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Transcriptional Regulator Leu3 of *Saccharomyces cerevisiae*: Separation of Activator and Repressor Functions†

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The Leu3 protein of *Saccharomyces cerevisiae* binds to specific DNA sequences present in the 5′ noncoding region of at least five RNA polymerase II-transcribed genes. Leu3 functions as a transcriptional activator only when the metabolic intermediate α-isopropylmalate is also present. In the absence of α-isopropylmalate, Leu3 causes transcription to be repressed below basal levels. We show here that different portions of the Leu3 protein are responsible for activation and repression. Fusion of the 30 C-terminal residues of Leu3 to the DNA-binding domain of the Gal4 protein created a strong cross-species activator, demonstrating that the short C-terminal region is not only required but also sufficient for transcriptional activation. Using a recently developed Leu3-responsive in vitro transcription assay as a test system for repression (J. Sze, M. Woontner, J. Jaehning, and G. B. Kohlhaw, Science 258:1143–1145, 1992), we show that mutant forms of the Leu3 protein that lack the activation domain still function as repressors. The shortest repressor thus identified had only about 15% of the mass of the full-length Leu3 protein and was centered on the DNA-binding region of Leu3. Implications of this finding for the mechanism of repression are discussed.

The Leu3 protein (Leu3p) of *Saccharomyces cerevisiae* interacts with a *cis*-acting element (UAS_{LEU}) found upstream of several unlinked genes involved in branched-chain amino acid biosynthesis (*LEU1*, *LEU2*, *LEU4*, and *ILV2*) and nitrogen metabolism (*GDH1*) (5, 10, 25a, 32). Leu3p is the major regulator for *LEU1* and *LEU2* gene expression (3) and acts in concert with the *GCN4* gene product to regulate *LEU4* (11a, 23).

Leu3p has several remarkable features. First, its action as a transcriptional activator depends entirely on the presence of a low-molecular-weight metabolite (5, 26). This metabolite, α-isopropylmalate (α-IPM), is an early intermediate in leucine biosynthesis and is part of an intricate feedback loop that focuses on α-IPM synthase (14). Second, transcriptional activation by Leu3p and the dependence of that activation on α-IPM can be reproduced in an in vitro system (26). Third, in the absence of α-IPM, Leu3p acts as a repressor of transcription (5, 26). Leu3p thus appears to be a molecular on-off switch, where "off" means not just the absence of activation, but actual suppression of transcription below basal levels.

Three major functions of the 886-residue-long Leu3p (DNA binding, transcriptional activation, and modulation by α-IPM) have been identified, and preliminary assignments for the location of regions responsible for these functions along the primary structure of the protein have been made. The DNA-binding domain is located near the N terminus where residues 37 to 67 conform to the $Zn(II)_2Cys_6$ binuclear cluster found in a number of lower eukaryotic DNA-binding proteins, including the well-studied Gal4 protein (Gal4p) (2). A recent X-ray crystallographic analysis of a Gal4p-UAS_{GAL} complex showed that dimeric Gal4p interacts with a dyad-symmetrical CGG. . .CCG motif (19). Direct contact is established via a short helical segment of the zinc-cysteine cluster. The Leu3p-binding element, UAS_{LEU}, contains a similar motif (GCC. . .GGC), although the distance between the two contact triplets is significantly shorter than that seen with UAS_{GAL} (10). Given the fact that the designated DNA contact region of Gal4p, Leu3p, and other lower eukaryotic activators is highly conserved, it is to be expected that their interaction with DNA will follow similar principles. At the same time, there is enough variability both within the zinc-cysteine cluster (especially in its central and C-terminal portions [2]) and in the flanking regions to account for the specificity of the interaction, which may also be aided by helper proteins such as the recently discovered Egd1 of *S. cerevisiae* (22).

The transcriptional activation function of Leu3p is located near the C terminus. Prior to the work to be presented here, the strongest support for this assignment came from an analysis of C-terminal deletion mutants (33). For example, a mutant protein lacking the C-terminal 32 residues was totally inactive but bound normally to DNA. In contrast, a mutant protein in which the C-terminal 32 residues were fused directly to the DNA-binding region retained activating capability. The suspected activation domain of Leu3p has a net charge of −4 at physiological pH. This domain also has a relatively large number of hydrophobic residues, some of which fit into a pattern recently recognized by Cress and Triezenberg (8) as being part of acidic, glutamine-rich, and proline-rich activation regions.

In contrast to the DNA-binding and transcription activation functions, the α-IPM response function of Leu3p has been mapped to at least two regions of the protein. One of these regions encompasses the middle portion of Leu3p, extending from residues 174 to 773; the other is identical with the activation domain (31–33). Certain mutations within these regions create constitutive activators, i.e., molecules that activate transcription even in the absence of α-IPM.

Here we show that the 30 C-terminal residues of Leu3p constitute a self-contained activation module that causes strong, cross-species transcriptional activation. We also show that a truncated Leu3p molecule that lacks the activation domain and a Leu3p molecule that contains little more

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than the DNA-binding domain still act as repressors of transcription.

MATERIALS AND METHODS

Strains and plasmids. The following *S. cerevisiae* strains were used: XK149-3D (*MATα leu3-Δ2::HIS3 his3-Δ1 ura3-52*), a non-Leu3-producing strain (5); XK160/p*GAL1-LEU3* (*reg1-501 gal1 pep4-3 leu1 LEU4*^{fbr} *ura3-52*), a Leu3p- and α-IPM-overproducing strain carrying a p*GAL1-LEU3* plasmid (25); and XK157-3C (*MATα leu3-Δ2::HIS3 trp1-289 ura3-52*), another non-Leu3-producing strain suited to carry pKZ plasmids (32). *Escherichia coli* HB101 (Bethesda Research Laboratories) was used to maintain plasmids. The mammalian cell line used was the mouse fibroblast cell line C3H10T½, obtained from the American Type Culture Collection.

