex of proteins at the Leu3p-binding sites, which is necessary for inhibition. (Such a complex could include general transcription factors such as adaptors or coactivators, proteins associated with RNA polymerase or components of the SWI/SNF complex, which are thought to influence chromatin structure [12]). It is also possible, however, that Leu3p binding downstream affects transcription initiation at the *RPL16* promoter, if the DNA is bent to allow interactions between proteins bound at distant sites. It is not possible to tell from the current data whether initiation or elongation of transcription is inhibited in this assay.

The assay described here may be more generally useful for investigating the localization of other DNA-binding proteins; a transcriptional activation domain might have to be fused to the protein of interest if it did not already contain such a domain. Both small and large Leu3p variants gave a signal in this assay (as long as the required functional regions were present), and the data therefore raise the possibility that the nuclear local-

ization signal of Leu3p is within the N-terminal 112 amino acids. The principal advantage of this method is its sensitivity to low levels of protein; in the case of Leu3p, it is more sensitive than immunofluorescence with antibodies against an introduced epitope tag.

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REFERENCES

1. **Alani, E., L. Cao, and N. Kleckner.** 1987. Method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics **116:**541–545.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York.
3. **Axelrod, J. D., J. Majors, and M. C. Brandriss.** 1991. Proline-independent binding of PUT3 transcriptional activator protein detected by footprinting in vivo. Mol. Cell. Biol. **11:**564–567.
4. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
5. **Bai, Y., and G. B. Kohlhaw.** 1991. Manipulation of the "zinc cluster" region of transcriptional activator *LEU3* by site-directed mutagenesis. Nucleic Acids Res. **19:**5991–5997.
6. **Baichwal, V. R., T. S. Cunningham, P. R. Gatzek, and G. B. Kohlhaw.** 1983. Leucine biosynthesis in yeast: identification of two genes (LEU4, LEU5) that affect α-isopropylmalate synthase activity and evidence that LEU1 and LEU2 gene expression is controlled by α-isopropylmalate and the product of a regulatory gene. Curr. Genet. **7:**369–377.
7. **Baleja, J. D., R. Marmorstein, S. C. Harrison, and G. Wagner.** 1992. Solution structure of the DNA-binding domain of Cd_2-GAL4 from *S. cerevisiae*. Nature (London) **356:**450–453.
8. **Berger, S. L., B. Piña, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg, and L. Guarente.** 1992. Genetic isolation of ADA2: a potential transcriptional adaptor required for function of certain acidic activation domains. Cell **70:**251–265.
9. **Brent, R., and M. Ptashne.** 1984. A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. Nature (London) **312:**612–615.
10. **Brisco, P. R. G., T. S. Cunningham, and G. B. Kohlhaw.** 1987. Cloning, disruption and chromosomal mapping of yeast *LEU3*, a putative regulatory gene. Genetics **115:**91–99.
11. **Brisco, P. R. G., and G. B. Kohlhaw.** 1990. Regulation of yeast *LEU2*: total deletion of regulatory gene *LEU3* unmasks *GCN4*-dependent basal level expression of *LEU2*. J. Biol. Chem. **265:**11667–11675.
12. **Carlson, M., and B. C. Laurent.** 1994. The SNF/SWI family of global transcriptional activators. Curr. Opin. Cell Biol. **6:**396–402.
13. **Cress, W. D., and S. J. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251:**87–90.
14. **Deuschle, U., R. A. Hipskind, and H. Bujard.** 1990. RNA polymerase II transcription blocked by *Escherichia coli Lac* repressor. Science **248:**480–483.
15. **Dingwall, C., and R. A. Laskey.** 1986. Protein import into the cell nucleus. Annu. Rev. Cell Biol. **2:**367–390.
16. **Dohrmann, P. R., G. Butler, K. Tamai, S. Dorland, J. R. Greene, D. J. Thiele, and D. J. Stillman.** 1992. Parallel pathways of gene regulation: homologous regulators *SWI5* and *ACE2* differentially control transcription of *HO* and chitinase. Genes Dev. **6:**93–104.
17. **Drain, P., and P. Schimmel.** 1988. Multiple new genes that determine activity for the first step of leucine biosynthesis in *Saccharomyces cerevisiae*. Genetics **119:**13–20.
18. **Friden, P., C. Reynolds, and P. Schimmel.** 1989. A large internal deletion converts yeast LEU3 to a constitutive transcriptional activator. Mol. Cell. Biol. **9:**4056–4060.
19. **Friden, P., and P. Schimmel.** 1987. *LEU3* of *Saccharomyces cerevisiae* encodes a factor for control of RNA levels of a group of leucine-specific genes. Mol. Cell. Biol. **7:**2708–2717.
20. **Friden, P., and P. Schimmel.** 1988. LEU3 of *Saccharomyces cerevisiae* activates multiple genes for branched-chain amino acid biosynthesis by binding to a common decanucleotide core sequence. Mol. Cell. Biol. **8:**2690–2697.
21. **Gill, G., and M. Ptashne.** 1988. Negative effect of the transcriptional activator GAL4. Nature (London) **334:**721–724.
22. **Guarente, L.** 1983. Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. Methods Enzymol. **101:**181–191.
23. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Hinnebusch, A. G.** 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52:**248–273.
25. **Jakobsen, B. K., and H. R. B. Pelham.** 1988. Constitutive binding of yeast heat shock factor to DNA in vivo. Mol. Cell. Biol. **8:**5040–5042.
26. **Keegan, L., G. Gill, and M. Ptashne.** 1986. Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science **231:**699–704.
27. **Kirkpatrick, C. A. R.** 1994. Ph.D. thesis. Massachusetts Institute of Technology, Cambridge.

