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The C6 Zinc Finger and Adjacent Amino Acids Determine DNA-Binding Specificity and Affinity in the Yeast Activator Proteins LAC9 and PPR1

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LAC9 is a DNA-binding protein that regulates transcription of the lactose-galactose regulon in *Kluyveromyces lactis*. The DNA-binding domain is composed of a zinc finger and nearby amino acids (M. M. Witte and R. C. Dickson, Mol. Cell. Biol. 8:3726–3733, 1988). The single zinc finger appears to be structurally related to the zinc finger of many other fungal transcription activator proteins that contain positively charged residues and six conserved cysteines with the general form Cys-Xaa$_2$-Cys-Xaa$_6$-Cys-Xaa$_{6-9}$-Cys-Xaa$_2$-Cys-Xaa$_6$-Cys, where Xaa$_n$ indicates a stretch of the indicated number of any amino acids (R. M. Evans and S. M. Hollenberg, Cell 52:1–3, 1988). The function(s) of the zinc finger and other amino acids in DNA-binding remains unclear. To determine which portion of the LAC9 DNA-binding domain mediates sequence recognition, we replaced the C6 zinc finger, amino acids adjacent to the carboxyl side of the zinc finger, or both with the analogous region from the *Saccharomyces cerevisiae* PPR1 or LEU3 protein. A chimeric LAC9 protein, LAC9(PPR1 34-61), carrying only the PPR1 zinc finger, retained the DNA-binding specificity of LAC9. However, LAC9(PPR1 34-75), carrying the PPR1 zinc finger and 14 amino acids on the carboxyl side of the zinc finger, gained the DNA-binding specificity of PPR1, indicating that these 14 amino acids are necessary for specific DNA binding. Our data show that C6 fingers can substitute for each other and allow DNA binding, but binding affinity is reduced. Thus, in a qualitative sense C6 fingers perform a similar function(s). However, the high-affinity binding required by natural C6 finger proteins demands a unique C6 finger with a specific amino acid sequence. This requirement may reflect conformational constraints, including interactions between the C6 finger and the carboxyl-adjacent amino acids; alternatively or in addition, it may indicate that unique, nonconserved amino acid residues in zinc fingers make sequence-specifying or stabilizing contacts with DNA.

Many yeast transcriptional activator proteins (reviewed in reference 16) as well as the QUTA regulatory protein of *Aspergillus nidulans* (4) and the qa-1F regulatory protein of *Neurospora crassa* (2) contain a similar sequence of 28 to 31 amino acid residues whose most striking feature is six conserved cysteines. The Cys residues are thought to chelate two Zn^{2+} ions and form a zinc finger motif that is part of the DNA-binding domain (3, 29; Halverson et al., unpublished results). A major question about the region containing the six cysteines, the C6 zinc finger, is whether it contacts DNA and thus plays a direct role in the recognition of a specific DNA sequence, or whether amino acids outside of the C6 region determine DNA sequence specificity.

Studies on proteins with zinc fingers of the C2H2 class have shown that the zinc finger region determines DNA-binding specificity (27, 34). More recently it has been shown (26) that the amino acids that determine the binding specificity of the estrogen-glucocorticoid receptor family are located on the carboxyl side of the C4 zinc finger. These reports however, deal with proteins that contain two or more zinc fingers. It is not clear how DNA-binding specificity is determined in proteins with only a single zinc finger of the C6 class.

Much of our current understanding of the function of the C6 zinc finger region comes from studies of the GAL4 protein of *Saccharomyces cerevisiae* and the LAC9 protein of *Kluyveromyces lactis*. These two positive regulatory proteins are structurally (33, 36) and functionally (32, 36) homologous. They activate transcription of genes in the galactose-melibiose regulon of *S. cerevisiae* and the galactose-lactose regulon of *K. lactis* (reviewed in reference 6). That zinc is actually part of the C6 finger region and is necessary for DNA-binding activity has been inferred from genetic studies of *gal4* mutants (17) and from physical studies of GAL4 peptides containing the DNA-binding domain (29). Recent biochemical studies on LAC9 peptides containing the DNA-binding domain show that two zinc atoms are necessary for DNA-binding activity (Halvorsen et al., unpublished results).

We (35, 36) and others (18, 33) have suggested that while the C6 zinc finger is essential for DNA binding, it may not play a direct role in the recognition of a specific DNA sequence. This proposal is based on genetic and biochemical experiments showing that some, but not all, amino acid changes on the carboxyl side of the zinc finger of GAL4 (18) and LAC9 (35) reduce DNA-binding activity. Another line of reasoning argues that since there is considerable amino acid sequence similarity between the C6 zinc fingers, this region is unlikely to determine DNA-binding specificity. This hypothesis was supported when Corton and Johnston (5) demonstrated that the C6 zinc finger region of GAL4 could be replaced by the analogous region from another yeast activator protein, PPR1, without changing the DNA-binding specificity of GAL4. They also demonstrated that a 14-amino-acid region adjacent to the carboxyl side of the zinc finger was necessary for specific DNA binding. The amino acids that determine DNA binding in proteins containing a

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C6 zinc finger are likely to be unique, since these proteins, except GAL4 and LAC9, recognize unique DNA sequences; the amino acid residues in GAL4 and LAC9 that contact DNA are probably identical or nearly so, since the proteins recognize similar DNA sequences (6).

