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Molecular Architecture of a Leu3p-DNA Complex in Solution: a Biochemical Approach†

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The Leu3 protein (Leu3p) of *Saccharomyces cerevisiae* is a pleiotropic transregulator that can function both as an activator and as a repressor of transcription. It binds to upstream promoter elements (UAS_{LEU}) with the consensus sequence 5′-GCCGGNNCCGGC-3′. The DNA-binding motif of Leu3p belongs to the family of $Zn(II)_2$-Cys_6 clusters. The motif is located between amino acid residues 37 and 67 of the 886-residue protein. In this study, we used a recombinant peptide consisting of residues 17 to 147 to explore the interaction between Leu3p and its cognate DNA. We found that the Leu3p(17-147) peptide is a monomer in the absence of UAS_{LEU} but assumes a dimeric structure when the DNA is present. Results of protein-DNA cross-linking and methylation and ethylation interference footprinting experiments show that the Leu3p(17-147) dimer interacts symmetrically with two contact triplets separated by 6 bp and suggest that the peptide approaches its target DNA in such a way that each subunit is positioned closer to one DNA strand than to the other. The binding of Leu3p is strongly affected by the spacing between the contact triplets of the UAS_{LEU} and by the type of triplet. Binding occurs when the triplets are 6 bp apart (normal spacing) but fails to occur when the triplets are 0, 5, or 8 bp apart. Weak binding occurs when the triplets are 7 bp apart. Binding does not occur when the UAS_{LEU} triplets (GCC....GGC) are replaced with triplets found in the UAS elements for Gal4p, Put3p, and Ppr1p (CGG....CCG). The apparent K_d for the normal Leu3p(17-147)–UAS_{LEU} complex is about 3 nM. A mutant form of Leu3p(17-147) in which the histidine at position 50 has been replaced with cysteine binds UAS_{LEU} with significantly greater affinity (apparent K_d of about 0.7 nM), even though the interaction between the mutant peptide and target DNA appears to be unchanged. Interestingly, repression of basal-level transcription, which is a hallmark property of the wild-type Leu3p(17-147) peptide, is largely lost with the mutant peptide, indicating that there is no direct correlation between strength of binding and repression.

The Leu3 protein (Leu3p) made by the yeast *Saccharomyces cerevisiae* occupies an important regulatory position in amino acid metabolism. It binds to at least four promoters of genes involved in the biosynthesis of branched-chain amino acids (4, 8). It also binds to the promoter of *GDH1*, the gene encoding $NADP^+$-dependent glutamate dehydrogenase (18), and participates in the regulation of this important step in nitrogen assimilation (13). Leu3p may therefore have a more general function, potentially acting in concert with Gcn4p, the transactivating protein that is synthesized in response to amino acid starvation (12). Interestingly, expression of the *LEU3* gene is itself subject to regulation by Gcn4p (28).

It is a unique feature of Leu3p that it requires the presence of a metabolic signal to become a transcriptional activator and that in the absence of that signal it acts as a repressor (4, 20, 21). The signal molecule is α-isopropylmalate (α-IPM), the product of the first specific step in the leucine pathway. α-IPM is part of a sophisticated feedback loop that is dominated by the tightly regulated, *LEU4*-encoded enzyme α-IPM synthase. Transcriptional activation by the Leu3p–α-IPM complex and repression by Leu3p in the absence of α-IPM have been demonstrated in vitro by using a transcriptionally competent extract from *leu3*-null cells and highly purified Leu3 protein (21).

Three-dimensional structural information is not available for any part of Leu3p. However, mapping of the functional domains along the primary structure of Leu3p has yielded several important clues with respect to overall structure-function relationships. Thus, the DNA-binding function of Leu3p is located near the N terminus, where amino acid residues 37 to 67 resemble a motif found in at least a dozen fungal transactivators (3), including the well-studied Gal4 protein. Nuclear magnetic resonance and X-ray crystallographic analyses of the Gal4 protein identified the motif as a $Zn(II)_2$-Cys_6 binuclear cluster (9, 10, 14). Mutations within the $Zn(II)_2$-Cys_6 cluster of Leu3p were shown either to abolish specific DNA binding (ΔCys-47 or Cys-47→Ser) or to make DNA binding dependent on exogenously added Zn(II) (ΔHis-50 and ΔAla-53) (3). One mutation in this region (His-50→Cys) actually created a Leu3p with significantly improved activator efficiency (3). The consensus sequence with which Leu3p interacts, designated UAS_{LEU}, has been identified as a G+C-rich, palindromic dodecamer with the sequence 5′-GCCGGNNCCGGC-3′ (4, 8, 15, 22, 28). The transcriptional activation function of Leu3p was found to reside exclusively within the 30 C-terminal residues of the protein and to be active across species (20, 21, 29). In the native Leu3p molecule, the activation domain appears to be masked when α-IPM is absent (21, 29). The fact that the α-IPM response is lost in Leu3 mutant proteins that either lack portions of the central region or contain modified C-terminal regions has led to the idea that masking of the activation domain might occur through contacts between the activation domain and the central region (26, 29). The form of Leu3p in which the activation domain is masked is also the form that acts as a repressor. The repressor function has been mapped to a short region near the N terminus by demonstrating that a peptide consisting of residues 17 to 147 of Leu3p [Leu3p(17-147)] can repress basal-level transcription to the same extent as full-

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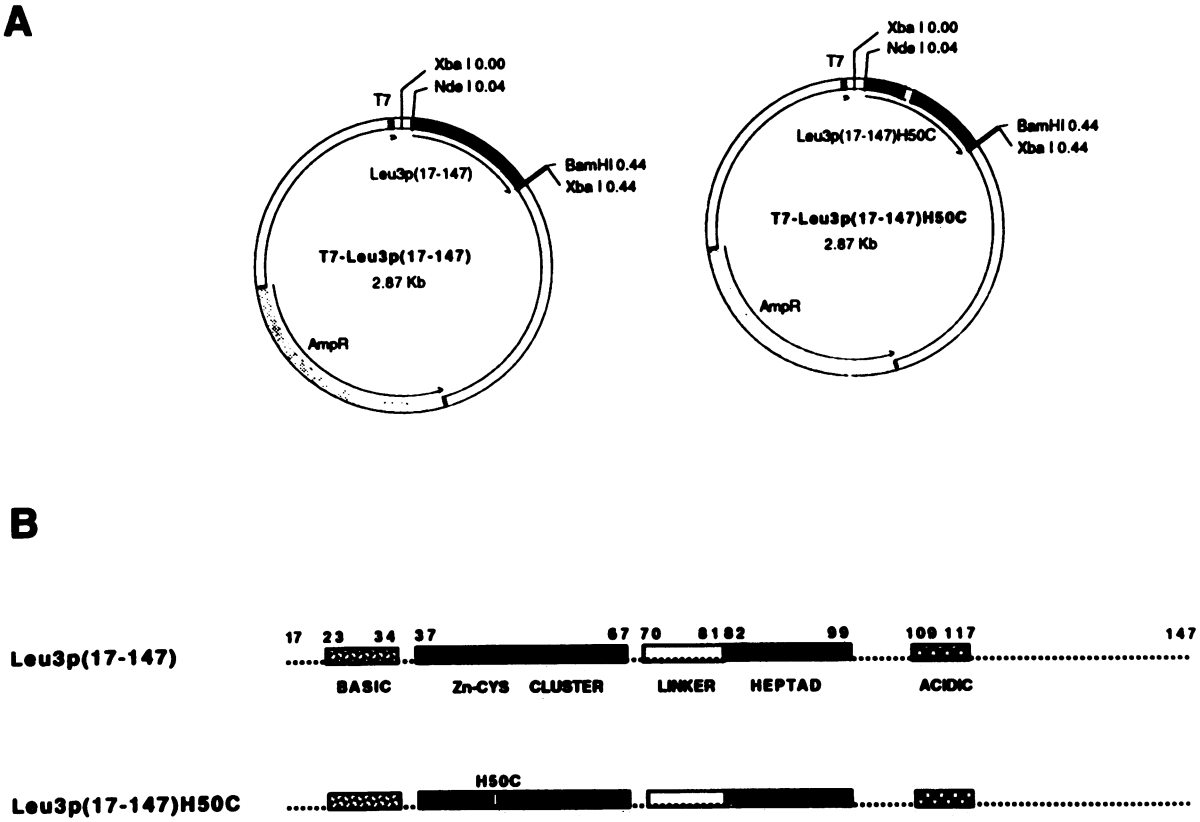


FIG. 1. (A) Partial restriction map of plasmids derived from *E. coli* expression vector pT7-7 showing the Leu3p(17-147)- and Leu3p(17-147)H50C-coding regions. (B) Structural features of the Leu3p(17-147) and Leu3p(17-147)H50C peptides.

length Leu3 (20). The most prominent feature of Leu3p(17-147) is the DNA-binding domain.

