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Manipulation of the 'zinc cluster' region of transcriptional activator *LEU3* by site-directed mutagenesis

Yinglin Bai and Gunter B.Kohlhaw*
Department of Biochemistry, Purdue University, West Lafayette, IN 47907, USA

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ABSTRACT

The transcriptional activator *LEU3* of *Saccharomyces cerevisiae* belongs to a family of lower eukaryotic DNA binding proteins with a well-conserved DNA binding motif known as the $Zn(II)_2Cys_6$ binuclear cluster. We have constructed mutations in *LEU3* that affect either one of the conserved cysteines (Cys47) or one of several amino acids located within a variable subregion of the DNA binding motif. *LEU3* proteins with a mutation at Cys47 were very poor activators which could not be rescued by supplying Zn(II) to the growth medium. Mutations within the variable subregion were generally well-tolerated. Only two of seven mutations in this region generated poor activators, and both could be reactivated by Zn(II) supplements. Three of the other five mutations gave rise to activators that were better than wild type. One of these, His50Cys, exhibited a 1.5 fold increase in *in vivo* target gene activation and a notable increase in the affinity for target DNA. The properties of the His50Cys mutant are discussed in terms of a variant structure of the DNA binding motif. During the course of this work, evidence was obtained suggesting that only one of the two *LEU3* protein-DNA complexes routinely seen actually activates transcription. The other (which may contain an additional protein factor) does not.

INTRODUCTION

The *LEU3* gene product of *S.cerevisiae* has been implicated in the transcriptional activation of several genes of the branched-chain amino acid pathways, most notably *LEU1*, *LEU2*, *LEU4*, and *ILV2* (1–4). Analysis of *LEU3* mutants in this laboratory (5, 6) and elsewhere (7) has led to a tentative mapping of three major functions to circumscribed regions of the 886 residues long protein. The region responsible for specific binding to the upstream activating sequence (UAS_L) of *LEU2* is contained within the N-terminal 110 amino acid residues, although other regions may have a stabilizing function (5). The C-terminal 32 amino acid residues are essential for target gene activation, and structures within this region are believed to interact with the transcription machinery (6). A large central portion of the protein, extending from residue 174 to residue 773, is important for the modulation function of *LEU3*, i.e. the conversion of *LEU3* from an inactive (yet DNA binding-competent) to an active form in response to α-isopropylmalate (α-IPM), an intermediate in leucine biosynthesis. Mutant proteins lacking the central portion are constitutive activators (5, 7). Certain residues within the designated activation domain are also important for modulation (6, and K. Zhou and G. Kohlhaw, unpublished observations). Based on this evidence, a model has been proposed according to which DNA-bound *LEU3* protein exists in an equilibrium between an inactive, 'folded-back' and an active, 'extended' conformation (6). The model predicts that the equilibrium would be shifted towards the active form whenever the intranuclear concentration of the signal molecule α-isopropylmalate reaches a certain threshold value.

The *LEU3* protein contains a cysteine- and basic amino acid-rich motif within the DNA binding region that is also found in at least eleven other transcriptional activators from lower eukaryotes (Figure 1). The cysteine motif of *GAL4*, the best-studied member of this family of proteins, had initially been likened to the 'zinc finger' identified in a number of other proteins including factor TFIIIA of *X.laevis* and the regulator *ADR1* of yeast (8, 9). Zn(II) was supposed to be complexed by two pairs of cysteine residues, creating a 'loop' of amino acids (16 residues in the case of the *GAL4* protein) believed to make contact with DNA. We rejected the zinc finger analogy for *LEU3* and similar proteins since it did not assign any function to two of the six highly conserved cysteines or other highly conserved residues (10). Indeed, recent NMR structure analyses of the *GAL4* protein showed the motif to form a 'cloverleaf'-like $Zn(II)_2Cys_6$ binuclear cluster (11, 12), with the third and fifth cysteine of the cluster postulated to serve as bridging molecules between the two zinc atoms (13).

This paper describes the properties of nine amino acid deletions or substitutions within the Cys_6 motif of the *LEU3* protein. Two of the mutant proteins with poor DNA binding capabilities can be made more efficient by adding Zn(II) to the growth medium or to the DNA binding assay. Another mutant protein which contains a seventh cysteine (replacing a wild type histidine) shows increased affinity for the target DNA and significantly greater efficiency as activator. The results are interpreted in terms of a variant Zn(II)-cysteine cluster.

* To whom correspondence should be addressed

MATERIALS AND METHODS

Strains and growth conditions

S.cerevisiae strain XK157−3C (*MATα* leu3-Δ2::*HIS3 trp1*-289 *ura3*-52), kindly provided by P.R.G.Brisco of this laboratory, was used as transformation recipient. It contains a total *LEU3* deletion (2). The *E.coli* strain used for amplification was TG1, genotype *supE hsd*Δ5 *thi* Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. Yeast cultures were grown at 30°C in SD minimal medium (14). When needed, amino acid supplements were added at 30 μg/ml. Cells were harvested at an $O.D._{600}$ of about 1.0. *E.coli* cultures were grown at 37°C in 2×TY medium (1.6% Bacto tryptone, 1% Bacto yeast extract, 0.5% NaCl). Ampicillin was added at 50 μg/ml.