In the experiments reported here, we have examined three C6 zinc fingers to determine whether they perform similar functions, whether their primary function is recognition of a specific DNA sequence, and if this is not their primary function, then which region of the DNA-binding domain determines DNA-binding specificity. To determine domain structure-function relationships, we replaced the C6 zinc finger of LAC9 with the corresponding region from the *S. cerevisiae* PPR1 (20) or LEU3 (37) protein. These proteins activate transcription of genes involved in pyrimidine and leucine biosynthesis, respectively, and are members of the C6 family of zinc finger proteins. Our results demonstrate that at least some of the amino acids necessary for specific recognition of DNA by PPR1 are located within the 14 amino acids adjacent to the carboxyl side of the C6 zinc finger. We also show that chimeric LAC9 proteins carrying the PPR1 or the LEU3 C6 zinc finger retain the DNA-binding specificity of LAC9, indicating that these C6 zinc fingers have some functional similarity.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study were SJ21R2 (a *gal4::ura3 leu2-3,112 ade1 ura3-52 MEL1*), a Ura$^-$ derivative of a strain in which the region between the *Sna*BI (51 base pairs [bp] in front of the ATG initiation codon) and the *Nru*I (at bp 999) restriction sites in the *GAL4* gene was deleted and replaced with the *URA3* gene by transplacement (35); YM2631 (a *ura3-52 his3D200 ade2-101 trp1D901 met gal4D542 LEU2::GAL1-lacZ*) (Mark Johnston, Washington University, St. Louis, Mo.); YM709:: PPR104 (a *gal4D542 gal80D538 ura3-52 his3D200 ade2-101 lys2-801 trp1D901 tyr1-501 met CAN^r ppr1::HIS3 URA3:: GAL1-lacZ*), in which the four GAL4- or LAC9-binding sites, UAS_G (CGGAAGACTCTCCTCCG), in the promoter region of the *GAL1-lacZ* fusion were replaced by four PPR1-binding sites, UAS_U (TTCGGTAATCTCCGAA; L. Keegan, Ph.D. thesis, Harvard University, Cambridge, Mass.); PFY420 (a *ura3-52 leu3::HIS3 LEU4^r-103 his3D1*) (9, 10); and PFY423 (α *leu3::HIS3 LEU4^r-103 his3 ura3-52 leu2-3,112 TRP1::LEU2-lacZ*), a strain carrying a *LEU2* promoter fused to the *lacZ* coding region and integrated at *TRP1* (8). The media for growing yeast strains were as described before (31). *Escherichia coli* DH5α and XA90 were used for maintaining plasmids.

Construction of LAC9 variants carrying zinc finger replacements. To replace the zinc finger of LAC9 with the corresponding region from other proteins, we first introduced an *Sph*I restriction site in front of the first Cys residue of the zinc finger region. This was done by using oligonucleotide-directed mutagenesis (22) to introduce a G→A substitution at nucleotide +282 (relative to the A in the ATG initiation codon) of the *LAC9* gene. As a consequence, the *LAC9* zinc finger, encoding amino acids 94 to 123, could be removed and replaced by the corresponding coding region from *LEU3* or *PPR1* as an *Sph*I-*Acc*I restriction fragment. The *Acc*I site is present naturally in *LAC9*. To replace amino acids adjacent to the zinc finger of LAC9 with the corresponding region from other proteins, we introduced a *Dra*I restriction site at nucleotide +456 by using oligonucleotide-directed mutagenesis. This allowed the region adjacent to the zinc finger, encoding residues 124 to 152, to be removed and replaced by the corresponding region of *LEU3* or *PPR1* as an *Acc*I-*Dra*I restriction fragment.

For these DNA constructions we used the vector pMW1, which is pTZ18R (Pharmacia) with the following modifications: the Ap^r gene was deleted between the three *Dra*I sites, a 1.4-kilobase (kb) DNA fragment containing the kanamycin resistance gene of transposon Tn*903* was inserted at the *Pst*I site in the polylinker, a 1.4-kb DNA fragment carrying the *TRP1-ARS1* region of *S. cerevisiae* was inserted by blunt-end ligation into the *Sal*I site of the polylinker, and *LAC9* was inserted into the *Sac*I site of the polylinker. The *Sph*I site in the polylinker was destroyed by treatment with mung bean nuclease. The *Acc*I site in *TRP1* was destroyed by filling in the ends with the Klenow fragment of DNA polymerase I. Double-stranded DNAs coding for the *LEU3* and *PPR1* zinc fingers or adjacent regions were prepared by the method of mutually primed synthesis (14). DNAs used for the zinc finger replacements were digested first with *Acc*I and then with *Sph*I. DNAs used for replacement of the region adjacent to the C6 zinc finger were used without restriction digestion. These DNA samples were extracted with phenol, phenol-chloroform, and chloroform and then ethanol precipitated in the presence of ammonium acetate. The DNA was washed once with 70% ethanol, dried, suspended in 10 μl of deionized water, and ligated into pMW1 cut with the appropriate restriction endonucleases. Chimeric *LAC9* genes carrying both the *LEU3* or *PPR1* zinc finger and the adjacent region were constructed by first replacing the zinc finger region and then replacing the adjacent region. The DNA sequence of chimeric *LAC9* genes was confirmed by sequence analysis.

Chimeric *LAC9* genes were joined to the normal *LAC9* promoter by replacing the wild-type 2.7-kb *Bgl*II-*Bst*EII fragment of pRS2 (36) with the analogous fragment from the pMW1 chimeric constructs. Single-copy *S. cerevisiae* vectors carrying the wild-type or chimeric *LAC9* coding sequences under control of the normal *LAC9* promoter were constructed by inserting the 5.4-kb *Eco*RI fragment from the pRS2 constructs into the *Eco*RI site of the vector YCp50 to generate YCp50-*LAC9*. Multicopy *S. cerevisiae* expression vectors containing wild-type or chimeric *LAC9* genes under the control of the *LAC9* promoter were constructed by inserting a 5.4-kb *Eco*RI fragment, from either wild-type or chimeric pRS2, into the *Eco*RI site of YEp352LK (L. Keegan, Harvard University, Cambridge, Mass.).