Here we report on some aspects of the Leu3p(17-147)–DNA interaction in solution. We present evidence showing that Leu3p(17-147) exists in monomeric form when DNA is absent but assumes a dimeric configuration when bound to UAS_{LEU}. Results of protein-DNA cross-linking and methylation and ethylation interference experiments suggest that each subunit of the Leu3p(17-147) dimer is positioned such that it is closer to one DNA strand than to the other. Like full-length Leu3p, Leu3p(17-147), makes specific contact with a symmetrical set of triplets (GCC. . . .GGC) that is different from the triplets found in the DNA targets of several other members of the $Zn(II)_2$-Cys_6 cluster family (CGG. . . .CCG). Contact specificity is influenced by the distance between the triplets. Any deviation from the normal 6-bp distance severely affects DNA binding. We also address the repressor function of Leu3p(17-147) and show that this function is essentially lost as a consequence of a single amino acid replacement (H-50→C) that increases the affinity of the peptide for DNA. We expect that the information communicated here will contribute to a greater appreciation of the differences among members of the zinc cluster family.

MATERIALS AND METHODS

Cloning of Leu3p(17-147)- and Leu3p(17-147)H50C-encoding DNAs. An *Xho*I-*Sal*I fragment encoding the first 171 amino acids of Leu3p was obtained from plasmid pZRL (26). The PCR was utilized to introduce unique *Eco*RI and *Nde*I sites near the 5′ end and a unique *Bam*HI site near the 3′ end of the clones. The *Bam*HI site was preceded by a stop codon. The final construct encodes amino acids 17 to 147 of Leu3p. No non-Leu3p residues are present. A mutant clone encoding Leu3p(17-147)H50C was prepared in an identical fashion from an M13 clone specifying cysteine instead of histidine at position 50 of Leu3p (3).

Expression and purification of Leu3p(17-147) and Leu3(17-147)H50C. The following procedure was employed for both peptides Leu3p(17-147) and Leu3p(17-147)H50C. A 5-ml volume of LB medium (2) containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) was inoculated from a glycerol stock of *Escherichia coli* BL21(DE3)pLysS cells containing either the T-7–Leu3p(17-147) or the T-7–Leu3(17-147)H50C plasmid (Fig. 1) and incubated at 37°C for 4 h. A 200-μl of volume of the inoculum was plated and grown overnight. A single colony was picked to inoculate 100 ml of LB-antibiotic medium. After overnight growth, the preculture was used to inoculate 1 liter of the main culture by using the same medium. The main culture was grown to an optical density at 600 nm of about 0.6, induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside, and then incubated for an additional 5 h. Cell pellets were recovered by centrifugation at 4,000 × *g* for 15 min and frozen at −80°C. To prepare a cell extract, frozen pellets were thawed on ice and resuspended in 200 mM Tris-HCl buffer (pH 7.9) containing 400 mM $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM dithiothreitol, 50 μM $ZnSO_4$, 20% glycerol, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 μg of pepstatin per ml, and 1 μg of leupeptin per ml (buffer L). The suspensions were passed twice through a French pressure cell at 1,260 lb/in^2, and the clarified extract was recovered by centrifugation at 27,000 × *g* for 15 min. The DNA was precipitated by adding

polyethylenimine P (Sigma) to a final concentration of 0.15% and stirring the mixture for 10 min in an ice bath. The supernatant solution was recovered after 10 min of centrifugation at 27,000 × *g* and adjusted to a final $(NH_4)_2SO_4$ concentration of 65%. The mixture was stirred on ice for 10 min and centrifugation at 27,000 × *g* for 15 min. The pellet was redissolved in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH buffer (pH 7.6) containing 1 mM dithiothreitol, 50 μM $ZnSO_4$, 0.1% Nonidet P-40, 20% glycerol, 2 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine (buffer D). The solution was divided into 0.5-ml aliquots. To each aliquot, an equal volume of buffer D preheated at 71°C for 15 min was added, and the mixture was further incubated at 71°C for 2 min before being rapidly cooled on ice. After the mixture had been on ice for 20 min, denatured protein was removed by centrifugation in a Microfuge (Eppendorf) at 14,000 rpm for 20 min at 4°C and the supernatant solution was dialyzed against buffer D containing 100 mM KCl. The dialysate was loaded onto a 5-ml immobilized heparin column (Pierce) that had been equilibrated with dialysis buffer, and the column was washed with a step gradient of buffer D containing 100, 250, 500, and 750 mM and 1 M KCl. The Leu3 peptides were eluted mainly in one fraction of buffer containing 500 mM KCl. For further purification, the peptide-containing fraction from the heparin-agarose column was dialyzed against buffer D containing 100 mM KCl and applied to a 5-ml immobilized Cibacron Blue F 3GA column (Pierce) equilibrated with the same buffer. The column was washed as outlined above for the heparin column. The Leu3 peptides were eluted quantitatively in buffer D containing 750 mM to 1 M KCl. The pooled fractions were dialyzed against buffer D containing 50% glycerol and stored at −80°C. DNA-binding activity was monitored by electrophoretic mobility shift assays. The peptides were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE; 15% acrylamide) followed by Coomassie brilliant blue staining. The method outlined yielded about 2 mg of peptide per liter of culture, with a purity of close to 95%. Figure 2 summarizes the purification steps. The overall purification level was 8.4-fold. More recently, the yield of purified peptide has been increased to about 8 mg/liter of culture by collecting protein that precipitates between 50 and 75% saturation with $(NH_4)_2SO_4$ and by omitting the heat step (16a). Rabbit anti-Leu3p(17-147) antibodies raised against the highly purified peptide recognize full-length Leu3p (19).

Electrophoretic mobility shift assays. DNA-binding assays were performed as described previously (19), except that DNA and protein were incubated at 23°C and acrylamide concentration was 6% with a ratio of acrylamide to methylene bisacrylamide of 19:1. The DNA probes were synthesized by the Laboratory for Macromolecular Structure of Purdue University. Gel-purified oligonucleotides were labeled by T4 polynucleotide kinase by using [γ-^{32}P]ATP in a buffer that contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM spermidine, 0.1 mM EDTA, 15 mM dithiothreitol, and 80 μg of bovine serum albumin per ml. The labeled probe was separated from unincorporated label by two subsequent ethanol precipitations and resuspension of the pellet in 10 mM Tris-HCl buffer (pH 8.0) containing 0.14 M KCl and 1 mM EDTA. The amount of probe used for the shift assays was about 1.4 ng, unless otherwise specified.

Protein-protein cross-linking. For protein-protein cross-linking, dimethylsuberimidate (Pierce) was dissolved in 0.2 M triethanolamine-HCl buffer (pH 8.5) at a concentration of 30 mg/ml just prior to use. The final reaction volume of 100 μl contained 120 mM triethanolamine-HCl buffer (pH 8.5), 3 mg of dimethylsuberimidate per ml, and 10 μg of purified protein. The mixture was incubated for 3 h at 23°C prior to SDS-PAGE. For reactions in the presence of DNA, 20 μg of double-stranded probe was added to create a molar ratio of DNA to peptide equal to 2.5:1. Peptides were incubated with DNA for 15 min at 23°C before addition of the cross-linking reagent. Following SDS-PAGE, protein was visualized with Coomassie brilliant blue.