The pKZ type plasmids employed in this study are multicopy yeast-*E. coli* shuttle vectors expressing *LEU3* and derivatives of *LEU3* under the control of the yeast *PHO5* promoter; their construction has been described previously (31–33). Wild-type Leu3p is encoded by pKZ5, Leu3p-Δ12 is encoded by a simple derivative of pKZ5, and Leu3p-Δ15 is encoded by pKZ51. The exact amino acid composition of the mutant Leu3 proteins is given in the appropriate figure legends. *E. coli* plasmid pT19-LEU3(131), used for the production of the Leu3p(17-147) peptide, was constructed as follows. An *Xho*I-*Sal*I fragment obtained from plasmid pZRL (31) and encoding the 171 N-terminal residues of Leu3p was subjected to polymerase chain reaction treatment to introduce unique *Nde*I and *Bam*HI sites and to obtain a 398-bp clone encoding residues 17 to 147 of Leu3p followed by a stop codon (UGA). Leu3p(17-147) does not contain any non-Leu3 residues. After verification of the structure by DNA sequencing, the *Nde*I-*Bam*HI fragment was inserted into the polylinker region of expression vector pT7-7 (1) to give pT19-LEU3(131), which was then expressed in *E. coli* BL21(DE3)pLysS. Vector pSGL30e, used to express a *GAL4-LEU3* fusion in mouse fibroblasts, was constructed as follows. The desired *LEU3* fragment was obtained from pKZ51, the plasmid carrying the *LEU3* mutant allele *Δ15* (31, 33). This mutant allele contained an additional G following nucleotide position +2562 of *LEU3*, which created a unique *Eco*RI site. After pKZ51 was cut with *Eco*RI and *Sac*I restriction enzymes, the fragment containing the *LEU3* 3′ region from positions +2563 to +3063 (which included the stop codon centered on position +2660) was inserted into the polylinker region of vector pSG424 (20). The polylinker region of this eukaryotic expression vector adjoins the 3′ end of a truncated *GAL4* gene that encodes the Gal4p DNA-binding region [*GAL4*(1-147)] and is under the control of the simian virus 40 promoter. Following insertion, the vector was reopened at the *Eco*RI site, treated with S1 nuclease to bring the *GAL4* and *LEU3* fragments in frame, and religated. The final construct, designated pSGL30e, contained amino acid residues 1 to 147 of Gal4p and 857 to 886 of Leu3p, linked by two additional residues (Pro and Ala) that arose from the cloning procedure. The *GAL4-LEU3* fragment was subcloned in pBluescript SK(−) (Stratagene Cloning Systems, La Jolla, Calif.) and sequenced to confirm the in-frame junction.

Growth conditions. Yeast cells were grown aerobically at 30°C. Strain XK149-3D was grown in YPD medium (1% yeast extract, 2% Bacto Peptone, 2% D-glucose). Strain XK160/p*GAL1-LEU3* was grown and induced as described elsewhere (25). Strain XK157-3C was grown in SD medium (0.67% Bacto yeast nitrogen base without amino acids, 2% D-glucose) supplemented with 2 mM L-leucine and 1 mM L-isoleucine. After an optical density at 600 nm of about 1.0 was reached, the cells were harvested, washed once with sterile distilled water, and resuspended in low-phosphate medium to activate the *PHO5* promoter (32). Growth was continued for two to three more generations.

E. coli cells were grown in L broth (25) containing ampicillin (100 μg/ml) alone or with chloramphenicol (50 μg/ml), as needed.

Mouse fibroblast C3H10T½ cells were maintained as described previously (29).

Preparation of cell extracts. Extracts from yeast cells for the purification of Leu3p (25) or for in vitro transcription assays (26) were prepared as described previously. Mouse fibroblast extracts were likewise prepared by published procedures (21, 28).

Protein purification. Wild-type Leu3p was purified from strain XK160/p*GAL1-LEU3* by a procedure, described in detail elsewhere (25), that included polyethylenimine treatment, ammonium sulfate fractionation, heat treatment, and DNA affinity chromatography. Leu3p(17-147) was expressed in *E. coli* BL21(DE3)pLysS under the control of the T7 promoter. A 1-liter culture was grown at 37°C to an optical density at 600 nm of 0.7, and the expression of Leu3p(17-147) was induced by adding isopropylthiogalactoside to a final concentration of 0.4 mM. After 3 h, the cells were harvested (optical density at 600 nm of ~1.5) and resuspended in 30 ml of lysis buffer (200 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] · NaOH [pH 7.9], 5 mM $MgCl_2$, 50 μM $ZnSO_4$, 1 mM EDTA, 20% [vol/vol] glycerol, 4 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 3 mg of lysozyme per ml, and 0.5% Triton X-100), referred to hereafter as buffer A. The cells were further disrupted by two passages through a French pressure cell at 138 MPa. All subsequent procedures, except the heat treatment, were performed at 0 to 4°C. The lysate was clarified by centrifugation for 1 h at 41,000 × *g*. A 5% solution of polyethylenimine was added to the supernatant solution to a final concentration of 0.15%. After the solution was stirred for 10 min, the precipitate was removed by centrifugation for 10 min at 12,000 × *g*. A saturated $(NH_4)_2SO_4$ solution containing 25 mM HEPES · NaOH (pH 7.9) and 1 mM EDTA was added to the supernatant solution until a concentration of $(NH_4)_2SO_4$ of 65% was reached. After the solution was stirred overnight, the precipitate was collected by centrifugation (30 min at 31,000 × *g*) and redissolved in buffer B (identical to buffer A except that dithiothreitol was omitted and lysozyme and Triton X-100 were replaced with 0.02% Nonidet P-40). The resulting solution was divided into 0.5-ml aliquots. An equal volume of buffer B preequilibrated at 85°C was added to each aliquot, and the mixture was further incubated at 85°C for 3 min before being rapidly cooled on ice. Denatured protein was removed by centrifugation at 31,000 × *g* for 20 min. The supernatant solution was dialyzed against several changes of buffer C (identical to buffer A except that dithiothreitol was replaced with 0.1% β-mercaptoethanol and $MgCl_2$ was omitted) containing 80 mM NaCl. Sonicated salmon sperm DNA was added at 12.5 μg/ml of dialysate. After the mixture was allowed to stand for 20 min, the mixture was loaded onto a UAS_{LEU} affinity column (25) preequilibrated with buffer C containing 80 mM NaCl. The column was eluted with 10 column volumes (each) of 80, 200, and 300 mM NaCl in buffer C and then with 6 column volumes (each) of 1 and 2 M NaCl in buffer C. The appearance of Leu3p(17-147) was