To overproduce LAC9 proteins in yeast cells, the wild-type or chimeric *LAC9* genes carrying the *LEU3* or *PPR1* zinc finger were fused to the strong *ADH1* promoter by inserting a 4.5-kb *Bgl*II-*Hin*dIII DNA fragment, from either wild-type *LAC9* or chimeric pRS2 constructs, into the *Bam*HI-*Hin*dIII site of the multicopy expression vector p1AJ(S) (Oh and Hopper, University of Pennsylvania, Hershey, Pa.). Plasmids were introduced into *S. cerevisiae* strains by a lithium acetate transformation procedure (15). Yeast extracts used for DNA mobility shift assays were prepared as described before (30). β-Galactosidase assays of whole cells were performed as described before (13).

Expression of LAC9 peptides in *E. coli*. DNA sequences encoding amino acids 84 to 228 of LAC9, as well as the analogous regions of the zinc finger replacement constructs, were each expressed from the *tac* promoter in *E. coli* XA90 (1; Yuan-di Halvorsen, University of Kentucky, Lexington). Briefly, cells carrying an expression plasmid were grown in LB medium with 50 μg of ampicillin per ml and 10 μM $ZnCl_2$ to an A_{600} of 0.7 and then induced with 1 mM IPTG

(isopropyl-β-D-thiogalactopyranoside) for 3 h. After induction, cells were centrifuged for 10 min at 5,000 × *g* at 4°C and washed once with 0.5 volume of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5) containing 200 mM NaCl. Cells were recentrifuged and resuspended in 1/20 volume of ice-cold buffer A (20 mM HEPES [pH 7.5], 200 mM NaCl, 10 mM β-mercaptoethanol, 10 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, 20 μg of leupeptin per ml, 20 μg of pepstatin A per ml). Cells were treated with 1 mg of lysozyme per ml for 10 min on ice and sonicated at 0°C for 2 min (four times for 30 s each), which reduced the A_{600} by 80 to 90%. Cellular debris was removed by centrifuging at 4°C for 15 min at 15,000 × *g*. The supernatant fluid, termed the lysate, was divided into portions and stored at −80°C. The protein concentration of the lysates was determined by the method of Lowry et al. (25). Production of LAC9 peptide in each lysate was examined by electrophoresis in a 10% polyacrylamide gel (acrylamide-bisacrylamide, 30:0.8), containing sodium dodecyl sulfate, as described by Laemmli (23). After electrophoresis, the gel was stained with 0.1% Coomassie brilliant blue R in 5% acetic acid–5% methanol for 30 min, and the gel was destained in 5% acetic acid–5% methanol overnight. The LAC9 peptide in each lysate constituted 10 to 15% of the total protein as determined by densitometry of stained polyacrylamide gels.

DNA-binding assays. Plasmid DNAs were purified by equilibrium centrifugation in CsCl. DNA binding was monitored by using a gel mobility shift assay (11). Binding experiments used DNA fragments containing UAS_G, UAS_L, or UAS_U. Specific binding to UAS_G (CGGAAATTTGTG GTCCG [24]) was determined by using pIC20R-UAS DNA (35). Both pIC20R-UAS and pIC20R were digested with *Eco*RI and radioactively labeled by filling in the 5′ ends with [α-^{32}P]dATP with the Klenow fragment of DNA polymerase I. The 110- or 88-bp fragment, respectively, was isolated by electroelution from a 2% agarose gel. Specific binding to UAS_L was determined by using a 279-bp *Hin*cII fragment isolated from the *LEU2* promoter (8). The fragment was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase and isolated by electroelution from a 2% agarose gel. To determine specific binding to UAS_U, a 200-bp *Hin*dIII-*Pst*I fragment was isolated from the *URA3* promoter. The fragment was radioactively labeled by filling in the *Hin*dIII site with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase I.

Binding reaction mixes contained 20 mM HEPES (pH 7.5), 0.1 mM EDTA, 7 mM β-mercaptoethanol, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM phenylmethylsulfonyl floride, 1 mM Na_2PO_3F, 5 mM AMP, 10% glycerol, 0.5 ng of radiolabeled DNA (10 fmol), and the indicated amount of protein in a total volume of 15 μl. Reaction mixes were incubated for 15 min at 23°C and loaded, with the current on, onto 5% polyacrylamide gels (acrylamide-bisacrylamide, 30:0.8) in TBE buffer (90 mM Tris hydrochloride, 90 mM H_3BO_3, 2.5 mM EDTA). Gels were preelectrophoresed at 180 V for 1 h, and then the sample was electrophoresed at 180 V until the bromphenol blue reached the bottom of the gel. After electrophoresis, gels were fixed in 10% methanol–10% acetic acid for 1 h, transferred to Whatman 3MM paper, dried, and autoradiographed.

RESULTS

Production of LAC9 protein. Previously we studied the effect of single amino acid changes on LAC9 DNA-binding activity by using a DNA mobility shift assay in which the LAC9 protein was made in vitro in the presence of [^{35}S]methionine and incubated with a DNA fragment carrying the LAC9 DNA-binding sequence, UAS_G (35). Initially we used this experimental approach for the studies described here. Two problems continually hampered progress. First, the concentration of LAC9 protein was always low and limited the sensitivity of the assay; even variant LAC9 proteins with a small, 75% reduction in binding affinity would appear not to bind the UAS_G. Second, reproducibility was poor.