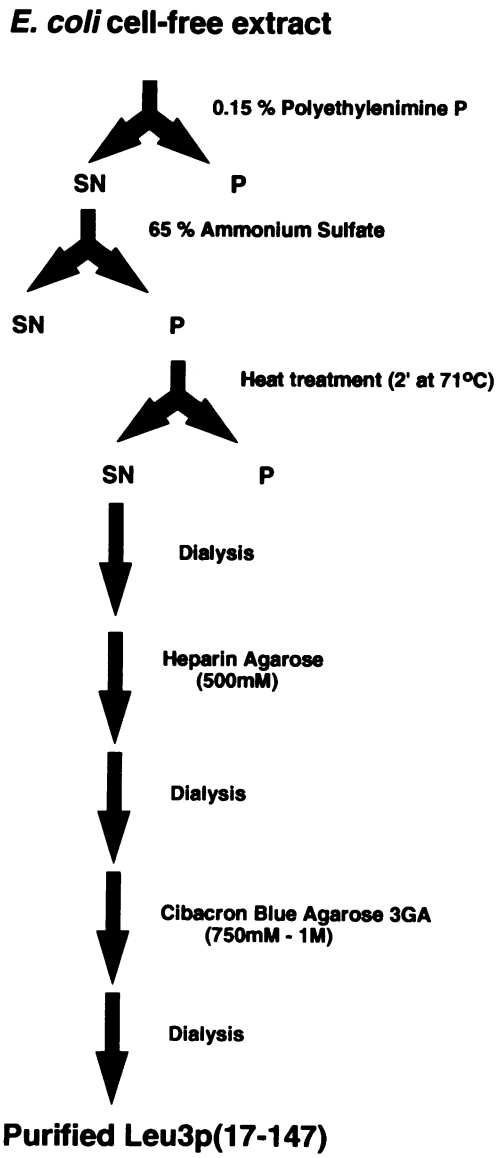


FIG. 2. Leu3p(17-147) peptide purification scheme. The same scheme was used to purify Leu3p(17-147)H50C. See Materials and Methods for further details. SN, supernatant; P, pellet.

Protein-DNA cross-linking. For protein-DNA cross-linking, 10 μg of purified protein was incubated in the presence of 14 ng of a ^{32}P-labeled UAS_{LEU} probe for 15 min at 23°C. The probe was a 30-mer containing the UAS_{LEU} and UAS_{LEU}-flanking regions of the *LEU2* promoter (4). Poly(dI-dC) was omitted from the binding reaction. The buffer was identical to the one used for protein-protein cross-linking. UV irradiation (maximum emission at 254 nm) was used to cross-link the protein to the DNA (2). Exposure lasted 15 min. The samples were kept on ice at a distance of 5 cm from the light source

(UVB1 UV light; Medical Specialities, Inc., Baltimore, Md.). When protein-DNA cross-linking was to be followed or preceded by protein-protein cross-linking, dimethylsuberimidate was added at a final concentration of 3 mg/ml and the mixture was incubated for an additional 3 h at 23°C. The total volume was 100 μl. At the end of the cross-linking period, a 15-μl volume was loaded onto a nondenaturing 6% polyacrylamide gel for electrophoretic mobility shift assays and a 50-μl volume was loaded onto an SDS–15% polyacrylamide gel for identification of the molecular species present. Protein bands were visualized with Coomassie brilliant blue before autoradiography. For the comparison of Leu3p(17-147) and Leu3p(17-147)H50C, UV cross-linking was performed in situ (25) with crude extracts from *E. coli* (5 μg of total protein). The protein-DNA complexes were separated by SDS-PAGE (18% acrylamide).

Ethylation and methylation interference footprinting. Ethylation and methylation interference experiments were performed essentially as described by Siebenlist and Gilbert (17). The UAS_{LEU}-containing probe (a 30-bp probe containing the UAS_{LEU} and UAS_{LEU}-flanking regions of the *LEU2* promoter [1, 4]) was labeled with ^{32}P at the 5′ end of the coding or noncoding strand and subjected to dimethyl sulfate (methylation) or *N*-ethyl-*N*-nitrosourea (ethylation) treatment. We incubated 2×10^6 cpm of the labeled, modified UAS_{LEU} probe with saturating amounts of the Leu3p(17-147) peptide. The proteins used were highly purified (19; this report). Following electrophoretic mobility shift assays, the bands representing the bound DNA and unbound DNA were excised and the DNA was eluted, purified, and subjected to piperidine cleavage at the methylated residues or alkali cleavage at the ethylated residues. The DNA fragments were then separated on a 20% polyacrylamide sequencing gel.

In vitro transcription assays. In vitro transcription with a whole-cell yeast extract was performed as previously described (20, 21). Purified peptides were preincubated for 15 min at 23°C with the transcription reaction mixture containing the templates. Reactions were started by extract addition and continued for 30 min at 23°C.

RESULTS

The Leu3p(17-147) peptide is a monomer in the absence of DNA but assumes a dimeric structure when UAS_{LEU} is present. The fact that the UAS_{LEU} consensus sequence shows hyphenated dyad symmetry is consistent with the idea that Leu3p binds to its target as a dimer. To explore the quaternary structure of Leu3p(17-147), we first subjected the peptide to gel filtration analysis (Fig. 3A). On Superdex 75, a composite of cross-linked agarose and dextran, functionally intact Leu3p(17-147) appeared at a position corresponding to an apparent molecular weight of about 16,000 compared with globular protein standards. The molecular weight of the monomeric peptide, calculated from its amino acid composition, is 15,543. No functional peptide was detected at any position larger than the monomer. Next, the peptide was subjected to protein-protein cross-linking by dimethylsuberimidate, following a preincubation period during which UAS_{LEU} was either present or absent. PAGE under denaturing conditions showed nothing but monomers when the DNA was absent (Fig. 3B). When UAS_{LEU} was present, Leu3p(17-147) dimers did appear, albeit at a relatively low yield. Increasing the DNA/peptide ratio did not improve the dimer yield (Fig. 3C). One possible explanation for the low yield is that DNA-bound Leu3p(17-147) was modified (amidinated but not cross-linked) at lysine residues outside the presumed dimerization region (heptad region in Fig. 2B) and that this modification diminished the cross-linking efficiency. The presence of the DNA seemed to expose more lysine residues to amidination since the Leu3p(17-147) monomers showed slightly retarded mobilities when UAS_{LEU} was present during the preincubation period (Fig. 3C). No evidence for species other than monomers and dimers was obtained in the cross-linking experiments. The question of higher-order oligomerization was also addressed by using mobility shift assays (Fig. 3D). Formation of only one Leu3p(17-147)–UAS_{LEU} complex was observed, even though the Leu3p(17-147) concentration was varied over 3 orders of magnitude (from 21 nM to 21 μM). We conclude that Leu3p(17-147) assumes a dimeric (but no higher-order) structure in the presence of its cognate DNA. The lack of dimer formation in the absence of UAS_{LEU} contrasts with an earlier observation showing that full-length Leu3p does exist in dimeric form even in the absence of DNA (19). Apparently, full-length monomers possess additional sites that stabilize the dimeric structure.

The Leu3p(17-147) dimer interacts symmetrically with two contact sites separated by 6 bp, with each subunit apparently approaching one DNA strand more closely than the other. UV-induced protein-DNA cross-linking (in combination with protein-protein cross-linking) and methylation and ethylation interference were used to gain more insight into the relative spatial arrangement of the Leu3p(17-147) dimer and its DNA target. The results of a cross-linking experiment are shown in Fig. 4. In this experiment, the purified Leu3p(17-147) peptide was incubated together with a ^{32}P-labeled UAS_{LEU} probe for 15 min before cross-linking was begun. At the end of the cross-linking period, a portion of each incubation mixture was subjected to an electrophoretic mobility shift assay. The results (Fig. 4A) demonstrated that the cross-linking reactions did not significantly affect UAS_{LEU}–Leu3p(17-147) complex formation. Another portion of each incubation mixture was used to identify the cross-linked species with SDS-PAGE (Fig. 4B). When the UAS_{LEU}–Leu3p(17-147) complex was subjected to both protein-DNA cross-linking and protein-protein cross-linking (Fig. 4B, lanes 2 and 3), two major SDS-resistant radioactive species with apparent molecular weights of about 54,000 and about 25,000 were observed. The size of the larger of the two species is close to that of a full complex consisting of a Leu3p(17-147) dimer linked to double-stranded DNA (Fig. 5). The calculated molecular weight of the full complex is close to 50,000, to which one would have to add the weight of an unknown number of dimethylsuberimidate moieties [each Leu3p(17-147) monomer has 18 lysyl residues, 2 of which are located within the presumed dimerization domain]. The appearance of a full complex is in agreement with the results of Fig. 3, showing that UAS_{LEU} elicits dimer formation. The size of the smaller of the two species in lanes 2 and 3 of Fig. 4B is close to that predicted for a half complex consisting of a Leu3p(17-147) monomer linked to single-stranded DNA (Fig. 5); it likely resulted from incomplete protein-protein cross-linking. When the UAS_{LEU}–Leu3p(17-147) complex was subjected to protein-DNA cross-linking only (Fig. 4B, lane 1), the only SDS-resistant species observed was the half complex (note that the half complex of lane 1 is slightly smaller than that of lanes 2 and 3 because there was no dimethylsuberimidate modification). The failure to form a stable full complex under these conditions is consistent with the idea that each subunit of the Leu3p(17-147) dimer is in closer proximity to one DNA strand than to the other, allowing cross-linking only to the proximal strand. The results are also consistent with the notion that the two subunits of the dimer approach opposite strands; otherwise, a species consisting of one DNA strand and a