27a. **Kirkpatrick, C. A. R.** Unpublished data.

28. **Kohlhaw, G. B.** 1983. Regulation of leucine biosynthesis in lower eukaryotes, p. 285–299. *In* K. M. Herrmann and R. L. Somerville (ed.), Amino acids: biosynthesis and genetic regulation. Addison-Wesley Publishing Co., Inc., Reading, Mass.
29. **Kolodziej, P. A., and R. A. Young.** 1991. Epitope tagging and protein surveillance. Methods Enzymol. **194:**508–519.
30. **Kraulis, P. J., A. R. C. Raine, P. L. Gadhavi, and E. D. Laue.** 1992. Structure of the DNA-binding domain of zinc GAL4. Nature (London) **356:**448–450.
31. **Leuther, K. K., J. M. Salmeron, and S. A. Johnston.** 1993. Genetic evidence that an activation domain of GAL4 does not require acidity and may form a β sheet. Cell **72:**575–585.

31a. **Loeb, J.** Personal communication.

32. **Ma, J., and M. Ptashne.** 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. Cell **48:**847–853.
33. **Marmorstein, R., M. Carey, M. Ptashne, and S. C. Harrison.** 1992. DNA recognition by GAL4: structure of a protein-DNA complex. Nature (London) **356:**408–414.
34. **Nasmyth, K., G. Adolf, D. Lydall, and A. Seddon.** 1990. The identification of a second cell cycle control on the *HO* promoter in yeast: cell cycle regulation of SWI5 nuclear entry. Cell **62:**631–647.
35. **Pavco, P. A., and D. A. Steege.** 1990. Elongation by *Escherichia coli* RNA polymerase is blocked *in vitro* by a site-specific DNA binding protein. J. Biol. Chem. **265:**9960–9969.
36. **Pfeifer, K., B. Arcangioli, and L. Guarente.** 1987. Yeast *HAP1* activator competes with the factor RC2 for binding to the upstream activation site UAS1 of the *CYC1* gene. Cell **49:**9–18.
37. **Pfeifer, K., K.-S. Kim, S. Kogan, and L. Guarente.** 1989. Functional dissection and sequence of yeast HAP1 activator. Cell **56:**291–301.
38. **Picard, D., and K. R. Yamamoto.** 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. **6:**3333–3340.
39. **Remboutsika, E., and G. B. Kohlhaw.** 1994. Molecular architecture of a Leu3p-DNA complex in solution: a biochemical approach. Mol. Cell. Biol. **14:**5547–5557.
40. **Rotenberg, M. O., and J. L. Woolford, Jr.** 1986. Tripartite upstream promoter element essential for expression of *Saccharomyces cerevisiae* ribosomal protein genes. Mol. Cell. Biol. **6:**674–687.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Selleck, S. B., and J. Majors.** 1987. In vivo DNA-binding properties of a yeast transcription activator protein. Mol. Cell. Biol. **7:**3260–3267.
43. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
45. **Silve, S., C. Volland, C. Garnier, R. Jund, M. R. Chevallier, and R. Haguenauer-Tsapis.** 1991. Membrane insertion of uracil permease, a polytopic yeast plasma membrane protein. Mol. Cell. Biol. **11:**1114–1124.
46. **Solomon, F., L. Connell, D. Kirkpatrick, V. Praitis, and B. Weinstein.** 1992. Methods for studying the cytoskeleton in yeast, p. 197–221. *In* K. L. Carraway and C. A. C. Carraway (ed.), The cytoskeleton: a practical approach. IRL Press, Oxford.
47. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. Cell **54:**855–864.
48. **Sze, J.-Y., E. Remboutsika, and G. B. Kohlhaw.** 1993. Transcriptional regulator Leu3 of *Saccharomyces cerevisiae*: separation of activator and repressor functions. Mol. Cell. Biol. **13:**5702–5709.
49. **Sze, J.-Y., M. Woontner, J. A. Jaehning, and G. B. Kohlhaw.** 1992. In vitro transcriptional activation by a metabolic intermediate: activation by Leu3

depends on α-isopropylmalate. Science **258:**1143–1145.
50. **Taylor, W. E., and E. T. Young.** 1990. cAMP-dependent phosphorylation and inactivation of yeast transcription factor ADR1 does not affect DNA binding. Proc. Natl. Acad. Sci. USA **87:**4098–4102.
51. **Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight.** 1988. Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. Genes Dev. **2:**718–729.
52. **Van Hoy, M., K. K. Leuther, T. Kodadek, and S. A. Johnston.** 1993. The acidic activation domains of the GCN4 and GAL4 proteins are not α helical but form β sheets. Cell **72:**587–594.
53. **Zhou, K., Y. Bai, and G. B. Kohlhaw.** 1990. Yeast regulatory protein LEU3: a structure-function analysis. Nucleic Acids Res. **18:**291–298.
54. **Zhou, K., P. R. G. Brisco, A. E. Hinkkanen, and G. B. Kohlhaw.** 1987. Structure of yeast regulatory gene *LEU3* and evidence that *LEU3* itself is under general amino acid control. Nucleic Acids Res. **15:**5261–5273.
55. **Zhou, K., and G. B. Kohlhaw.** 1990. Transcriptional activator *LEU3* of yeast: mapping of the transcriptional activation function and significance of activation domain tryptophans. J. Biol. Chem. **265:**17409–17412.