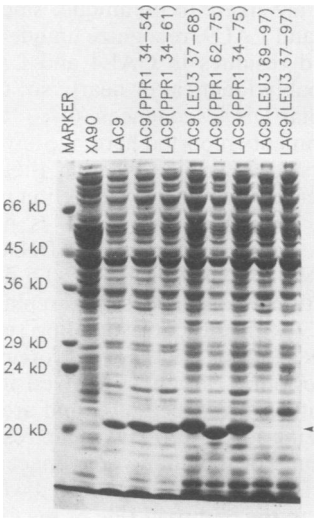

FIG. 1. Production of LAC9 Peptides in *E. coli*. Lysates from *E. coli* XA90, producing wild-type LAC9 or chimeric LAC9 peptides (indicated by the arrow), were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The lane marked XA90 represents a lysate from cells lacking an expression vector. Equal amounts of total protein (50 μg) were loaded per lane. Size markers are shown in kilodaltons (kD).

To overcome these problems we needed a way to make large amounts of wild-type and several variant proteins. Production of a wild-type LAC9 peptide containing the DNA-binding domain, amino acids 84 to 223, and variant peptides was finally obtained in *E. coli* by using the inducible *tac* promoter to drive *LAC9* expression. With this approach, about 10% of cellular protein was the LAC9 peptide (Fig. 1). The high yield of LAC9 peptide allowed rapid detection and quantitation of small changes in DNA-binding affinity with cell lysates. The sensitivity of our procedure enabled detection of important binding activities that would have been overlooked by less-sensitive procedures.

PPR1 zinc finger replacements. The role of the C6 zinc finger in DNA binding remains elusive. Does the finger determine the DNA-binding specificity by contacting specific bases, or does it perform a more general role, such as displacing histones from the DNA or contacting phosphates in the DNA backbone? To begin to understand the function of the single C6 zinc finger domain, defined here as residues 94 to 123 of LAC9 (Fig. 2), in yeast transcriptional activator proteins, we replaced the zinc finger of LAC9 with the corresponding region of PPR1. Two types of replacements were made. In one experiment, amino acids 95 to 115 of LAC9 were replaced by amino acids 34 to 54 of PPR1 to create LAC9(PPR1 34-54) (Fig. 2). This replacement includes the four cysteine residues Cys-95, Cys-98, Cys-112, and Cys-115 that are thought to chelate Zn^{2+}. In the other

	Zinc finger		Adjacent region	
	34			91
PPR1	CKRCRLKKIKCDQE--FPS-CKRC	AKLEVPC	VSLDPATGKDVPRS	YVFFLEDRLAVMMRVL
LAC9(PPR1 34-54)	CKRCRLKKIKCDQE--FPS-CKRC	LKYNLDC	VYSPQVVRTFLTRA	NLTENENRVAELEQFL
LAC9(PPR1 34-61)	CKRCRLKKIKCDQE--FPS-CKRC	AKLEVPC	VYSPQVVRTPLTRA	NLTENENRVAELEQFL
C9(PPR1 62-75)	CDACRKKKWKCSKT--VPT-CTNC	LKYNLDC	VSLDPATGKDVPRS	NLTENENRVAELEQFL
AC9(PPR1 34-75)	CKRCRLKKIKCDQE--FPS-CKRC	AKLEVPC	VSLDPATGKDVPRS	NLTENENRVAELEQFL
	95			152
LAC9	CDACRKKKWKCSKT--VPT-CTNC	LKYNLDC	VYSPQVVRTPLTRA	NLTENENRVAELEQFL
LAC9(LEU3 37-68)	CVECRQQKSKCDAHERAPEPCTKC	AKKNVPC	IYSPQVVRTPLTRA	NLTENENRVAELEQFL
LAC9(LEU3 69-97)	CDACRKKKWKCSKT--VPT-CTNC	LKYNLDC	VLKRDFRRTYKRAR	NEAIEKRFKELTRTLT
LAC9(LEU3 37-97)	CVECRQQKSKCDAHERAPEPCTKC	AKKNVPC	ILKRDFRRTYKRAR	NEAIEKRFKELTRTLT
	37			97
LEU3	CVECRQQKSKCDAHERAPEPCTKC	AKKNVPC	ILKRDFRRTYKRAR	NEAIEKRFKELTRTLT

FIG. 2. Comparison of the zinc finger regions of LAC9, PPR1, LEU3, and each chimeric LAC9 protein. PPR1 and LEU3 sequences are shown in boldface type. LAC9 sequences are shown in standard type. The C6 zinc finger and the adjacent region are indicated.

experiment, amino acids 95 to 122 of LAC9 were replaced by amino acids 34 to 61 of PPR1 to create LAC9(PPR1 34-61). This replacement includes all six of the conserved cysteines in the zinc finger. The PPR1 protein was chosen for study because it binds to a different DNA sequence, UAS_U, than does LAC9, yet its zinc finger domain has an amino acid sequence similar to that of LAC9, including the number of amino acids between the six conserved cysteine residues (Fig. 2).