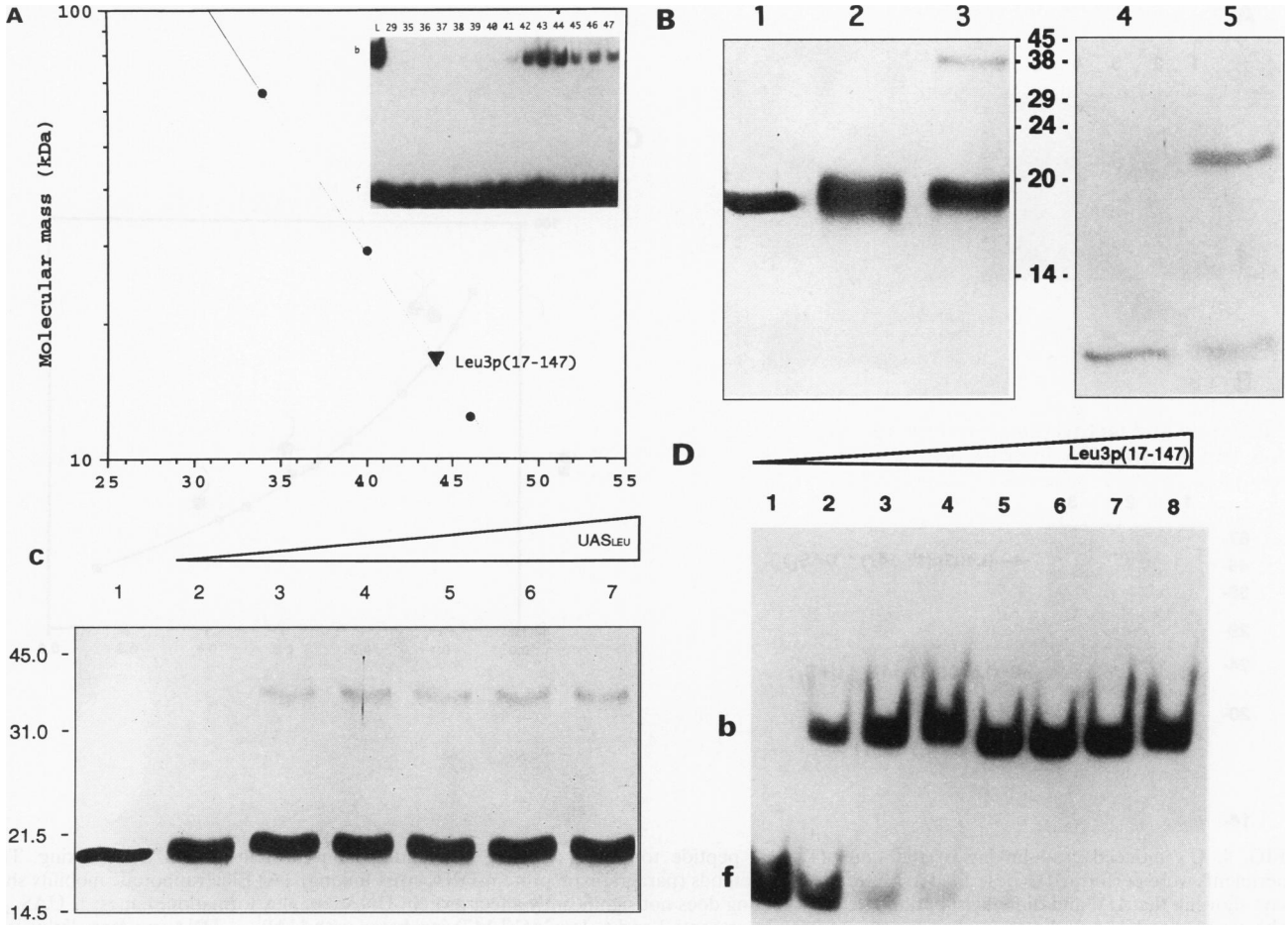


FIG. 3. Oligomeric state of Leu3p(17-147) in the presence and absence of UAS_{LEU}-containing DNA. (A) Gel exclusion chromatography of the Leu3p(17-147) peptide. A 25-μg sample of the highly purified Leu3p(17-147) peptide was applied to a Superdex-75 size exclusion column (20 by 1 cm) (Sigma). The column was equilibrated and eluted with 25 mM HEPES-KOH buffer (pH 7.6) containing 0.1% β-mercaptoethanol, 5% glycerol, 50 μM $ZnSO_4$, and 100 mM KCl. Fractions (230 μl) were monitored for DNA-binding activity by electrophoretic mobility shift assays (inset). Lane L of the inset represents the material loaded onto the column. b and f, bound oligonucleotide probe and free oligonucleotide probe, respectively. The molecular size standards, indicated by the filled dots, were bovine serum albumin (66 kDa), carbonic anydrase (29 kDa), and cytochrome *c* (12.4 kDa) (Sigma). The filled triangle indicates the Leu3p(17-147) peak position with an estimated molecular mass of about 16 kDa. (B) Protein-protein cross-linking of Leu3p(17-147) in the presence and absence of UAS_{LEU}-containing DNA. The pattern obtained after SDS-PAGE and Coomassie brilliant blue staining is shown. Lanes: 1, Leu3p(17-147) protein only, no treatment; 2, protein only, dimethylsuberimidate treatment; 3, protein preincubated with DNA (a 30-bp probe containing the UAS_{LEU} and UAS_{LEU}-flanking regions of the *LEU2* promoter [4]) and then treated with dimethylsuberimidate; 4 and 5, cross-linking control using the TrpR protein of *E. coli* (the TrpR protein is a dimer with a mass of about 24 kDa [11]): 4, *E. coli* TrpR protein, no treatment; 5, TrpR protein, dimethylsuberimidate treatment. The numbers between lanes 3 and 4 refer to the molecular weights (10^3) of protein standards. See Materials and Methods for further details. (C) Protein-protein cross-linking in the presence of increasing amounts of DNA. The pattern obtained after SDS-PAGE and Coomassie brilliant blue staining is shown. Lanes: 1, Leu3p(17-147) protein only; 2, protein only, dimethylsuberimidate treatment; 3 to 7, protein preincubated with DNA and then treated with dimethylsuberimidate. The protein-DNA molar ratios were 1:2.5, 1:3.75, 1:5, 1:7.5, and 1:10, respectively. The dimer-monomer ratio observed was about 1:10 throughout. The numbers on the left are molecular weights (10^3). (D) Electrophoretic mobility shift assays with increasing amounts of the Leu3p(17-147) peptide, indicating the lack of multimeric complex formation. Lanes: 1, UAS_{LEU} probe only (2.8 ng); 2 to 8, increasing amounts of purified peptide incubated with the radioactively labeled UAS_{LEU} probe (see B for a description of the probe). The amounts of protein used were 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 μg, respectively. b and f, bound probe and free probe, respectively.

peptide dimer (molecular weight, about 40,000) should have appeared in lane 1 of Fig. 4B.