monitored by electrophoretic mobility shift assays. The majority of the peptide eluted at the 1 M step. The appropriate fractions were pooled and dialyzed against two changes of buffer D [25 mM HEPES · NaOH (pH 7.9), 50 μM $ZnSO_4$, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 60 mM monopotassium glutamate, 50% (vol/vol) glycerol, and 0.2% β-mercaptoethanol]. The purified protein was stored at −70°C and was stable for at least 6 months, even when thawed and frozen repeatedly. About 2 to 3 mg of Leu3p(17-147) could be obtained from 1 liter of culture. The final preparation was approximately 95% pure.

Transformation and transfection procedures. Yeast cells were transformed as described by Ito et al. (12). *E. coli* cells were transformed by the calcium chloride procedure essentially by the method of Maniatis et al. (18). Transfection of fibroblasts was performed by published procedures (30).

DNA manipulation. Plasmid isolation, restriction enzyme digestion, Klenow enzyme treatment, S1 nuclease digestion, DNA ligation, and DNA sequencing were performed as described previously (4, 5, 18, 32).

Electrophoretic mobility shift assays. DNA binding of Leu3p and derivatives was assayed as described elsewhere (32, 33). $UAS_{L\text{-}2a}$, a 30-bp oligomer containing the UAS_{LEU} sequence of the *LEU2* promoter, was used as a probe.

In vitro transcription assays. In vitro transcription using whole-cell yeast preparations was performed as previously described (26, 27). Two DNA templates were used. The first, or UAS^- template, was identical to plasmid pJJ469 (26). pJJ469 contained a polylinker region followed by a modified *CYC1* promoter fused to a 377-bp sequence lacking guanine nucleotides in the nontranscribed strand (G-less cassette). The modified *CYC1* promoter consisted of positions −139 to −35 (15), except the G at position −68 was missing and there was a substitution of C for G at position −35. The promoter contained a TATA box designated 2α (15). The second template, designated the $(UAS_{LEU})_2$ template, was identical to plasmid pJJ482 (26). pJJ482 differed from pJJ469 in two ways: it contained a head-to-head repeat of the Leu3p binding element of yeast *LEU2* in the polylinker region, and its G-less cassette was shorter by 99 bp. This latter feature allowed the simultaneous use of UAS^- and $(UAS_{LEU})_2$ templates in transcription assays. The distance from the center of the proximal UAS_{LEU} element to the center of the TATA box in pJJ482 was 45 bp. Plasmids pJJ469 and pJJ482 were gifts from J. Jaehning and M. Woontner, Indiana University. When purified proteins were used, they were preincubated for 15 min at 25°C with the transcription reaction mixture (27). Reactions were started with the addition of extract and were continued for 30 min at 25°C.

Oligonucleotide synthesis and purification. Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer by the Laboratory for Macromolecular Structure, Purdue University. They were purified as previously described (5).

RESULTS

The 30 C-terminal amino acid residues of Leu3p are sufficient for strong, self-contained, cross-species transcriptional activation. It had previously been shown that a mutant form of Leu3p that lacked the 32 C-terminal residues had lost its ability to stimulate expression of the *LEU2* gene while still binding normally to the UAS_{LEU} target (33). This result suggested that the C-terminal region was the only sequence of Leu3p necessary for transcriptional activation and might be a self-contained transcription activation domain. We therefore asked whether the C-terminal region of Leu3p would stimulate transcription in a heterologous setting, e.g., when attached to a non-Leu3 DNA-binding region, and in eukaryotic cells other than *S. cerevisiae*. To answer these questions, residues 857 to 886 (i.e., the 30 C-terminal residues) of Leu3p were fused to a peptide containing residues 1 to 147 of Gal4p. This part of Gal4p contains the DNA-binding domain (13). A plasmid encoding either the fusion peptide or the Gal4 peptide under the control of the simian virus 40 promoter was then introduced into mouse fibroblast cells together with a plasmid containing the bacterial chloramphenicol acetyltransferase (CAT) gene as a reporter gene. The CAT gene was under the control of a promoter that consisted of the E1b TATA box and five upstream Gal4p-binding sites (Fig. 1A). The presence of the 30 C-terminal residues of Leu3p elicited a clear CAT gene activation response (Fig. 1C) that was similar to the response seen with some mammalian activators such as E1a and MRF (data not shown), indicating that the 30 C-terminal residues of Leu3p constitute a strong general eukaryotic activation domain. This domain is now one of the shortest natural eukaryotic activation domains known.