Transformation procedures

Yeast cells were transformed as described previously (2). To show that transformants harbored both pYB1 and pYB6 or its derivatives (see below for description), plasmid segregation was carried out by inoculating 5 ml of YEPD broth with a single transformed colony, growing to saturation, plating on YEPD and replica-plating to minimal medium lacking uracil or tryptophan to score for plasmid loss. *E.coli* cells were transformed by established procedures (15).

General DNA manipulation

Isolation of plasmid DNA, agarose gel electrophoresis, oligonucleotide purification, 5' end labeling, and DNA sequencing were carried out as described (2, 5). DNA fragments were purified with the silica-based Geneclean kit from Bio 101, Inc. Restriction endonuclease digestions, the Klenow reaction, and ligations with T4 DNA ligase were performed essentially according to the instructions provided by the supplier (New England Biolabs, Boehringer-Mannheim).

Plasmid construction

Plasmid pYB1 contains a *LEU2'-'lacZ* translational fusion for determining the degree of *LEU2* expression. It also contains yeast *URA3* as selectable marker, an ampicillin-resistance marker and yeast centromere *CEN4*. Its construction has been described (2). Plasmid pYB6 contains the *LEU3* gene and adjacent regions. It carries yeast *TRP1* as selectable marker and also contains an ampicillin-resistance marker and yeast centromere *CEN4*. It is identical to the previously described pYB5 (5) except that the *LEU3* region is present in the opposite orientation. Plasmid pYB2 was obtained by deleting *LEU3* from pYB6. It served as negative control.

The following procedure was used to construct a series of pYB6 derivatives containing mutations in the DNA binding region of *LEU3* (Figure 2). A vector, designated M13mp19−4, was constructed by inserting a *LEU3* fragment ranging from nucleotide position −561 to position +518 (10) into M13mp19. The *LEU3* region of this vector was then subjected to site-directed mutagenesis. This was followed by digestion with *Sac*I and *Sal*I endonucleases and isolation of the smaller of the two resulting fragments. Plasmid pYB6 was likewise digested with *Sac*I and *Sal*I and the larger fragment was isolated. Legation of the two isolated fragments resulted in plasmid pYB6M, one for each mutation generated.

Site-directed mutagenesis

The two alternative methods used were the gapped duplex method offered as 'site-directed mutagenesis kit' by Boehringer-Mannheim and the method offered as 'Muta-Gene' mutagenesis kit by Bio-Rad, which is based on the procedure of Kunkel (16). Oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer by the Laboratory for Macromolecular Structure, Purdue University. The following sequences were used: 5'-GTGAGCATCACTTTTCGAC-3' (19 mer, to change Cys47 to Ser); 5'-CTTTCGTGACATCTTTCGATTCTGCT-G-3' (29 mer, to delete Cys47); 5'-CTCCGGTGCTCTTTCA-GCATCACATTTCG-3' (29 mer, to delete His50); 5'-GCT-CTTTCGGCAGCATCAC-3' (19 mer, to change His50 to Ala); 5'-GCTCTTTCGGAAGCATCAC-3' (19 mer, to change His50 to Ser); 5'-GCTCTTTCGCAAGCATCAC-3' (19 mer, to change His50 to Cys); 5'-GTGCATGGCTCCGGTCTTTCGTGAGC-ATC-3' (29 mer, to delete Ala53); 5'-CACTTAGTGCATGG-CGGTGCTCTTTCGTG-3' (29 mer, to delete Glu55), and 5'-CTTAGTGCATCGCTCCGGT (19 mer, to change Pro56 to Arg). To establish the identity of the mutations, a stretch of about 450 bp covering and flanking the mutated region of each mutant was sequenced using the Sequenase version 2.0 kit (United States Biochemical Corp.). The primer used was 5'-GAAGAATCC-GTATGTCTTCTGTTGGTACTG-3', corresponding to positions 491−462 of the coding region of the *LEU3* gene.

Yeast extract preparation and partial purification of *LEU3* protein

Cell-free yeast extracts were prepared by twice passing a cell suspension through a French pressure cell and centrifuging for 20 minutes at 27,000×g. Cells were suspended at a ratio of 1.5 ml of extraction buffer to 1 g of cells (wet weight). Extraction buffer was 200 mM Tris−HCl (pH 8.0) containing 400 mM $(NH_4)_2$ SO_4, 10 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 4 mM dithiothreitol, and 1 mM phenylmethane sulfonylfluoride. Partial purification was achieved by saturating the extract with ammonium sulfate to a final concentration of 40% (4°C) and redissolving the ensuing precipitate in a small volume of 20 mM HEPES buffer pH 8.0 containing 5 mM EDTA, 20% (v/v) glycerol, 4 mM dithiothreitol, and 1 mM phenylmethane sulfonylfluoride. The partially purified preparation was divided into aliquots and stored at −70°C.