The DNA-binding activity of the LAC9(PPR1 34-54) and LAC9(PPR1 34-61) chimeras was measured by using a DNA mobility shift assay. If the zinc finger region plays a key role in DNA-binding specificity, then one or both chimeras should behave like PPR1 and bind UAS_U. On the other hand, if the zinc finger region is not the primary determinant of DNA sequence recognition, then the chimeras should behave like LAC9 and bind UAS_G. Both chimeric proteins behaved qualitatively like wild-type LAC9 and bound to UAS_G (Fig. 3). However, the affinity of the chimeras for UAS_G was reduced about 10-fold relative to the wild-type LAC9 peptide as estimated from densitometry of the autoradiograms shown in Fig. 3. Since the chimeras retained LAC9 DNA-binding specificity, it seems unlikely that the C6 zinc finger region of LAC9 is solely responsible for sequence-specific binding to UAS_G.

To determine whether the chimeras acquired any PPR1 DNA-binding properties, their affinity for UAS_U was measured. Surprisingly, the wild-type LAC9 peptide bound UAS_U. However, its affinity for UAS_U was 100-fold lower than its affinity for UAS_G (Fig. 4 and Table 1). Likewise, LAC9(PPR1 34-54) had 100-fold and LAC9(PPR1 34-61) had 20-fold less affinity for UAS_U than for UAS_G. Therefore, the chimeras did not gain DNA-binding affinity for UAS_U over that shown by wild-type LAC9.

To further define the function of the C6 zinc finger region, we examined the wild-type and chimeric *LAC9* genes for their ability to complement an *S. cerevisiae* strain defective in *gal4*. Complementation was measured by cell growth on plates containing galactose as the sole carbon source. Growth would indicate that the wild-type and chimeric

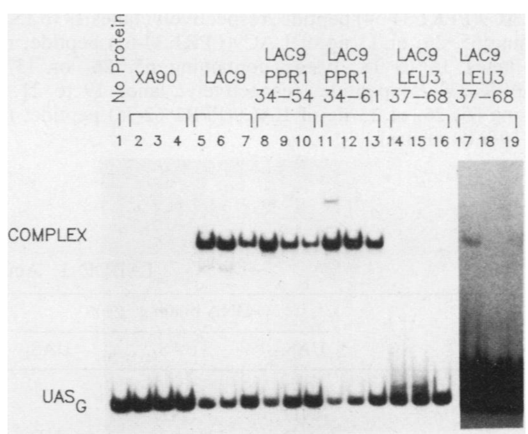

FIG. 3. LAC9(PPR1 34-54), LAC9(PPR1 34-61), and LAC9 (LEU3 34-68) retain affinity for UAS_G. Gel electrophoresis mobility shift assays were performed with ^{32}P-labeled UAS_G DNA. Protein-DNA complex formation (indicated by COMPLEX) was analyzed by native polyacrylamide gel electrophoresis and autoradiography (2-h exposure). UAS_G indicates the mobility of the unbound DNA. All lysates are from *E. coli*. Samples: lane 1, no protein lysate; lanes 2 to 4, lysate from *E. coli* XA90 lacking an expression vector (650, 260, and 130 ng of total lysate protein, respectively); lanes 5 to 7, lysate containing 4, 2, or 1 ng of wild-type LAC9 peptide, respectively; lanes 8 to 10, lysate containing 65, 26, or 13 ng of LAC9(PPR1 34-54) peptide, respectively; lanes 11 to 13, lysate containing 65, 26, or 13 ng of LAC9(PPR1 34-61) peptide, respectively; lanes 14 to 16, lysate containing 65, 26, or 13 ng of LAC9(LEU3 37-68) peptide, respectively; lanes 17 to 19, 20-h reexposure of lanes 14 to 16.

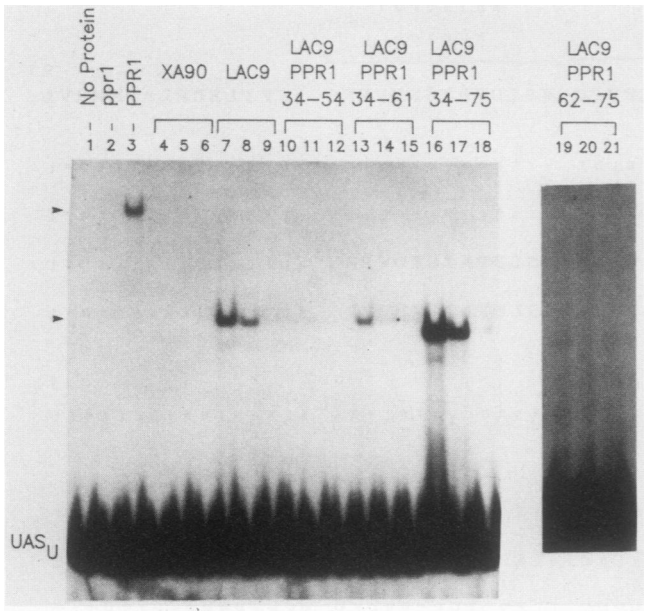

FIG. 4. LAC9 and chimeric LAC9-PPR1 peptides show binding affinity for UAS_U. Gel electrophoresis mobility shift assays were performed with ^{32}P-labeled UAS_U DNA. Protein-DNA complex formation (indicated by the arrows) was analyzed by native polyacrylamide gel electrophoresis and autoradiography (2-h exposure). UAS_U indicates the mobility of the unbound DNA. Unless indicated otherwise, the lysates were from *E. coli*. Samples: lane 1, no protein lysate; lane 2, 2 μg of yeast extract lacking PPR1 (strain YM709::PPR104); lane 3, 2 μg of yeast extract containing PPR1 (strain YM709::PPR104 transformed with a plasmid encoding wild-type PPR1); lanes 4 to 6, lysate from *E. coli* strain XA90 lacking an expression vector (650, 260, and 130 ng of total lysate protein, respectively); lanes 7 to 9, lysate containing 65, 26, or 13 ng of LAC9 peptide, respectively; lanes 10 to 12, lysate containing 65, 26, or 13 ng of LAC9(PPR1 34-54) peptide, respectively; lanes 13 to 15, lysate containing 65, 26, or 13 ng of LAC9(PPR1 34-61) peptide, respectively; lanes 16 to 18, lysate containing 65, 26, or 13 ng of LAC9(PPR1 34-75) peptide, respectively; lanes 19 to 21, lysate containing 65, 26, or 13 ng of LAC9(PPR1 62-75) peptide, respectively (10-h exposure).