A severely truncated form of Leu3p is still a repressor. Leu3p-dependent transcriptional activation can be successfully reconstituted in vitro (26). By using a transcriptionally competent extract from non-Leu3-producing yeast cells and UAS_{LEU}-containing templates, it was shown previously that the addition of highly purified Leu3p strongly stimulated transcription when α-IPM was also present. In the absence of α-IPM, Leu3p caused significant (up to fivefold) suppression of transcription (26). In an attempt to further exploit the in vitro transcription system for the functional analysis of Leu3p, we tested the behavior in vitro of several mutant forms of Leu3p. The mutants are depicted in Fig. 2. Initially, their DNA-binding potential was examined by electrophoretic mobility shift assays (Fig. 3). As expected, non-Leu3-producing cells did not exhibit a shift. Leu3-overproducing cells, purified Leu3p protein, and Leu3-Δ15 cells gave rise to the two shifted bands typically seen with wild-type Leu3p (32, 33); Leu3-Δ12 gave rise to one shifted band, as did the Leu3p(17-147) peptide. Potential reasons for these differences in Leu3p-DNA complex formation include different phosphorylation states of Leu3p (25) and/or different oligomeric states.

In vitro transcription tests of the mutant proteins were performed as previously described (26, 27; see also Materials and Methods). The DNA templates used contained either no UAS element or a pair of UAS_{LEU} elements upstream from a *CYC1* TATA box which in turn was linked to a G-less cassette. The presence of the G-less cassettes allowed the specific identification of RNase T_1-resistant transcripts. To compare basal-level and UAS_{LEU}-dependent transcription, both UAS^- and UAS_{LEU}-containing templates were included in the same assay mixture. Figure 4 shows the in vitro behavior of wild-type Leu3p and two deletion proteins. When an extract from cells producing wild-type Leu3p (labeled $LEU3^{++}$ in Fig. 4) was compared with an extract from cells lacking the *LEU3* gene (*leu3-Δ2*), it was evident that Leu3p by itself caused inhibition of basal-level transcription (compare lane 2 with lane 1), while Leu3p in the presence of α-IPM caused strong activation (lane 3). The mutant protein Leu3-Δ15 lacks the 32 C-terminal residues of Leu3p (33). In vivo assays showed that this protein has lost its ability to activate the *LEU2* gene. In the in vitro assay, Leu3-Δ15 extract not only failed to function as an activator

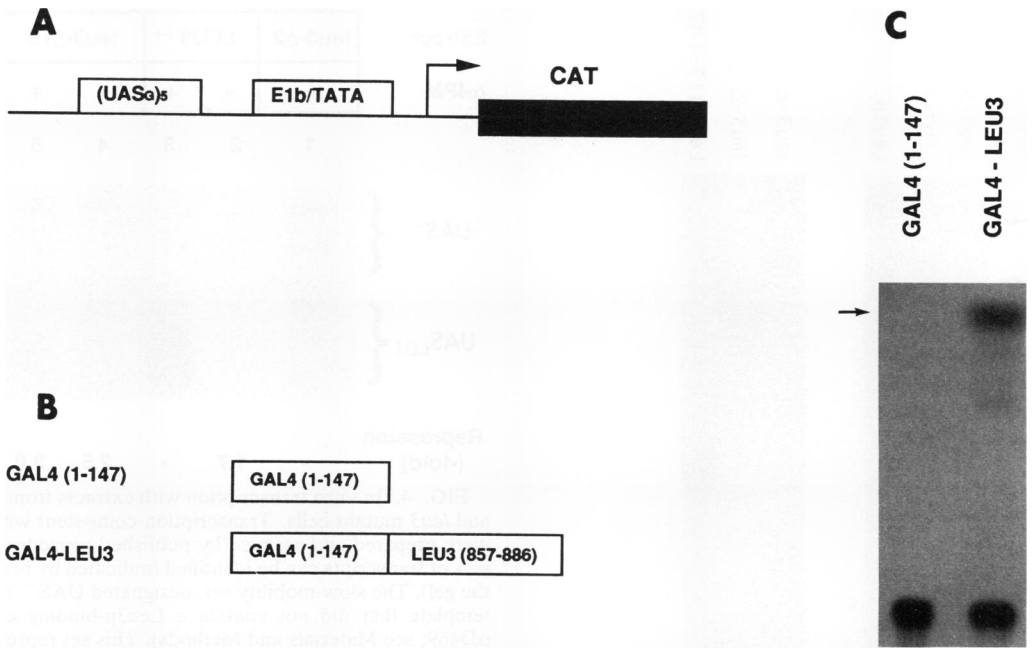

FIG. 1. A peptide containing the 30 C-terminal residues of Leu3p causes transcriptional activation in mouse fibroblasts. (A) Elements of the reporter plasmid (20). $(UAS_G)_5$, cluster of five binding sites for Gal4p; E1b/TATA, TATA box of the E1b promoter. (B) Fibroblast-expressed peptides. The GAL4-LEU3 peptide contains two additional amino acids (Pro and Ala) at the junction (see Materials and Methods). (C) Result of CAT assays. Transfections and assays were performed as described by Yutzey and Konieczny (29). The arrow indicates the position of acetylated [^{14}C]chloramphenicol.

but behaved as a repressor, causing a 2.5- to 3-fold reduction of gene expression, irrespective of the presence or absence of α-IPM (Fig. 4, lanes 4 and 5). Figure 4 also shows the behavior of the extract from *leu3-Δ12* cells (lanes 6 and 7). Leu3-Δ12 contains the DNA-binding region and the activation domain of Leu3p but lacks 600 residues of the central portion of the protein (32). In vitro and in vivo, Leu3-Δ12 behaves as a strong, α-IPM-independent activator.