DNA binding assay

Electrophoretic mobility shift assays were performed as follows: 20 μg of partially purified protein was added to 4 mM Tris−HCl buffer (pH 8.0) containing 1 mM EDTA, 5% (v/v) glycerol, 4 mM dithiothreitol, 40 mM NaCl, 5 mM $MgCl_2$, 2 μg of poly(dI-dC)·poly(dI-dC), 6 μg of bovine serum albumin, and 0.5−1 ng of ^{32}P-5' end labeled oligonucleotide (UAS_L-30mer, 5'-AGGTGAGAGGCCGGAACCGGCTTTTCATAT-3') in a total volume of 20 μl. In some experiments, a non-binding UAS_L-24 mer, 5'-AGGTGAGAGGCCGGCTTTTCATAT-3', was used. The reaction mixture was routinely incubated at 30°C for 15 min. and then immediately applied to a pre-electrophoresed (2h) 4% non-denaturing polyacrylamide gel. Electrophoresis was performed for 1.5 h at 30 mA in buffer containing 90 mM Tris base, 90 mM H_3BO_3, and 25 mM EDTA. Gels were dried and subjected to autoradiography.

β-Galactosidase assay

β-Galactosidase activity in cell-free extracts was measured as described (2), the units of activity being nmoles/min×mg protein. The protein concentration was determined by the method of Bradford (17). In some instances, a rapid assay utilizing

permeabilized cells was performed, as follows: Cells from 5 ml of cell culture was resuspended in 1 ml of Z buffer (18), then 20 μl of a 0.1% sodium dodecyl sulfate solution and 40 μl of chloroform was added and the suspension was vortexed for 30 sec. For this assay, the β-galactosidase activity units are expressed as $OD_{420} \times 10^3 / OD_{600} \times min \times ml$.

RESULTS

The Cys_6 motif of lower eukaryotic activators has distinct subregions

The Cys_6 region located near the N-terminus of the *LEU3* protein shows extensive sequence similarity with corresponding regions of at least eleven other activators from lower eukaryotes (Figure 1). It is possible to distinguish three subregions within the sequences shown. The first, at the N-terminal side, encompasses 13 residues and appears to be highly conserved. It includes the first three cysteines of the motif, four strongly conserved basic residues, and a conserved small residue (alanine, glycine, or serine) preceding the first cysteine. The second subregion, adjacent to the first, consists of as many as 8 residues (*LEU3*) and as few as 4 residues (*MAL63*, *PDR1*, *PUT3*). It is variable, with the exception of a rigidly conserved proline. Finally, there are 11 residues on the C-terminal side (14 in the case of *HAP1*) which may be described as moderately conserved. This subregion includes the remaining three cysteines of the motif.

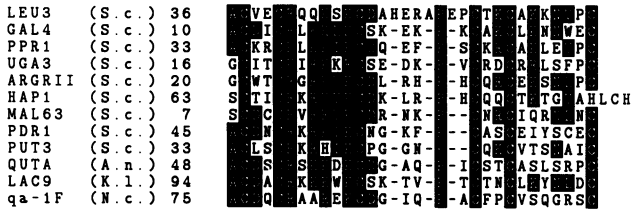

Figure 1: Comparison of the Cys_6 DNA binding motif of 12 lower eukaryotic transcriptional activators. One-letter symbols of the amino acids are used. Identities and conservative changes are shaded when they appear in six cases or more. Numbers refer to the first amino acid shown where 1 = the deduced N-terminus. S.c. = *Saccharomyces cerevisiae*; A.n. = *Aspergillus nidulans*; K.l. = *Kluyveromyces lactis*; N.c. = *Neurospora crassa*. Sequences are from the following references: *LEU3* (10), *GAL4* (21), *PPR1* (22), *UGA3* (23), *ARGRII* (24), *HAP1* (25), *MAL63* (26), *PDR1* (27), *PUT3* (28), *qutA* (29), *LAC9* (30), qa-IF (31).