LAC9 proteins could bind to UAS_G and activate transcription of the four genes that form the galactose regulon (16). For these experiments the entire *LAC9* coding region and promoter were incorporated into a single-copy *CEN* vector or a high-copy-number 2μm vector and transformed into yeast cells. *S. cerevisiae* was used as a host instead of *K. lactis* because the only available *K. lactis* vectors are medium copy number and are highly unstable compared with the *S. cerevisiae* vectors used here. It has been shown previously that *LAC9* can substitute for *GAL4* and allow *S. cerevisiae* to grow on galactose (36).

When chimeric genes for LAC9(PPR1 34-54) or LAC9 (PPR1 34-61) were carried on a single-copy *CEN* vector, the transformed cells grew on galactose as well as did cells having a wild-type *LAC9* gene (Fig. 5). These results support the DNA mobility shift data, and together they show that the C6 zinc finger of PPR1 can substitute for the LAC9 zinc finger to yield chimeric proteins that retain LAC9 DNA-binding specificity. Thus, C6 zinc fingers must perform a similar function(s). The function is not solely to determine DNA-binding specificity.

LEU3 zinc finger replacements. The considerable amino acid similarity and spacing of cysteines in the LAC9, GAL4, PPR1, and LEU3 zinc fingers suggests functional similarity, and data for the LAC9(PPR1 34-54) and LAC9(PPR1 34-61) chimeric proteins support this idea. To gain further support for the functional similarity of C6 zinc fingers, we replaced the LAC9 zinc finger, amino acids 95 to 122, with the corresponding region from LEU3, amino acids 37 to 68, to create LAC9(LEU3 37-68). The LEU3 C6 zinc finger was chosen because it has the least sequence similarity to the LAC9 C6 region and because the six cysteines that are thought to chelate zinc are separated by three more amino acids than in the corresponding region in LAC9 or PPR1 (Fig. 2). Thus, the LEU3 zinc finger provides a more critical test of the similarity of the C6 zinc fingers than does the PPR1 C6 finger. Gel mobility shift assays (Fig. 3) showed that the chimeric LAC9(LEU3 37-68) peptide bound to UAS_G but with reduced affinity. The reduction in DNA-binding affinity was estimated to be 500-fold by using two different X-ray film exposures to quantitate the amount of UAS_G complexed to peptide. No protein-DNA complex was seen when the same amount of chimeric peptide was incu-

TABLE 1. Activities of chimeric LAC9 proteins

Protein	DNA binding[a] (%)			Cell growth[b]			β-Galactosidase activity (nmol/min)[c]		
	UAS_G	UAS_U	UAS_L	UAS_G	UAS_U	UAS_L	UAS_G	UAS_U	UAS_L
LAC9	100	1	−	++	−	−	71	0.4	0.2
LEU3	ND[d]	ND		−	ND	++	0.2	ND	84
PPR1	ND		ND	−	++	ND	0.1	73	ND
LAC9(LEU3 37-68)	0.2	−	−	+	−	−	6.4	0.4	0.2
LAC9(LEU3 69-97)	−	−	−	−	−	−	0.1	0.3	0.2
LAC9(LEU3 37-97)	−	−	−	−	−	−	0.1	0.4	0.2
LAC9(PPR1 34-54)	10	0.1	−	++	−	−	37	11	0.2
LAC9(PPR1 34-61)	10	0.5	−	++	−	−	33	9	0.2
LAC9(PPR1 62-75)	0.1	−	−	−	−	−	0.2	0.4	0.2
LAC9(PPR1 34-75)	0.1	1.4	−	−	+	−	0.2	230	0.2

[a] DNA-binding activity is expressed as a percentage of the dissociation constant for the LAC9 peptide. −, No DNA binding was detectable. Crude yeast extracts were used as positive controls for wild-type PPR1 and LEU3 binding activities; therefore, dissociation constants for these samples could not be determined.

[b] The ability to complement *gal4*, *ppr1*, or *leu3* deletion strains of *S. cerevisiae* is shown as + or − as determined by growth on plates containing minimal medium and appropriate supplements.

[c] β-Galactosidase activities are shown as nanomoles of substrate hydrolyzed per minute per A_{600} unit. Values shown are averages of three independent experiments, each done in duplicate, in which the standard deviation was less than 20%.

[d] ND, Not determined.