To further delimit the region of Leu3p responsible for repression, we used a recombinant peptide, consisting of residues 17 to 147 of Leu3p, that contained little more than the DNA-binding region of Leu3p (Fig. 2). We found that even this severely truncated form of Leu3p caused repression. Since the Leu3p(17-147) peptide could be purified to near homogeneity, we were able to use it in a titration experiment where a transcription-competent Leu3 null extract was supplied with increasing concentrations of the peptide. Figure 5 shows the results of such an experiment. Repression, expressed as the ratio of UAS_{LEU} to UAS^- transcripts, increased with increasing peptide concentrations and appeared to level off at about 20% of the basal-level transcription seen in the absence of Leu3p(17-147). A very

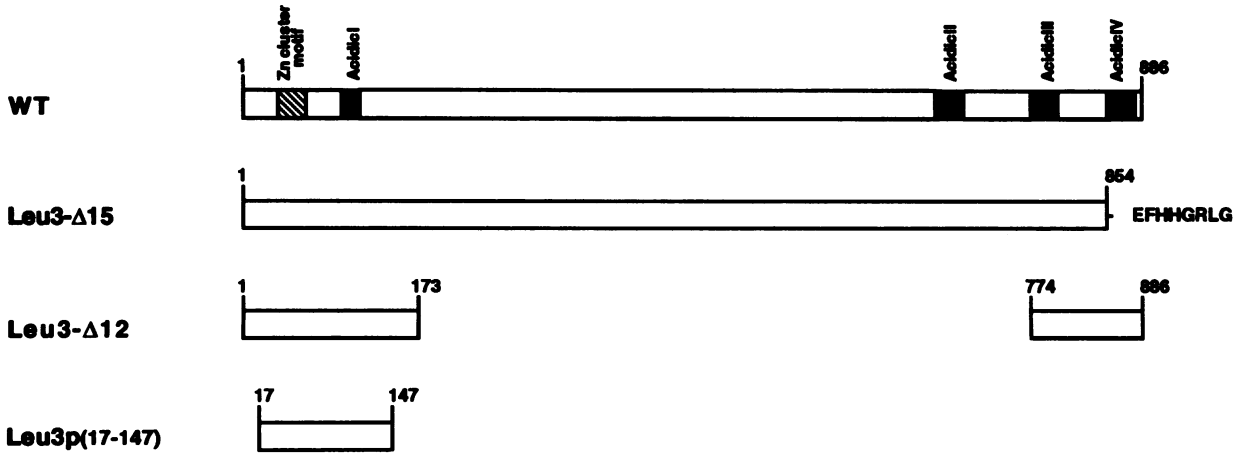

FIG. 2. Wild-type and mutant forms of Leu3p. Numbers on the bars indicate amino acid positions. Leu3-Δ15 lacks the C-terminal 32 residues of Leu3p, having acquired eight non-Leu3p residues instead (33). Leu3-Δ12 contains 173 N-terminal residues of Leu3 attached directly to the C-terminal portion of the protein at residue 774; the total length of Leu3-Δ12 is 286 residues (32). The Leu3p(17-147) peptide contains residues 17 to 147 and was constructed as outlined in Materials and Methods. WT, wild type.

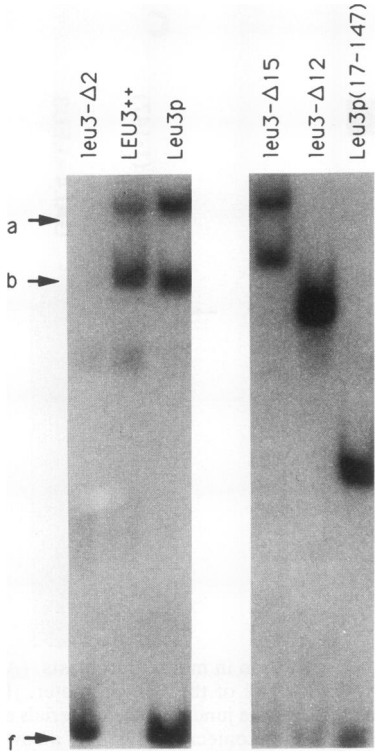

FIG. 3. Electrophoretic mobility shift assays with Leu3p and derivatives. The assays were performed as previously described (32, 33; see also Materials and Methods). The following amounts of proteins or cell extracts were used: *leu3-Δ2* cells (extract from non-Leu3-producing cells), 60 μg; *LEU3*$^{++}$ cells (extract from Leu3p-overproducing cells), 60 μg; Leu3p (purified protein), 1.7 ng; *leu3-Δ15* cells (extract from Leu3-Δ15-producing cells), 60 μg; *leu3-Δ12* cells (extract from Leu3-Δ12-producing cells), 60 μg; and Leu3p(17-147) (purified peptide), 1.2 ng. The amount of probe in each lane was 0.8 ng. The positions of the two shifted complexes routinely seen with full-length Leu3p (25, 32, 33) and of free DNA are indicated by the arrows labeled a, b, and f, respectively. The slight mobility differences between the crude and highly purified preparations of wild-type Leu3p are probably due to factors such as differences in salt concentration and total protein concentration.