Only two of seven *LEU3* mutants with an altered 'variable subregion' of the Cys_6 motif produce poor activators, and both can be stimulated by external Zn(II)

Table 1 lists nine single amino acid changes or deletions affecting residues 47, 50, 53, 55, and 56 of the *LEU3* protein. Two of the mutations changed the highly conserved third cysteine of the Cys_6 motif, the other seven were located within the variable subregion of the motif. After introducing each mutation into the host strain as a pYB6M plasmid, the functional competence of the mutant proteins was assessed in two ways: (i) their *in vivo* activation efficiency was monitored by measuring the level of expression of a *LEU2-lacZ* fusion carried on plasmid pYB1 (5); (ii) their ability to bind to target DNA was determined by electrophoretic mobility shift assays using the UAS_L of *LEU2* as a probe (see METHODS). A series of control experiments regarding DNA binding is shown in Figure 3. Of the bands that appeared when wild type extract was used, only those designated **a** and **b** were considered to represent *LEU3*-specific complexes, for the following reasons. Both bands disappeared when excess unlabeled UAS_L-30mer was present during preincubation (which competes with the labeled probe), but were unaffected when unlabeled, non-interactive UAS_L-24mer (2) was present. Moreover, neither the **a** nor the **b** complex appeared when extract from a strain carrying no *LEU3* sequences was used; in their place, a complex with an intermediate mobility appeared, designated **c** in Figure 3. Band **c**'s protein component also showed specificity for the UAS_L-30mer (J. Sze, unpublished observation). The other non-lettered bands visible in Figure 3

Table 1. Summary of properties of *LEU3* proteins mutated within the DNA binding domain

	LEU3 protein Residues 47-57	*In vivo* Activation of *LEU2*[a] High α-IPM	Low α-IPM	*In vitro* DNA binding[b]	*In vivo* Activator efficiency
Wild type	C D A H E R A P E P C	244	34	wild type	wild type
Cys 47Δ	- D A H E R A P E P C	25	25	very poor	very poor
Cys 47 Ser	s D A H E R A P E P C	23	15	poor	very poor
His 50Δ	C D A - E R A P E P C	47	12	poor, Zn-reversible	poor, Zn-reversible
His 50 Ala	C D A a E R A P E P C	188	27	good	good
His 50 Ser	C D A s E R A P E P C	278	9	very poor	very good
His 50 Cys	C D A c E R A P E P C	370	41	superb	superb
Ala 53Δ	C D A H E R - P E P C	38	16	poor, Zn-reversible	Poor, Zn-reversible
Glu 55Δ	C D A H E R A P - P C	160	20	very poor	good
Pro 56 Arg	C D A H E R A P E r C	306	57	very good	very good

a) *In vivo* activation of *LEU2* was determined by measuring the β-galactosidase activity of a *LEU2-lacZ* encoded fusion protein (see METHODS). 'High α-IPM' conditions were generated by growing cells under non-repressing conditions (supplemented with 0.2 mM leucine [initial concentration]); 'low α-IPM' conditions were generated by growing cells under repressing conditions (supplemented with 2 mM leucine plus 1 mM isoleucine [initial concentration]) (see ref. 2). Numbers are specific activities (nmoles/min×mg protein). They represent the average of at least two independent determinations. The error was <15%. Note that there is a basal level expression of *LEU2-lacZ* (specific activity of about 10) that is independent of *LEU3* (5).
b) From results shown in Figures 4–6.

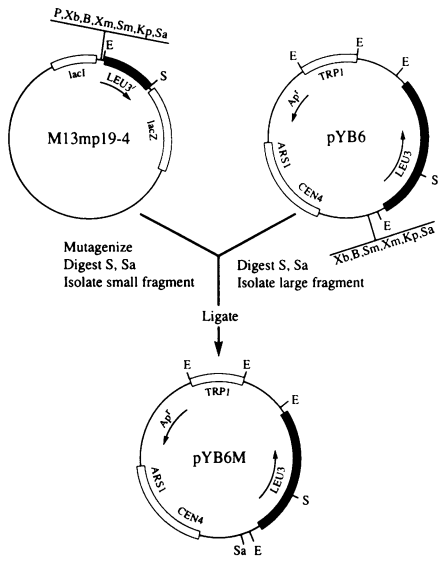

Figure 2: Construction of pYB6M plasmids by 'cassette exchange'. Mutants of the DNA binding region of the *LEU3* protein were constructed using a short segment of the *LEU3* gene. Mutated segments were then exchanged for the corresponding wild type segment resulting in one pYB6M plasmid for each mutant generated. See text for further details. Restriction sites: B, *BamHI*; E, *EcoRI*; Kp, *KpnI*; P, *PstI*; S, *SalI*; Sa, *SacI*; Sm, *SmaI*; Xb, *XbaI*; Xm, *XmaI*. Drawings are not to scale.