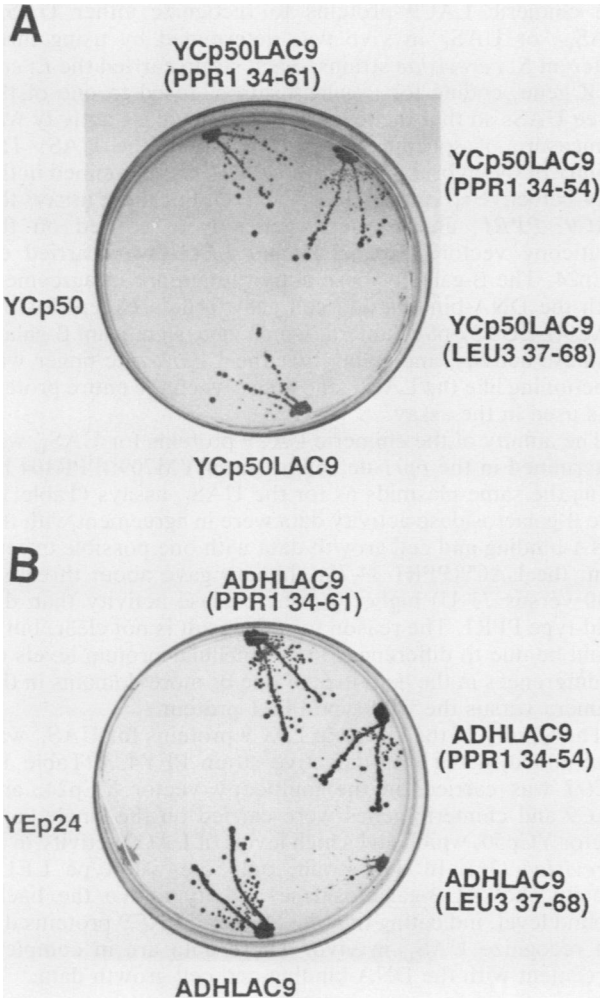

FIG. 5. In vivo complementation assays of chimeric LAC9 proteins. *S. cerevisiae* SJ21R2 (*gal4*) was transformed with chimeric *LAC9* genes fused to the *LAC9* promoter carried on the single-copy centromere vector YCp50 (A) or high-copy-number 2μm vectors having the chimeric *LAC9* genes fused to the strong *ADH1* promoter (B). Complementation of *gal4* was done by using plates containing galactose as the sole carbon source.

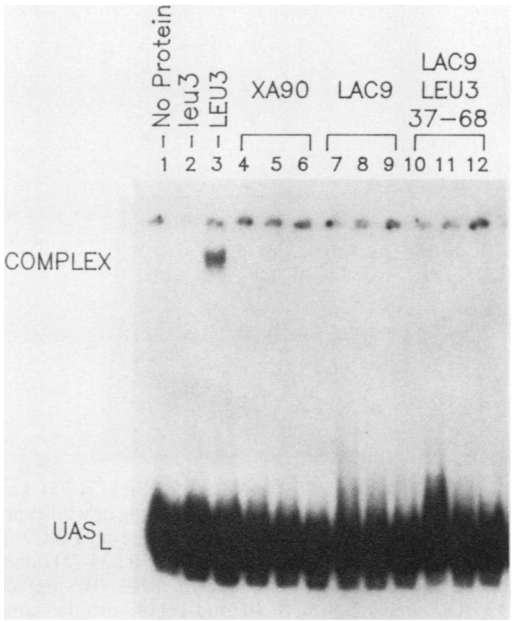

FIG. 6. LAC9(LEU3 37-68) shows no affinity for UAS_L. Gel electrophoresis mobility shift assays were performed with ^{32}P-labeled UAS_L DNA. Protein-DNA complex formation (indicated by COMPLEX) was analyzed by native polyacrylamide gel electrophoresis and autoradiography (2-h exposure). UAS_G indicates the mobility of the unbound DNA. Unless indicated otherwise, lysates were from *E. coli*. Samples: lane 1, no protein lysate; lane 2, 2 μg of yeast extract lacking LEU3 (strain PFY423); lane 3, 2 μg of yeast extract containing LEU3 (strain PFY423 transformed with a plasmid encoding wild-type LEU3); lanes 4 to 6, 650, 260, or 130 ng of total lysate protein from *E. coli* XA90, respectively; lanes 7 to 9, lysate containing 65, 26, or 13 ng of LAC9 peptide, respectively; lanes 10 to 12, lysate containing 65, 26, or 13 ng of LAC9(LEU3 37-68) peptide, respectively.

bated with the same DNA fragment lacking the UAS_G, indicating that complex formation was specific for UAS_G (data not shown). These results showed that the LAC9 and LEU3 zinc finger regions are functionally related even though they contain a different number of amino acids.

We also determined whether the LAC9(LEU3 37-68) chimera recognized the LEU3 binding site, UAS_L. The results (Fig. 6) demonstrate that the LAC9(LEU3 37-68) chimera did not bind to UAS_L under conditions in which it bound UAS_G. These data add further support to the hypothesis that the C6 zinc finger region is not the sole determinant of sequence-specific DNA binding in the LEU3 or LAC9 protein.

To verify the ability of the LAC9(LEU3 37-68) chimera to bind UAS_G, we examined this chimeric *LAC9* gene for its ability to complement an *S. cerevisiae* strain defective in *gal4* function. When carried on a 2μm vector in which the LAC9(LEU3 37-68) coding region was fused to the strong *ADH1* promoter, the chimeric protein allowed cells to grow slowly on plates containing galactose as the sole carbon source (Fig. 5B). No growth was seen when the chimeric gene, expressed from the LAC9 promoter, was carried on a low-copy-number *CEN* vector (Fig. 5A). These constructs were also introduced into a strain of *S. cerevisiae* defective in *leu3* function in order to verify that the chimeric protein does not bind UAS_L. When carried on a 2μm vector in which the LAC9(LEU3 37-68) coding region was fused to the strong *ADH1* promoter, the chimeric protein did not enable cells to grow on minimal medium lacking leucine (data not shown). These results, in agreement with those obtained from the DNA mobility shift assays, demonstrate that the LAC9 and LEU3 C6 zinc finger regions are functionally related and are not sufficient for determining the DNA-binding specificities of the LAC9 and LEU3 proteins.