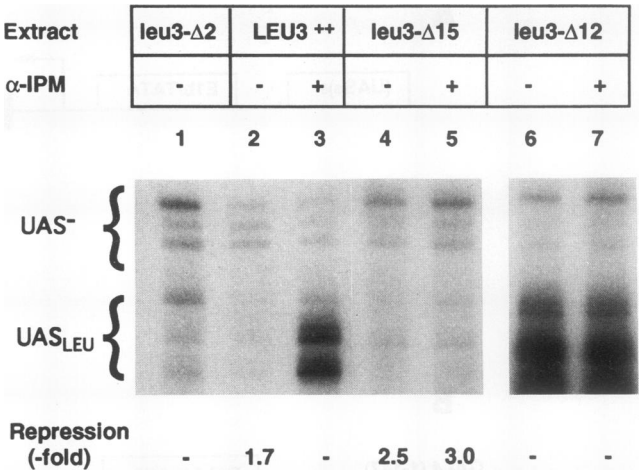

FIG. 4. In vitro transcription with extracts from wild-type *LEU3* and *leu3* mutant cells. Transcription-competent whole-cell extracts were prepared and assayed by published procedures (26, 27). Two sets of transcripts can be identified (indicated by braces to the left of the gel). The slow-mobility set, designated UAS$^-$, originated from a template that did not contain a Leu3p-binding element (plasmid pJJ469; see Materials and Methods). This set represents basal-level expression and served as an internal control in each lane. The fast-mobility set, designated UAS$_{LEU}$, originated from a template that contained two UAS$_{LEU}$ elements arranged in tandem (plasmid pJJ482; see Materials and Methods). The 30-μl reaction mixtures contained 0.3 μg of each template. α-IPM (natural isomer) was present at a final concentration of 1 mM where indicated (+ lanes). Lane 1, extract from *leu3-Δ2* cells (non-Leu3-producing); lanes 2 and 3, extract from cells overproducing Leu3p (wild-type form); lanes 4 and 5, extract from Leu3-Δ15-producing cells; lanes 6 and 7, extract from Leu3-Δ12-producing cells. The fold repression numbers underneath lanes 2, 4, and 5 were obtained from densitometric scanning and summing of the densities of each set of transcripts and represent the ratio of UAS$^-$ to UAS$_{LEU}$. The numbers were normalized with respect to the ratio of transcripts observed in lane 1.

similar repression plateau had been reached with full-length Leu3p (26).

DISCUSSION

RNA polymerase II-dependent transcription initiation is known to be subject to both positive and negative regulation. As a rule, activation of transcription is brought about by *trans*-acting factors that bind to *cis* elements located at variable distances from the point of transcription initiation. Activators are thought to increase the initiation rate by interacting with one or more components of the basal transcription apparatus, either directly or through coactivator molecules (for a recent review, see reference 11). In apparent contrast to activation, gene-specific repression of transcription is achieved in many different ways. Although the diversity of repression mechanisms defies easy categorization, certain distinctions can be made. One important question is whether a repressor acts mainly by preventing activation or by actually suppressing transcription below basal levels. The mammalian thyroid hormone receptor, TRα, and its oncogenic derivative, v-*erbA*, are examples of repressors that actively suppress transcription (9). TRα reduces basal-level expression of a reporter gene by about fivefold, as long as the cognate hormone is absent. In the presence of thyroid hormone, strong activation is observed. The v-*erbA*-encoded protein, which has lost the ability to bind thyroid hormone, is a constitutive repressor, reducing reporter gene expression below basal levels whether or not hormone is present. Another example of an active repressor is the "yin and yang" protein YY1, a member of the GLI-*Krüppel*-related family of human zinc finger proteins (24). The YY1 protein causes strong inhibition of transcription in different promoter contexts. Inhibition can be relieved and turned into activation by the adenovirus E1a protein. It is not clear whether the E1a protein brings about a conformational change in YY1, associates with YY1, or causes an inhibitory factor to dissociate from YY1.

It would appear that Leu3p has important features in common with TRα and YY1, as far as their dual function as repressor and activator is concerned. What makes Leu3p unique is that its repressor-to-activator conversion depends on the low-molecular-weight metabolite α-IPM (26). Since Leu3p binds to its target DNA irrespective of the presence of α-IPM (5), the switch from the repressing to activating mode probably takes place within the Leu3p-DNA complex. As a

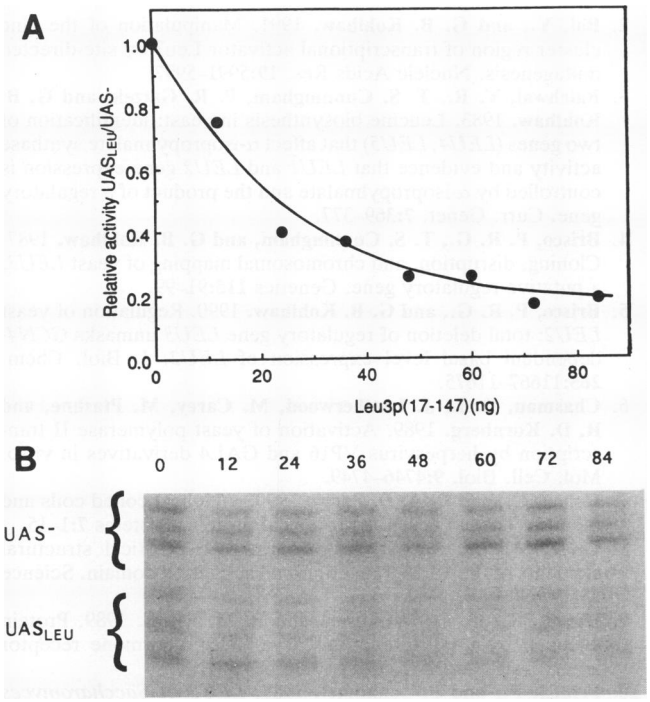

FIG. 5. Repression of basal-level transcription by Leu3p(17-147). In vitro transcription was performed with a constant amount of extract prepared from non-Leu3-producing cells and with the indicated amounts of a highly purified preparation of Leu3p(17-147). α-IPM was absent. The ratios of UAS_{LEU}-derived transcripts over UAS^--derived transcripts plotted (A) were obtained from the autoradiography results (B). The lanes of panel B are placed directly underneath the corresponding points of panel A. Transcription was quantitated by densitometric scanning and summing of each set of transcripts (see also the legend to Fig. 4 and Materials and Methods). The UAS_{LEU}/UAS^- ratios were plotted after normalization with respect to the ratio seen in the absence of Leu3p(17-147) (leftmost lane).