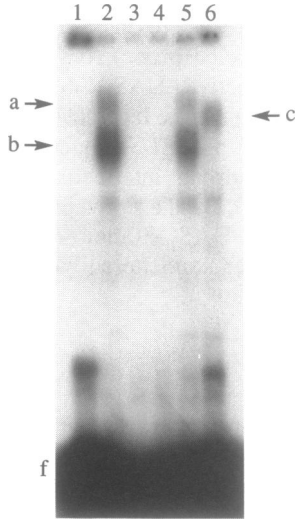

Figure 3: Electrophoretic mobility shift assays: Controls. See METHODS for details of the assay. Lane 1: assay buffer only; lane 2: protein from *LEU3* wild type cells (strain XK157−3C transformed with pYB6 [and pYB1]); lane 3: same as lane 2, but in presence of 50-fold excess of unlabeled UAS_L-30mer; lane 4: same as lane 2, but in presence of 100-fold excess of unlabeled UAS_L-30mer; lane 5: same as lane 2, but in presence of 50-fold excess of unlabeled UAS_L24mer; lane 6: protein from *leu3Δ* (total *LEU3* deletion) cells (strain XK157−3C transformed with pYB2 [and pYB1]). **a**, **b**, and **c**: Shifted bands with specificity for the UAS_L-30mer; f: free DNA probe. See text for further details.

were considered non-specific or artifactual since they appeared throughout and/or were seen even when no yeast protein had been added.

Deleting Cys47 from the *LEU3* protein or replacing it with serine resulted in almost total loss of activation efficiency (Table 1). Band shift assays showed that band **b** was either severely

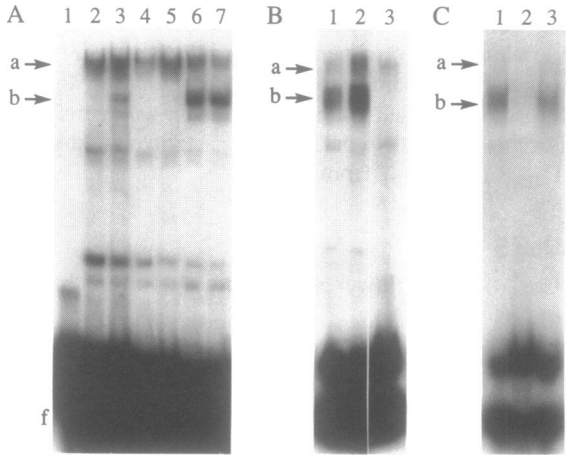

Figure 4: Electrophoretic mobility shift assays: *LEU3* mutants. See METHODS for details of the assay. Lane A1: assay buffer only; lane A2: His50Δ; lane A3: Cys47Ser; lane A4: Glu55Δ; lane A5: Ala53Δ; lane A6: His50Cys; lane A7: wild type; lane B1: wild type; lane B2: Pro56Arg; lane B3: Cys47Δ; lane C1: wild type; lane C2: His50Ser; lane C3: His50Ala. In all cases, partially purified preparations from strain XK157−3C transformed with pYB1 and the appropriate pYB6M plasmid (or pYB6 for wild type controls) was used. The DNA probe was UAS_L-30mer. Symbols **a**, **b**, and **f**: see Figure 3.

reduced in intensity (Cys47Ser, lane 3 of Figure 4A) or totally absent (Cys47Δ, lane 3 of Figure 4B). Surprisingly, both mutant proteins were still capable of forming band **a**, with an intensity at least as strong as that observed with wild type protein. The possibility that there might be a significant functional difference between complexes **a** and **b** (in addition to their obvious structural differences), with complex **b** representing the 'productive' *LEU3*-UAS_L complex, will be discussed below.

In contrast to the mutations at Cys47, mutations altering or deleting single amino acids within the variable subregion did not cause irreparable damage to the *LEU3* protein. Of the seven *LEU3* mutants of this kind investigated here, two were classified as having 'good' *in vivo* activator efficiency (His50Ala and Glu55Δ, Table 1), with 77% and 66% respectively, of the efficiency of wild type protein. Two were classified as 'very good' activators (His50Ser and Pro56Arg), being slightly more efficient than wild type protein. One mutant activator was classified as 'superb' (His50Cys); it was more than 1.5 times as efficient as wild type activator. Only two mutant proteins were classified as 'poor' activators (His50Δ and Ala53Δ). Since Zn(II) had been recognized as an important component of this type of transcriptional activator and had in some instances been shown to interact with the peptide regions displayed in Figure 1 (8, 12, 19), we wondered whether the deficiencies of His50Δ and Ala53Δ could be remedied by growing the mutants in media supplemented with Zn(II). Figure 5 shows that this was indeed the case. His50Δ could be stimulated to about 65%, and Ala53Δ to about 55% of wild type activation efficiency. The optimal initial concentration of Zn(II) in the medium was 8 mM for His50Δ and 6 mM for Ala53Δ. The latter mutant could also be stimulated by adding Cd(II) to the medium, with 10 μM $CdCl_2$ being optimal (data not shown). Activation of *LEU2-lacZ* expression by wild-type *LEU3* protein was unaffected by Zn(II) concentrations as high as 10 mM. There was also no measurable effect of Zn(II) on the very poor activation efficiency of mutants Cys47Ser and Cys47Δ.