The above interpretations of the cross-linking experiment were supported by results of methylation and ethylation interference studies. Methylation interference footprinting identifies guanines and, to a minor extent, adenines which, when methylated, interfere with protein binding; similarly, ethylation interference footprinting identifies backbone phosphates which, after treatment with *N*-ethyl-*N*-nitrosourea, impede protein binding (17, 23). Both methods rely on separate analyses of bound DNA and unbound DNA. Bands corresponding to positions at which modification interfered with protein binding are more prominent in unbound DNA. The same bands are underrepresented in bound DNA. The effect of methylation and ethylation of either strand of a UAS_{LEU}-containing probe on Leu3p(17-147) peptide binding is shown in Fig. 6. From the methylation interference experiment, the presence of two palindromic contact triplets that are 6 bp apart (5′-GCC. . . .GGC-3′) can be deduced. The central position of each triplet showed the strongest interference. The ethylation

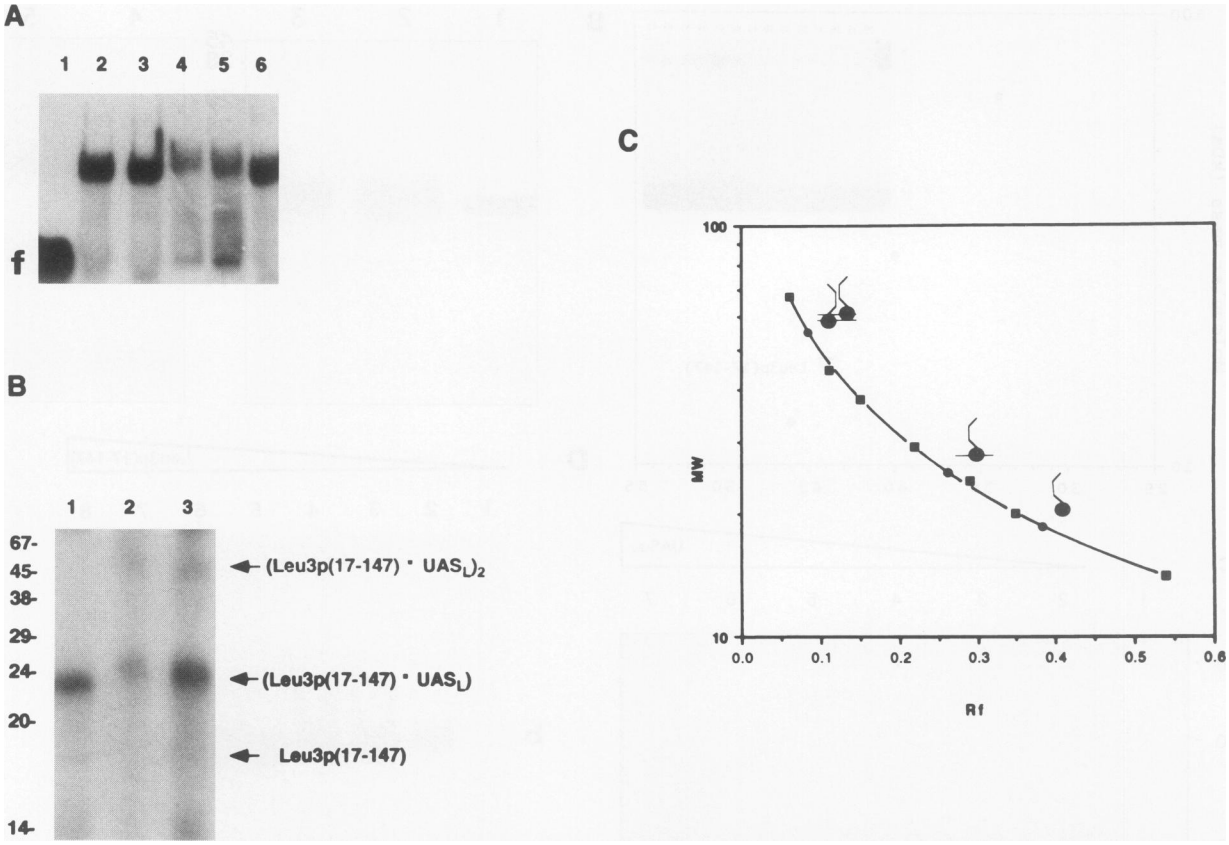


FIG. 4. UV-induced cross-linking of the Leu3p(17-147) peptide to UAS_{LEU} DNA with and without protein-protein cross-linking. The experiments were performed as described in Materials and Methods (paragraph on protein-DNA cross-linking). (A) Electrophoretic mobility shift assays showing that UV and dimethylsuberimidate cross-linking does not significantly affect protein-DNA complex formation. Lanes: 1, UAS_{LEU} probe exposed to UV and dimethylsuberimidate, no protein present; 2 and 6, Leu3p(17-147) incubated with UAS_{LEU} DNA, no cross-linking; 3, Leu3p(17-147) incubated with UAS_{LEU} DNA, UV treatment only; 4, Leu3p(17-147) incubated with UAS_{LEU} DNA and then subjected to dimethylsuberimidate cross-linking followed by UV treatment; 5, Leu3p(17-147) incubated with UAS_{LEU} DNA and then subjected to UV treatment followed by dimethylsuberimidate cross-linking. f, free probe. (B) Identification of SDS-resistant species formed during cross-linking. The bands represent ^{32}P-labeled DNA-containing complexes separated by SDS-PAGE and visualized by autoradiography. Lanes: 1, Leu3p(17-147) incubated with UAS_{LEU} DNA, UV treatment only; 2, Leu3p(17-147) incubated with UAS_{LEU} DNA, and then subjected to dimethylsuberimidate cross-linking followed by UV treatment; 3, Leu3p(17-147) incubated with UAS_{LEU} DNA, and then subjected to UV treatment followed by dimethylsuberimidate cross-linking. The numbers on the left are the molecular weights (10^3) of protein standards. The upper two arrows on the right point to the positions of the two major radioactive species observed and identify these species as either a full complex, i.e., a Leu3p dimer covalently linked to a double-stranded DNA probe [(Leu3p(17-147) · $UAS_L)_2$] or a half complex, i.e., a Leu3p monomer covalently linked to single-stranded DNA probe [(Leu3p(17-147) · UAS_L)], on the basis of their apparent molecular weights and other considerations spelled out in the text. The bottom arrow points to the position of the Leu3p(17-147) monomer, obtained separately by Coomassie brilliant blue staining. (C) Estimation of the apparent molecular weights (MW, 10^3) of the three species identified in panel B, lanes 2 and 3. The three species (filled dots on the curve) are symbolized as in Fig. 5. The squares show positions of molecular weight standards.

interference experiment showed only one clear contact point on each strand; it corresponded to the central position of the triplets. What is important in the present context is that interference with peptide binding at each subunit binding site involves two adjacent methylated guanines on one DNA strand but only one methylated guanine on the other strand. Ethylation interference appears to involve only one ethylated phosphate near one of the two adjacent guanines. These results are consistent with preferential participation of one DNA strand in the recognition of a Leu3p subunit.

An earlier methylation interference study with a crude extract from cells producing full-length Leu3p had given results similar to those obtained in this study with the Leu3p(17-147) peptide (8). Since full-length Leu3p gives rise to two shifted complexes (27, 29), we expanded that experiment to include the two individual complexes (a and b in Fig. 7A) by using highly purified Leu3p. The results (Fig. 7B) showed that complexes a and b had indistinguishable methylation interference patterns. We also analyzed a mutant peptide, Leu3p(17-147)H50C, that has significantly greater affinity for UAS_{LEU} than does the wild-type peptide (see below). There was no noticeable difference between the interference footprints of the wild-type and mutant peptides (compare lanes 3 and 4 of Fig. 7B).

Leu3p binding to UAS_{LEU} is very sensitive to the spacing between the contact triplets and to the type of triplet. The interference footprinting studies indicated that the Leu3p dimer binds to two symmetrically placed contact triplets with the sequence GCC. . . .GGC (coding strand). The normal (wild-type) space between the two triplets is 6 bp. To explore the stringency of triplet spacing, we generated several mutant forms of UAS_{LEU} (Fig. 8). Their behavior in electrophoretic mobility shift assays showed the importance of maintaining the correct distance between the contact triplets. Decreasing the

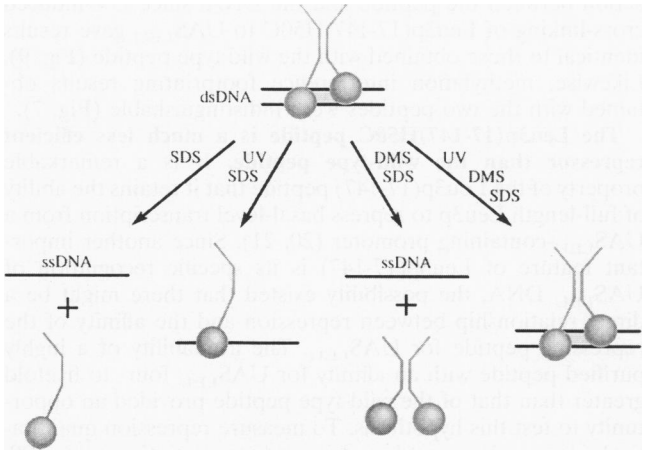


FIG. 5. Schematic representation of Leu3p(17-147) species obtained in the cross-linking experiments. The spheres represent $Zn(II)_2$-Cys_6 clusters. The central portions of the straight-line extensions represent dimerization domains. In Leu3p(17-147), a good potential dimerization element (heptad region) is located between residues 82 and 99 (Fig. 1B). The straight-line extensions proximal to the spheres represent the linkers (residues 70 to 81). The representation of the full protein-DNA complex is meant to reflect the idea that each Leu3p subunit is in closer proximity to one DNA strand and that the two subunits of a Leu3p dimer approach the DNA from the same direction. DMS, dimethylsuberimidate; ss, single stranded; ds, double stranded. See Results and Discussion for further details.