first step toward elucidating the mechanism of repression, we have shown here that a severely truncated form of the Leu3 protein [Leu3p(17-147)] can cause as much repression as full-length Leu3p. In the present context, repression is defined as a lowering of the rate of transcription below the basal level seen in the absence of the regulator. Both the truncated Leu3p and the full-length Leu3p repress transcription four- to fivefold, suggesting that the 131-residue peptide contains all the information necessary for repression. Some structural details of the repressing peptide are shown in Fig. 6. The most prominent feature is the cysteine-rich DNA-binding region, located between positions 37 and 67 (2, 32). This region is fully functional, since Leu3p(17-147) binds to UAS_{LEU} with an affinity constant that is similar to that of wild-type Leu3p (23a). On the C-terminal side of the DNA-binding motif, there is a basic region (residues 70 to 81; net charge, +6) that is followed by a heptad repeat (residues 85 to 99). The latter has the potential to form a stable amphipathic α-helix, with large hydrophobic residues (three leucines, one isoleucine, and one phenylalanine) occupying positions a and d (7). This arrangement is very similar to that seen with Gal4p (19), and the region containing residues 85 to 99 may therefore, by analogy to Gal4p, function as a dimerization element. Other recognizable features of Leu3p(17-147) are (i) a basic region near the N terminus (residues 23 to 34; net charge, +6) that shows some similarity to nuclear uptake signals and (ii) a short acidic stretch in the C-terminal half of the peptide (residues 109 to 117; net charge, −5) whose function is unknown; deletion of residues 110 to 117 from full-length Leu3p had only minor effects on transcriptional activation and modulation (32). The Leu3p (17-147) peptide does not contain alanine-rich regions or regions with a high density of proline and glutamine residues. Such regions have been shown to be essential for the repressor function of the *Drosophila Krüppel* protein (16) and of the WT1 Wilm's tumor gene product (17).

It has recently been shown that Leu3p is subject to phosphorylation and dephosphorylation (25). We believe, however, that phosphorylation is not directly involved in repression, since repression occurs even when the recombinant peptide Leu3p(17-147) is used. To explain repression by Leu3p(17-147), we are currently considering the following models. (i) The peptide (and, by implication, the full-length Leu3 protein in the absence of α-IPM) may interact directly with a component(s) of the transcription initiation apparatus, exerting an inhibitory effect. (ii) Repression may be an indirect effect brought about by protein-protein interactions. That is, Leu3p(17-147) may interact with another protein

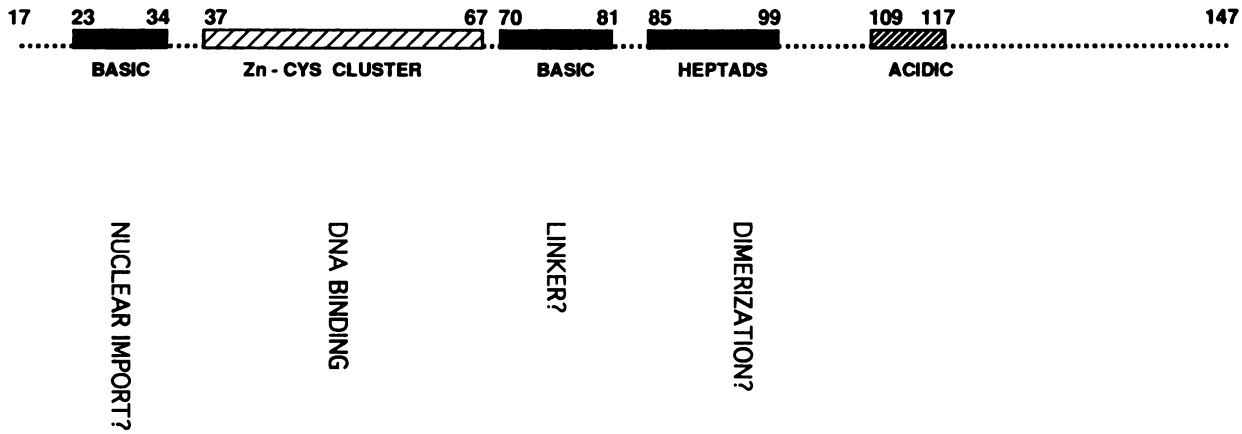

FIG. 6. Structural elements of the repressing peptide Leu3p(17-147). See the Discussion for further explanation. The numbers are amino acid positions.

which would then inhibit transcription. This model includes the possibility of heterodimer formation. (iii) Repression may be an indirect effect brought about by DNA-protein interactions. For example, Leu3p(17-147) may cause a conformational change, e.g., bending of the DNA to which it binds; the altered configuration might then cause inhibition of transcription.

Previous in vivo observations with the *LEU2* promoter led to the assumption that there are two basal levels of expression. The level seen in non-Leu3-producing cells was defined as basal level I. Under other conditions, e.g., in cells that produced normal Leu3p but no α-IPM, a further decreased level defined as basal level II was observed. In view of the in vitro transcription results, we can now equate basal level II expression with repression in vivo.

Two observations can be used to argue against the possibility that the Leu3p-mediated repression of transcription is an artifact of the in vitro transcription system. First, when in vitro transcription was performed essentially as described here but with a UAS_{GAL} template and a DNA-binding Gal4 peptide [Gal4p(1-147)], basal-level expression was diminished by only about 10% (12a). Independently, Gal4p(1-147) had been shown not to have any effect on transcription in a yeast nuclear extract (6). These results strongly suggest that the effect given by Leu3p(17-147) is specific, and since the UAS_{GAL} and UAS_{LEU} elements are located in similar relative positions on the templates, they also argue against the possibility that repression is due to simple steric hindrance of the basal transcription apparatus by the Leu3p peptide. The fact that the small Leu3p(17-147) peptide and the full-length Leu3p protein can both reduce basal transcription to about the same extent is likewise inconsistent with the steric hindrance idea.