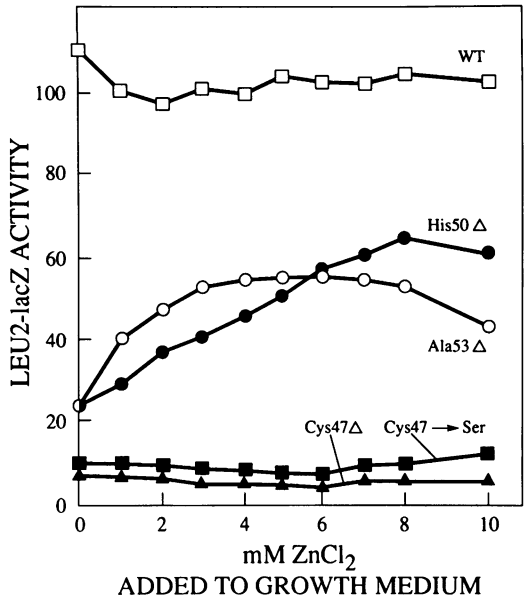

Figure 5: Effect of the presence of $ZnCl_2$ on the expression of *LEU2* in selected *LEU3* mutant strains. Cells were grown as described in METHODS with the indicated initial concentrations of $ZnCl_2$. The expression of *LEU2* was estimated by measuring β-galactosidase activity in permeabilized cells (see METHODS) of strain XK157−3C transformed with pYB1 and the appropriate pYB6M plasmid (or pYB6 for wild type).

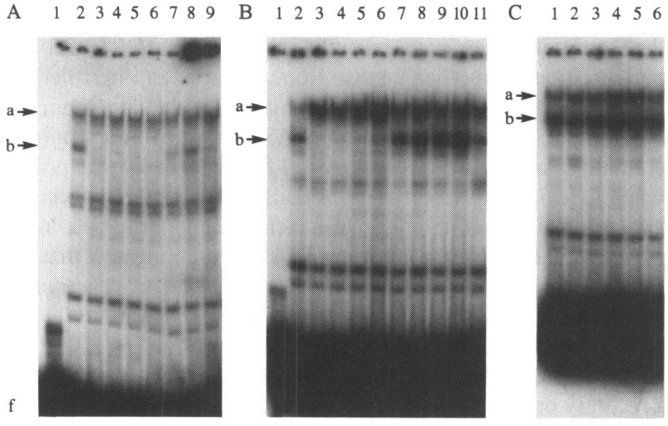

Figure 6: Electrophoretic mobility shift assays: Effect of $ZnCl_2$ addition on DNA binding pattern of His50Δ, Ala53Δ, and wild type *LEU3* proteins. See METHODS for details of the assay. **A**: Lane A1, assay buffer only; lane A2, wild type; lanes A3−A9, His50Δ protein pre-incubated with the following concentrations of $ZnCl_2$: 0, 25 μM, 50 μM, 100 μM, 500 μM, 1 mM, 2 mM. **B**: Lane B1, assay buffer only; lane B2, wild type; lanes B3−B11, Ala53Δ protein preincubated with the following concentrations of $ZnCl_2$: 0, 25 μM, 50 μM, 100 μM, 500 μM, 750 μM, 1 mM, 1.5 mM, 2 mM. **C**: Lanes C1−C6, wild type protein pre-incubated with the following concentrations of $ZnCl_2$: 0, 100 μM, 500 μM, 1 mM, 2 mM, 5 mM. In all cases, partially purified preparations from strain XK157−3C transformed with pYB1 and the appropriate pYB6M plasmid (or pYB6 for wild type) were used. The DNA probe was UAS_L-30mer. Pre-incubation time was 15 min. at 30°C. Symbols **a**, **b**, and **f**: see Figure 3.

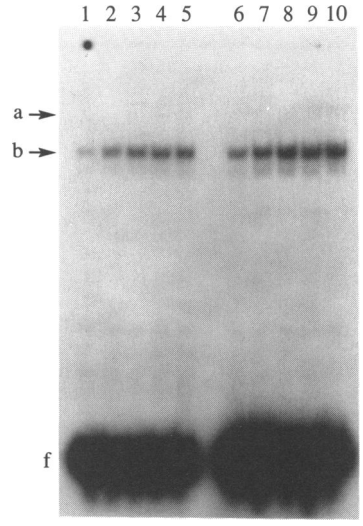

Figure 7: Electrophoretic mobility shift assays: Comparison of association rates (protein-DNA complex formation) of wild type and His50Cys protein. Seven μg of partially purified protein were added to assay buffer containing labeled UAS_L-30mer (see METHODS) at time zero. At various times thereafter, a 50-fold excess of unlabeled UAS_L-30mer was added to thwart incorporation of labeled probe. Lanes 1−5: wild type protein; lanes 6−10: His50Cys protein. Times of exposure to labeled probe: lanes 1 & 6, 15 sec; lanes 2 & 7, 30 sec; lanes 3 & 8, 60 sec; lanes 4 & 9, 90 sec; lanes 5 & 10, 120 sec. Symbols **a**, **b**, and **f**: see Figure 3.