distance by just 1 bp or increasing it by 2 bp (to 5 and 8 bp, respectively) led to total loss of DNA-protein complex formation under the conditions employed. Increasing the distance by 1 bp (to 7 bp) was tolerated, but the resulting interaction between the mutated UAS_{LEU} and Leu3p(17-147) was weak. The effect of the seventh base pair was the same irrespective of whether a G·C or a T·A base pair was inserted, showing that it was indeed the distance and not the particular sequence that caused the diminished binding. Within the context of the normal 6-bp spacing, the sequence of the central 4 bp could be modified without apparent penalty (Fig. 8B, lines 8 and 9). However, this was not true for the base pairs immediately adjacent to the contact triplets; whenever these base pairs were different from G·C and C·G, respectively, binding was impaired (Fig. 8B, lines 7 and 10; also, reference 8).

Since other members of the $Zn(II)_2$-Cys_6 cluster family, e.g., Gal4p, Put3p, and Ppr1p, recognize a different set of triplets (CGG. . . .CCG) (Fig. 8A, right-hand portion), we wondered whether Leu3p might also interact with that set of triplets. The results in Fig. 8 show that this is not the case: no complex formation was observed between Leu3p(17-147) and DNA containing the spacer of UAS_{LEU} but the triplets of UAS_{GAL} (Fig. 8B, line 12).

Oligonucleotide 2 of Fig. 8 (UAS_{LO}; zero spacing) actually contained two potential monomer binding sites (5′-GCC GG-3′ on each strand). The lack of any recognizable binding to this probe suggests that Leu3p(17-147) monomers do not stably interact with the half site.

The Leu3p(17-147)H50C peptide has greater affinity for UAS_{LEU} than does the wild-type peptide, but its overall interaction with the DNA appears to be unchanged. Previous work had shown that substitution of the histidine in position 50

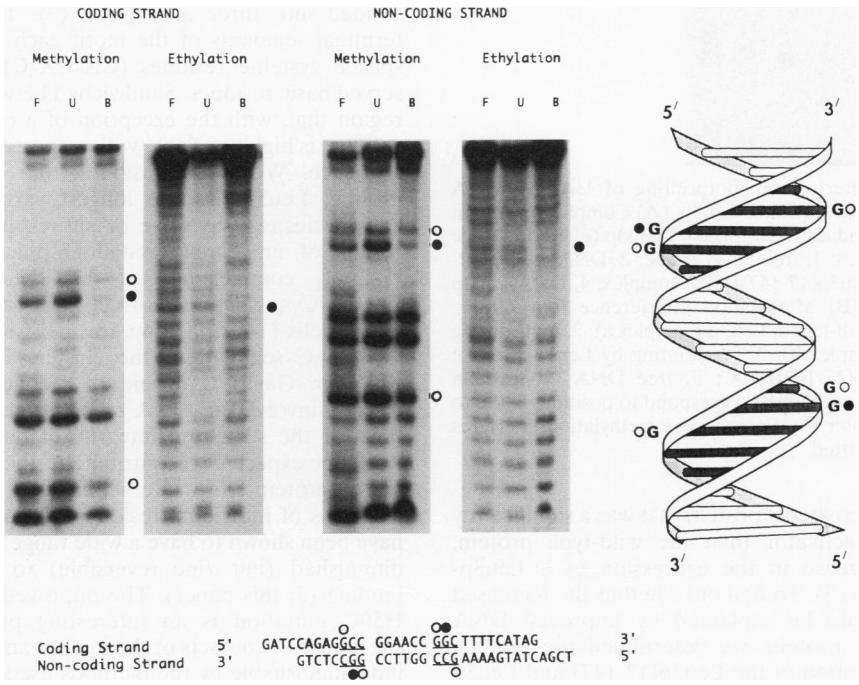


FIG. 6. Ethylation and methylation interference footprinting of UAS_{LEU} DNA with the Leu3p(17-147) peptide. Lanes F represent the free fraction, lanes U represent the unbound fraction, and lanes B represent the bound fraction of the modified DNA. Stronger contacts are indicated by filled circles, and weaker contacts are indicated by open circles. The coding and noncoding strands of the UAS_{LEU}-containing probes are shown at the bottom. A projection of the target DNA showing the contacts deduced by methylation and ethylation interference footprinting is shown on the right.

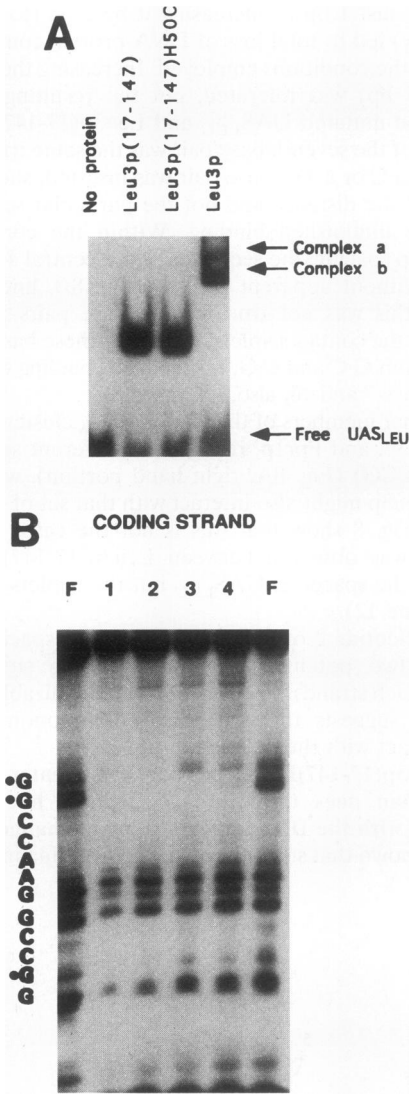


FIG. 7. Methylation interference footprinting of UAS_{LEU} DNA (coding strand) with different forms of Leu3p. (A) Complex formation between UAS_{LEU} DNA and different forms of Leu3p (electrophoretic mobility shift assays). Lanes: 1, free DNA probe; 2, DNA–Leu3p(17-147) complex; 3, DNA–Leu3p(17-147)H50C complex; 4, DNA-Leu3p (full-length) complexes. (B) Methylation interference footprinting. Lanes: 1, footprinting by full-length Leu3p (complex a); 2, footprinting by full-length Leu3p (complex b); 3, footprinting by Leu3p(17-147); 4, footprinting by Leu3p(17-147)H50C; F, free DNA (no protein present). The bases shown on the left correspond to positions −198 to −187 of the *LEU2* promoter (1). Bases whose methylation interferes with Leu3p binding are dotted.