The properties of *LEU3* mutant strains also provide clues for the physiological significance of Leu3p-mediated repression. Cells from which the *LEU3* gene had been deleted showed considerable leakiness, i.e., they still grew at about half the rate of wild-type cells (in the absence of extraneous leucine) (5). Cells elaborating a repressing form of the Leu3 protein grew much more slowly. These observations indicate that the mere absence of the Leu3p–α-IPM activating system does not suffice to arrest the biosynthesis of leucine and suggest that repression of *LEU2* (and possibly *LEU1*) expression is necessary to curb a potential waste of metabolic energy.

The ability of the in vitro transcription system employed here to describe the in vivo behavior of Leu3p lends credence to the notion that the in vitro assays are a good reflection of what goes on in the cell. The in vitro system is currently being utilized for an extensive study of the activation, repression, and α-IPM response functions of Leu3p.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology, vol. 2. John Wiley & Sons, Inc., New York.
2. **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.
3. **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.
4. **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.
5. **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.
6. **Chasman, D. I., J. Leatherwood, M. Carey, M. Ptashne, and R. D. Kornberg.** 1989. Activation of yeast polymerase II transcription by herpesvirus VP16 and GAL4 derivatives in vitro. Mol. Cell. Biol. **9**:4746–4749.
7. **Cohen, C., and D. A. D. Parry.** 1990. α-Helical coiled coils and bundles: how to design an α-helical protein. Proteins **7**:1–15.
8. **Cress, W. D., and S. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251**:87–90.
9. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by v-*erbA* functions as a thyroid-hormone receptor antagonist. Nature (London) **339**:593–597.
10. **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.
11. **Gill, G., and R. Tjian.** 1992. Eukaryotic coactivators associated with the TATA box binding protein. Curr. Opin. Genet. Dev. **2**:236–242.

11a. **Hu, Y., and G. Kohlhaw.** Unpublished results.

12. **Ito, H., Y. Fukaka, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.

12a. **Jaehning, J., and M. Woontner.** Personal communication.

13. **Johnston, M.** 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **51**:458–476.
14. **Kohlhaw, G. B.** 1990. The leucine biosynthetic pathway in yeast: compartmentation, enzyme regulation, gene expression, p. 33–42. *In* Z. Barak, D. M. Chipman, and J. V. Schloss (ed.), Biosynthesis of branched-chain amino acids. VCH, Weinheim, Germany.
15. **Li, W., and F. Sherman.** 1991. Two types of TATA elements for the *CYC1* gene of the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**:666–676.
16. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. Drosophila *Krüppel* protein is a transcriptional repressor. Nature (London) **346**:76–79.
17. **Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rausher.** 1991. Transcriptional repression by the WT1 Wilms tumor gene product. Science **253**:1550–1553.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **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.
20. **Martin, K. J., J. W. Lillie, and M. R. Green.** 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. Nature (London) **346**:147–152.
21. **Nielsen, D. A., J. Chou, A. J. Mackrell, M. J. Casadaban, and D. F. Steiner.** 1983. Expression of a preproinsulin-β-galactosidase gene fusion in mammalian cells. Proc. Natl. Acad. Sci. USA **80**:5198–5202.
22. **Parthun, M. R., D. A. Mangus, and J. A. Jaehning.** 1992. The *EGD1* product, a yeast homolog of human BTF3, may be involved in GAL4 DNA binding. Mol. Cell. Biol. **12**:5683–5689.

23. **Peters, M. H., J. P. Beltzer, and G. B. Kohlhaw.** 1990. Expression of the yeast *LEU4* gene is subject to four different modes of control. Arch. Biochem. Biophys. **276**:294–298.
23a. **Remboutsika, E.** Unpublished results.
24. **Shi, Y., E. Seto, L. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-*Krüppel*-related protein, and relief of repression by adenovirus E1a protein. Cell **67**:377–388.
25. **Sze, J., and G. B. Kohlhaw.** 1993. Purification and structural characterization of transcriptional regulator Leu3 of yeast. J. Biol. Chem. **268**:2505–2512.
25a. **Sze, J.** 1993. The Leu3 protein: a metabolite-dependent transcriptional activator that is also a repressor. Ph.D. thesis. Purdue University, West Lafayette, Ind.
26. **Sze, J., M. Woontner, J. 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.
27. **Woontner, M., P. A. Wade, J. Bonner, and J. A. Jaehning.** 1991. Transcriptional activation in an improved whole-cell extract from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**:4555–4560.
28. **Yutzey, K. E., R. L. Kline, and S. F. Konieczny.** 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. **9**:1397–1405.
29. **Yutzey, K. E., and S. F. Konieczny.** 1992. Different E-box regulatory sequences are functionally distinct when placed within the context of the troponin I enhancer. Nucleic Acids Res. **20**:5105–5113.
30. **Yutzey, K. E., S. J. Rhodes, and S. F. Konieczny.** 1990. Differential *trans*-activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. Mol. Cell. Biol. **10**:3934–3944.
31. **Zhou, K.** 1991. A molecular switch: structure-function study of yeast regulatory protein Leu3. Ph.D. thesis. Purdue University, West Lafayette, Ind.
32. **Zhou, K., Y. Bai, and G. B. Kohlhaw.** 1990. Yeast regulatory protein Leu3: a structure-function analysis. Nucleic Acids Res. **18**:291–298.
33. **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.