For most (but not all) variable-region mutants, the *in vivo* behavior was reflected in the DNA binding ability of the mutated proteins (Figure 4A−C). There were again differential effects on the formation of complexes **a** and **b**. For example, the His50Δ and Ala53Δ mutations affected complex **b** but not complex **a**. With both mutants, only complex **b** was absent; complex **a** was produced in apparently normal fashion. Complex **b** reappeared upon pre-incubation with Zn(II), with no diminishing effect on complex **a** (Figure 6A, B). The optimal Zn(II) concentration for the reappearance of band **b** was 1 mM for His50Δ and about 1.5 mM for Ala53Δ. By contrast, complex formation with wild type *LEU3* protein was virtually unaffected by concentrations of Zn(II) of up to 5 mM (Figure 6C).

Two mutants (Glu55Δ and His50Ser) produced activators that were efficient *in vivo*, but did not bind well to DNA *in vitro*. The Glu55Δ protein failed to form complex **b** (Figure 4A), and the His50Ser protein was virtually incapable of forming either complex **a** or complex **b** under the conditions of the experiment (Figure 4C). We assume that the Glu55Δ and the His50Ser proteins are unstable in crude extracts, the former with respect to the formation of complex **b** only, the latter to the extent of being unable to form any complex with UAS_L.

Changing His50 to Cys creates an improved activator with increased affinity for target DNA

As shown in Table 1, a *LEU3* molecule containing cysteine in place of histidine at position 50 became a significantly better activator of the *LEU2* gene. A closer look at the DNA binding properties of the His50Cys mutant relative to wild type revealed that the mutant protein had acquired an increased affinity for UAS_L. This was demonstrated by qualitatively comparing association and dissociation rates of the His50Cys mutant with those of wild type *LEU3* protein (Figures 7 & 8). Association rates were estimated by incubating fixed amounts of protein and ^{32}P-labeled DNA probe in binding buffer (Figure 7). At the indicated times, starting at 15 sec, further reaction with labeled probe was thwarted by adding a 50-fold excess of unlabeled probe. The samples were then immediately electrophoresed. Dissociation rates were estimated by incubating protein and ^{32}P-

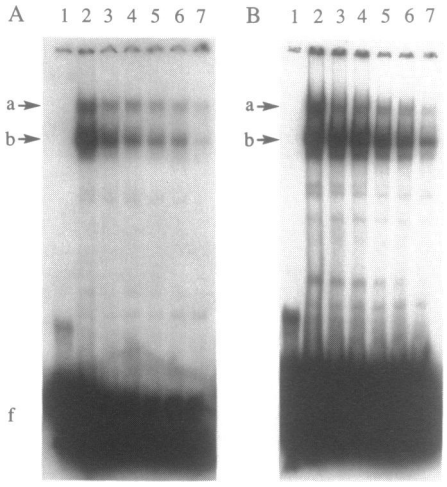

Figure 8: Electrophoretic mobility shift assays: Comparison of dissociation rates (protein-DNA complex dissociation) of wild type and His50Cys protein. Twenty μg of partially purified protein were added to assay buffer containing labeled UAS_L-30mer (see METHODS) and incubated for 15 min. At that point, a 50-fold excess of unlabeled UAS_L-30mer was added ('chase'), and samples were taken for electrophoresis at various times thereafter. **A**, wild type protein. **B**, His50Cys protein. Lanes 1, assay buffer only; lanes 2, no chase; lanes 3, 2 min chase; lanes 4, 5 min chase; lanes 5, 15 min chase; lanes 6, 30 min chase; lanes 7, 60 min chase. Symbols **a**, **b**, and **f**: see Figure 3.