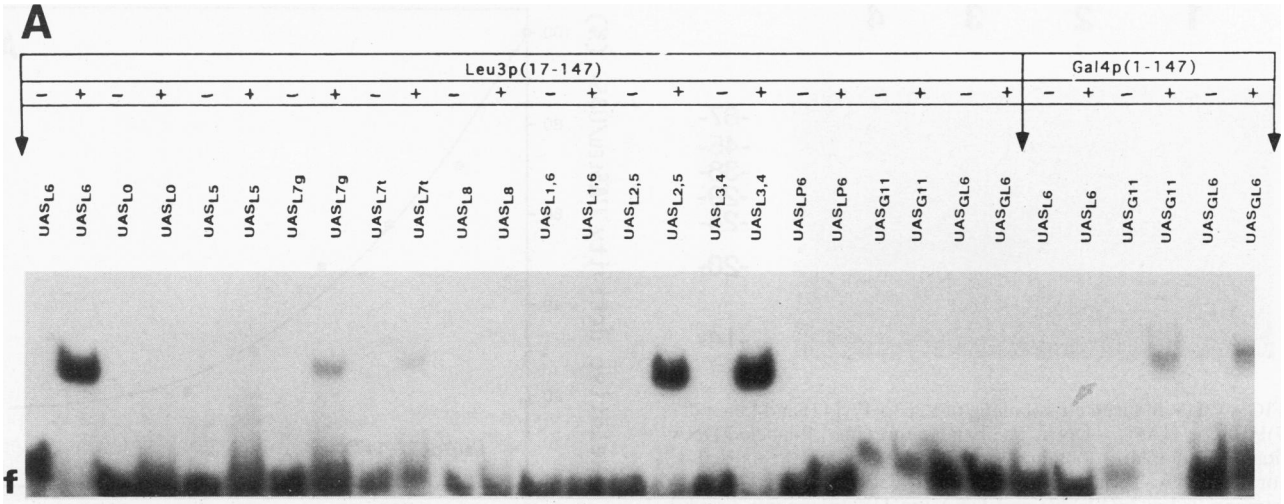
of Leu3p with cysteine created a protein that was a significantly better transcriptional activator than the wild-type protein, causing a 1.5-fold increase in the expression of a Leu3p-regulated reporter gene (3). To find out whether the increased activation capacity could be explained by improved DNA binding of the mutant protein, we determined the binding constants for the interaction of the Leu3p(17-147) and Leu3p (17-147)H50C peptides with UAS_{LEU}. The apparent dissociation constants, determined by quantitation of electrophoretic mobility shift assays (5), were 3.1 nM for the wild-type peptide and 0.7 nM for the mutant peptide. This four- to fivefold increase in affinity apparently did not affect the overall interaction between the peptide and the DNA, since UV-induced cross-linking of Leu3p(17-147)H50C to UAS_{LEU} gave results identical to those obtained with the wild type peptide (Fig. 9). Likewise, methylation interference footprinting results obtained with the two peptides were indistinguishable (Fig. 7).

The Leu3p(17-147)H50C peptide is a much less efficient repressor than the wild-type peptide. It is a remarkable property of the Leu3p(17-147) peptide that it retains the ability of full-length Leu3p to repress basal-level transcription from a UAS_{LEU}-containing promoter (20, 21). Since another important feature of Leu3p(17-147) is its specific recognition of UAS_{LEU} DNA, the possibility existed that there might be a direct relationship between repression and the affinity of the repressing peptide for UAS_{LEU}. The availability of a highly purified peptide with an affinity for UAS_{LEU} four- to fivefold greater than that of the wild-type peptide provided an opportunity to test this hypothesis. To measure repression quantitatively, we used a yeast-based in vitro transcription assay (20, 21). Interestingly, the results (Fig. 10) showed that Leu3p(17-147)H50C is a much weaker repressor than Leu3p(17-147), demonstrating that there is no direct, proportional correlation between strength of binding and repression.

DISCUSSION

The results presented here contribute in two ways to our understanding of the DNA-protein interactions within the family of $Zn(II)_2$-Cys_6 cluster proteins. First, they reinforce the special position of Leu3p within this class of proteins. Second, they allow some important conclusions to be drawn with respect to the relative spatial arrangement of Leu3p and its DNA target.

About a dozen members of the $Zn(II)_2$-Cys_6 cluster family are known. Their strongly conserved cluster motif can be divided into three subregions (3). The N-terminal and C-terminal segments of the motif each contain three distinctly spaced cysteine residues (CX_2CX_6C) and several well-conserved basic residues. Sandwiched between these segments is a region that, with the exception of a centrally located proline residue, is highly variable with respect to both its length and its sequence. With nine residues, one of them an additional proline, Leu3p has the longest variable region; the other known clusters have five or six residues in this region. The results of an X-ray crystallographic analysis of a Gal4p-UAS_{GAL} complex suggest that the main purpose of the $Zn(II)_2$-Cys_6 cluster is correct positioning of the DNA recognition helix (14). In Gal4p, this helix is part of the N-terminal CX_2CX_6C segment of the cluster. The variable subregion, which in Gal4p is devoid of secondary structure and not directly involved in DNA recognition, is nevertheless important for the structural integrity of the cluster and therefore would be expected to contribute to the strength and specificity of the protein-DNA interaction. In Leu3p, substitutions and deletions of individual residues within the variable subregion have been shown to have a wide range of effects, from strongly diminished (but zinc reversible) to significantly improved binding (3; this paper). The improved binding caused by the H50C mutation is an interesting phenomenon. Since the overall DNA contacts of the mutant and wild-type peptides are indistinguishable by the methods used here, it is possible that the zinc cluster of the mutant peptide has assumed a more flexible configuration that allows a better fit between peptide and DNA, without changing the actual contact points. Increased flexibility could have resulted from the formation of a less constrained $Zn(II)_2$-Cys_6 complex that would contain only



B

#	Designation	Oligonucleotide sequence (coding strand)	DNA Binding Activity
1	UAS_{L6}	5' AGG TGA GAG GCC **GGA ACC** GGC TTT TCA TAT 3'	+
2	UAS_{L0}	5' AGG TGA GAG GCC GGC TTT TCA TAT 3'	-
3	UAS_{L5}	5' AGG TGA GAG GCC **GG ACC** GGC TTT TCA TAT 3'	-
4	UAS_{L7g}	5' AGG TGA GAG GCC **GGA g ACC** GGC TTT TCA TA 3'	+/-
5	UAS_{L7t}	5' AGG TGA GAG GCC **GGA t ACC** GGC TTT TCA TA 3'	+/-
6	UAS_{L8}	5' GG TGA GAG GCC **GGA gc ACC** GGC TTT TCA TA 3'	-
7	$UAS_{L1,6}$	5' AGG TGA GAG GCC **aGA ACt** GGC TTT TCA TAT 3'	-/+
8	$UAS_{L2,5}$	5' AGG TGA GAG GCC **GaA AtC** GGC TTT TCA TAT 3'	+
9	$UAS_{L3,4}$	5' AGG TGA GAG GCC **GGt tCC** GGC TTT TCA TAT 3'	+
10	UAS_{LP6}	5' AGG TGA GAG GCC **caa ttg** GGC TTT TCA TAT 3'	-
11	UAS_{G11}	5' CCA AAA AGC GCT CGG **ACA ACT GTT GA** CCG TGA TCC GA 3'	-
12	UAS_{GL6}	5' CCA AAA AGC GCT CGG **gga acc** CCG TGA TCC GA 3'	-

FIG. 8. Effect of the spacing of the contact triplets of UAS_{LEU} and of UAS_{LEU} sequence changes on Leu3p(17-147) binding. (A) Electrophoretic mobility shift assays. In a total volume of 30 μl, 250 ng of the highly purified Leu3p(17-147) or Gal4p(1-147) peptide was incubated with 1.4 ng of a ^{32}P-labeled DNA probe. The sequences of the probes used are given in panel B. Each probe was incubated either with (+) or without (−) a peptide. f, free probe. (B) Correlation between oligonucleotide sequences and the binding activity of Leu3p. Binding strength is given in qualitative terms as + (wild-type-like binding), +/− (weak binding), −/+ (very weak binding), or − (no recognizable binding). The sequence on line 1 is that of the *LEU2* promoter (positions −206 to −177 [1]). The UAS_{LEU} contact triplets are underlined, and the spacer between the triplets is in bold letters. Lines 2 to 6 show variations in spacer length. Lines 7 to 10 show variations in the spacer sequence. The spacer shown in line 10 is identical to the Ppr1p-binding site. Line 11 shows the Gal4p-binding site from the *GAL7* promoter (6). Line 12 is the same as line 11, except that the spacer is that of the *LEU2* gene. Note that the contact triplets in lines 11 and 12 are those of the Gal4p-binding site, while all of the others are those of the Leu3p-binding site.

one bridging cysteine instead of the two believed to be present in the $Zn(II)_2$-Cys_6 complex (3).