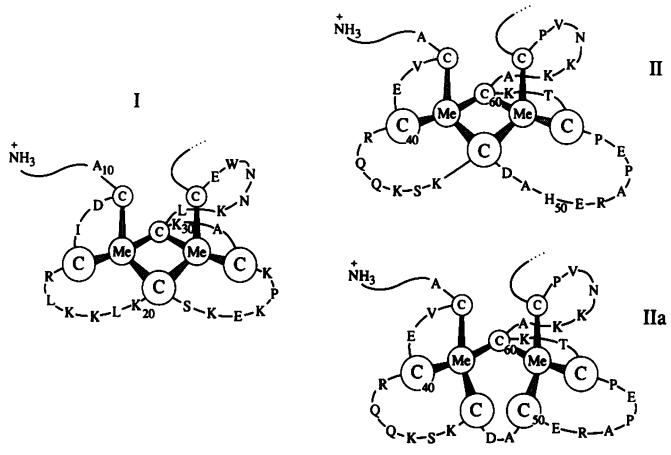

Figure 9: Proposed structures for the zinc cluster region of *GAL4* and *LEU3* proteins. I, structure of the *GAL4* protein cluster, adopted from refs. 11–13; II & IIa, proposed structures of the cluster as it might appear in *LEU3* wild type and *LEU3* His50Cys protein. Note: Recent refinements of the 3D structure of a *GAL4* DNA binding peptide (32) suggest that the bridging ligands within the zinc cluster of that peptide may be Cys11 and Cys28 instead of Cys21 and Cys31, as previously assumed. While transposition of the new structure to the *LEU3* protein would change the positions of its cysteines correspondingly (the bridging ligands would now be Cys37 and Cys57 instead of Cys47 and Cys60), the principles outlined for the variant motif of the His50Cys mutant protein would remain the same.

labeled DNA probe for 15 min, then chasing the label by adding a 50-fold excess of unlabeled probe. Samples were taken at the indicated times (Figure 8A, B) and electrophoresed immediately. The results showed that His50Cys protein bound to the UAS_L probe more rapidly than wild type protein. Likewise, His50Cys protein dissociated from the probe more slowly than wild type protein. These data are consistent with an increased association constant for the interaction of His50Cys protein and target DNA, compared with wild type protein. Figure 7 also shows that, for both wild type and His50Cys protein, the formation of complex **a** lags behind the formation of complex **b**, with almost no complex **a** having formed within 2 min under the conditions of the experiment. Likewise, complex **a** seems to be less stable once it has formed (Figure 8).

DISCUSSION

One of the more puzzling aspects of the interaction between the *LEU3* protein and target DNA (as measured by band shift assays) has been the consistent formation of two specific complexes, designated **a** and **b**. The relative amount of the two complexes is not very predictable, but in most experiments complex **b** predominates (2, 5, 6). Results obtained with the Cys47Δ and Cys47Ser proteins and especially with the Zn(II)-activatable mutants His50Δ and Ala53Δ are consistent with the idea that complex **b** stimulates transcription of *LEU2*, whereas complex **a** does not. It is not known at present whether complex **a** can also form *in vivo*, but it is clearly *LEU3*-specific. In addition to the evidence cited in this report, there is also the previous observation that the mobility of both bands **a** and **b** was affected similarly in a series of *LEU3* deletions located in the C-terminal region of the *LEU3* protein (6). There are several possible explanations for the presence of a second *LEU3* protein-DNA complex, such as post-translational modification of the *LEU3* protein, different oligomeric states, different stoichiometries of the same unit, as was recently shown for *ADR1* (20), or participation of another factor. None of these can be ruled out at this point. However, we are intrigued by the appearance of a non-*LEU3* protein specific for UAS_L (band **c**) in a strain not containing any *LEU3* sequences and the absence of this protein when *LEU3* is expressed. This is consistent with the notion that in complex **a** an additional protein factor might participate in and perhaps mediate *LEU3* protein-DNA interaction; and that this factor might antagonize *LEU3*'s function as activator. Experimental tests of this hypothesis are underway.

The present analysis of the DNA binding motif of *LEU3* has focused on the variable subregion of the $Zn(II)_2Cys_6$ cluster. In a recently proposed structure for this cluster as it exists in *GAL4* (11), the variable subregion occupies the second large loop of the 'cloverleaf', located between Cys21 and Cys28 (Figure 9). Given the strong conservation of key residues of the DNA binding motif, it is reasonable to assume that the overall configuration of the *LEU3* Cys_6 cluster would resemble that of *GAL4*, one major difference being that the variable loop would be larger by three residues (Figure 9II). This loop would also contain a histidine residue (His50). Since histidine is known to participate as metal binding ligand in some zinc fingers, we wondered whether it might be similarly involved in the DNA binding region of *LEU3*. Participation of His50 would permit a very regular arrangement of potential metal-complexing residues: [37]Cys Xaa_2 Cys Xaa_6 Cys Xaa_2 His Xaa_6 Cys Xaa_2 Cys Xaa_6 Cys [67], where Xaa signifies any amino acid. Results obtained with substitutions at His50 argue against a direct participation of His50 in metal binding since mutant proteins in which His50 had been replaced with serine or alanine were perfectly good activators. However, substitution of His50 with cysteine led to a significantly better activator with greater affinity for *LEU2* promoter DNA. While it is possible that this was due to improved direct contact

between the amino acids of the variable loop and DNA, cysteine is not known to be especially well-suited for this purpose. It is therefore conceivable that the His50Cys mutant protein contains a variant of the $Zn(II)_2Cys_6$ cluster exemplified by *GAL4*. The variant motif could be a $Zn(II)_2Cys_7$ cluster of the type shown in Figure 9IIa where seven cysteines are engaged in coordinating two Zn(II), with only one cysteine (Cys60) serving as a bridge. Whether this is indeed the case will have to be answered by direct structural analysis.

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