To understand the interaction of Leu3p and related activators with DNA, two additional segments of their DNA-binding region must be taken into account. These segments are the so-called linker region, located on the C-terminal side of the $Zn(II)_2$-Cys_6 cluster, and a coiled-coil dimerization element of the leucine zipper type that adjoins the linker. The importance of the linker for DNA-binding specificity has repeatedly been documented (7, 14, 16, 24). For example, in a recent comparison of the binding regions of the Gal4, Put3, and Ppr1 proteins, Reece and Ptashne demonstrated, with the help of chimeric constructs, that the binding specificity of these proteins is, to a very large degree, determined by a structural unit

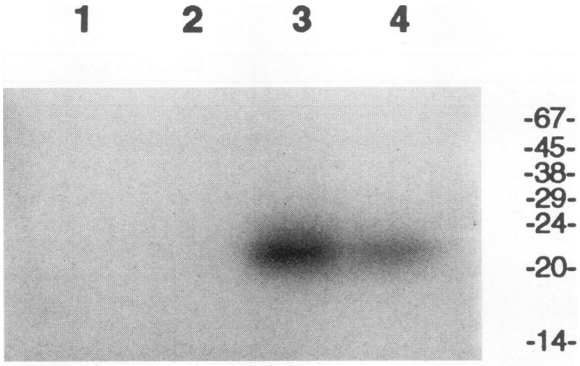
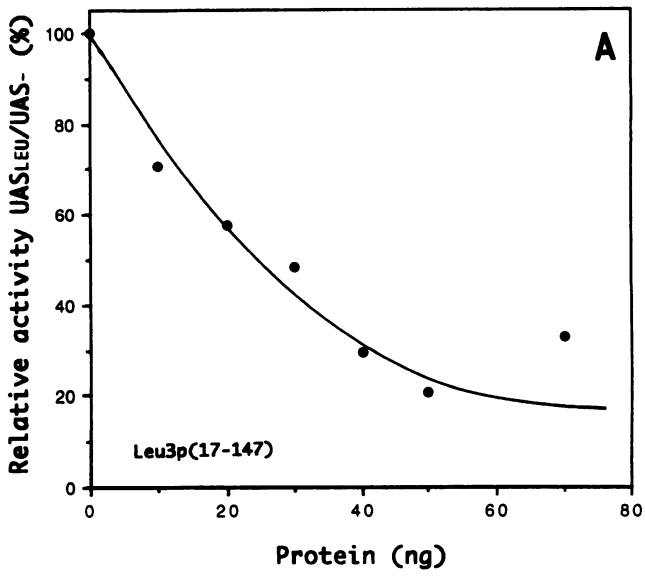


FIG. 9. UV-induced cross-linking of Leu3p(17-147) and Leu3p(17-147)H50C to UAS_{LEU} DNA. The bands represent ^{32}P-labeled DNA-containing complexes obtained by SDS-PAGE and visualized by autoradiography. Lanes: 1, Leu3p(17-147)H50C and DNA, no UV treatment; 2, Leu3p(17-147) and DNA, no UV treatment; 3 and 4, identical to lanes 1 and 2 but with UV treatment. The numbers on the right are molecular weights (10^3) of protein standards.

that encompasses the linker and the N-terminal end of the dimerization motif; as a consequence, the $Zn(II)_2$-Cys_6 clusters of these proteins are interchangeable (16). They proposed that other members of this family, especially Lac9, Leu3, and Hap1 (which also seem to be endowed with the cluster-linker-dimerization element structural triad), should bind to their DNA targets in a manner similar to that of Gal4, Put3, and Ppr1. We believe that, at least in Leu3, both the linker and the zinc cluster contribute to the specificity of DNA binding. The work of Witte and Dickson is pertinent here (24). They found that a peptide containing the $Zn(II)_2$-Cys_6 cluster of Leu3p and the linker region of Lac9 (a transcriptional activator from *Kluyveromyces lactis* that is structurally and functionally homologous to the Gal4 protein of *S. cerevisiae*) interacted with UAS_{GAL} much more weakly than did a peptide containing the $Zn(II)_2$-Cys_6 cluster of Ppr1 and the linker region of Lac9. By contrast, interaction of the latter with UAS_{GAL} was practically indistinguishable from the interaction of native Lac9 with UAS_{GAL}. These results indicate that while the Ppr1 and Lac9 zinc clusters are freely interchangeable, the Leu3 and Lac9 clusters are not. An obvious reason for this difference is that the clusters of Ppr1 and Lac9 (like the clusters of Gal4 and Put3) react with CGG....CCG triplets, whereas the Leu3 cluster reacts with GCC....GGC triplets.

On the basis of all of the available evidence, the following picture emerges for the interaction between Leu3p and UAS_{LEU}. First, Leu3p dimers approach the DNA in search of specific binding sites. Although no direct evidence of dimer formation in the absence of DNA was obtained for the peptide, it is possible that there exists a monomer-dimer equilibrium that is very much on the side of the monomers but is shifted in favor of the dimers by the target DNA. Alternatively, a Leu3p(17-147) monomer might transiently associate with the target DNA and be locked in place by the arrival of a second monomer, or the first monomer might facilitate the binding of the second monomer. Next, tight contact is established by interaction between the DNA recognition element of the $Zn(II)_2$-Cys_6 cluster and the GCC....GGC triplets. That contact takes place in the major groove and results in an arrangement in which each Leu3p subunit is in closer proximity to one of the two DNA strands. For tight binding to occur, it is imperative that the center-to-center distance between the contact triplets be 9 bp (corresponding to a spacer of 6 bp).



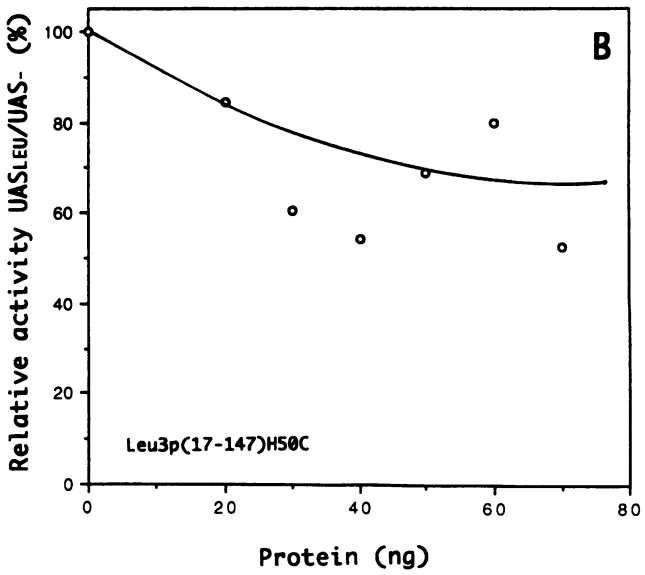


FIG. 10. Repression of basal-level transcription by Leu3p(17-147) (A) and Leu3p(17-147)H50C (B). In vitro transcription was performed with a *leu3*-null yeast extract and the indicated amounts of highly purified peptides in the absence of α-IPM. The ratios of UAS_{LEU}-derived transcripts over UAS^- derived transcripts plotted were obtained by densitometry scanning of autoradiography results. The values for UAS_{LEU}/UAS^- were normalized with respect to the value obtained without the peptides.

Since this center-to-center distance is close to one full turn of B-DNA, the two zinc clusters of the Leu3p dimer would be expected to approach the DNA from the same direction. This is in contrast to the Gal4 protein in which the center-to-center distance between the contact triplets in UAS_{GAL} is 14 bp, causing the Gal4p zinc clusters to grip the DNA from opposite directions (14). The postulated Leu3p linker region extends from residue 70 to residue 81 (Fig. 1) and is thus almost

identical in size to the Gal4p linker. The latter forms an extended chain that follows the contours of the DNA (14). Given the different spatial arrangements of the UAS_{GAL} and UAS_{LEU} contact triplets, one would expect the structure of the Leu3p linker to be more compact than that of the Gal4p linker and/or to point away from the DNA instead of hugging it. The lack of extensive contact of the Leu3p(17-147) peptide with the sugar-phosphate backbone suggested by the ethylation interference experiment is consistent with this view.

Since the Leu3p(17-147) peptide retains the transcriptional repression function of full-length Leu3p, understanding the molecular architecture of the Leu3p(17-147)–UAS_{LEU} complex may help to elucidate the mechanism of repression. We consider two major possibilities to explain the repression function of Leu3p(17-147): (i) repression may result from direct Leu3p-transcription factor interactions, or (ii) repression may stem from Leu3p-DNA interactions that result in conformational changes in the DNA. Interactions with transcription factors could occur through different regions of the Leu3p(17-147) molecule. Two distinct regions are being considered: (i) the DNA-binding region, defined as the zinc cluster and the adjoining linker, and (ii) the region(s) outside the DNA-binding domain, including the acidic region present in the last 30 amino acids of the peptide (Fig. 1). We cannot yet distinguish between the different models.

However, one question we were able to address was whether higher affinity for the DNA target would result in greater repression ability. The results obtained with the Leu3p(17-147)H50C mutant peptide show that, at least in this case, the opposite is true: higher affinity is accompanied by weaker repression capability. This nevertheless suggests that the DNA-binding and repression functions of Leu3p are interrelated and that an important determinant for repressor efficiency of Leu3p is a repression-competent conformation of the DNA-binding domain.

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