Function of amino acids adjacent to the carboxyl side of the zinc finger. To determine whether amino acids adjacent to the carboxyl side of the LAC9 C6 zinc finger are determinants of DNA sequence recognition, we made two chimeric LAC9 proteins. One construct, LAC9(PPR1 62-75), replaced the 14 amino acids on the carboxyl side of the C6 finger region, amino acids 123 to 136, with the corresponding region of PPR1, amino acids 62 to 75 (Fig. 2). The second construct, LAC9(PPR1 34-75), replaced amino acids 95 to 136 of LAC9 with residues 34 to 75 from PPR1. This construct carried both the PPR1 zinc finger and the 14 amino acids on the carboxyl side of the zinc finger. LAC9(PPR1 34-75) bound to UAS_U with 14-fold higher affinity than it bound to UAS_G (Fig. 4), indicating that the chimera, in

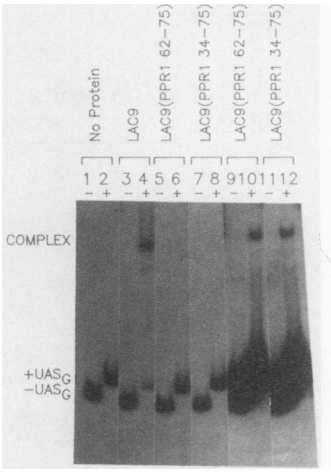

FIG. 7. LAC9(PPR1 62-75) and LAC9(PPR1 34-75) have low affinity for UAS_G. *E. coli* lysates containing 13 ng of wild-type LAC9 peptide (lanes 3 and 4) or 65 ng of chimeric LAC9 peptide LAC9(PPR1 62-75) (lanes 5 and 6) or LAC9(PPR1 34-75) (lanes 7 and 8) were each incubated with ^{32}P-labeled DNA (0.5 ng) carrying UAS_G (+UAS_G, lanes 2, 4, 6, 8, 10, and 12) or with the same DNA fragment lacking only the 17-bp UAS_G (−UAS_G, lanes 1, 3, 5, 7, 9, and 11). Protein-DNA complex formation (indicated by COMPLEX) was analyzed by native polyacrylamide gel electrophoresis and autoradiography (2-h exposure). Lanes 1 and 2 show the mobility of the unbound DNA fragments. Lanes 9 to 12 show a 5-day exposure of lanes 5 to 8.

contrast to LAC9, LAC9(PPR1 34-54), and LAC9(PPR1 34-61), had gained affinity for UAS_U. This gain in affinity is significant because LAC9(PPR1 34-75) had very low affinity for UAS_G (see below). LAC9(PPR1 62-75), carrying only the 14 amino acids adjacent to the zinc finger from PPR1, failed to show any detectable binding to UAS_U (Fig. 4), indicating that the 14-amino-acid region alone was not sufficient to determine the DNA-binding specificity.

To demonstrate that the increased affinity of LAC9(PPR1 34-75) for UAS_U was due to increased specificity of binding instead of increased affinity for any DNA, the affinity of the chimera for UAS_U was measured. Figure 7 shows a DNA mobility shift assay with UAS_G. LAC9(PPR1 34-75) and LAC9(PPR1 62-75) bound weakly to UAS_G (Fig. 7, Table 1); their binding affinity was reduced over 500-fold compared with the wild-type LAC9 peptide. Binding was specific for UAS_G, since no DNA-protein complex was seen when the same DNA fragment lacking the UAS_G was used in the assay (Fig. 7). These results demonstrate that the LAC9(PPR1 34-75) chimera gained affinity and specificity for UAS_U.

To determine whether amino acids adjacent to the carboxyl side of the LEU3 C6 zinc finger also contributed to DNA-binding specificity, two additional chimeras were made. One construct, LAC9(LEU3 69-97), replaced amino acids 123 to 152 of LAC9 with amino acids 69 to 97 from LEU3 (Fig. 2). This replaced the 29 amino acids adjacent to the LAC9 zinc finger with those from LEU3. Second, residues 95 to 152 of LAC9 were replaced with residues 37 to 97 from LEU3 to create LAC9(LEU3 34-97). This chimera carried the LEU3 zinc finger as well as the 29 amino acids on the carboxyl side of the zinc finger. Neither chimeric peptide was detectable in our *E. coli* expression system (Fig. 1, lanes 8 and 9) probably because they were unstable and degraded rapidly.

Function of LAC9 chimeric proteins in vivo. The ability of the chimeric LAC9 proteins to recognize either UAS_G, UAS_U, or UAS_L in vivo was determined by using three different *S. cerevisiae* strains. Each strain carried the *E. coli lacZ* gene, coding for β-galactosidase, fused to one of the three UASs so that the level of β-galactosidase activity was a measure of specific protein binding to the UAS. The affinity of the hybrid proteins for UAS_G was examined in the *gal4*-defective strain YM2631 (Table 1). For these assays the *LAC9*, *PPR1*, and chimeric genes were carried on the multicopy vector YEp352LK and *LEU3* was carried on YEp24. The β-galactosidase activity data are in agreement with the DNA-binding and cell growth data except for the LAC9(LEU3 37-68) chimera, which gave significant β-galactosidase activity, indicating that the LEU3 zinc finger was functioning like the LAC9 zinc finger when the entire protein was used in the assay.

The affinity of the chimeric LAC9 proteins for UAS_U was determined in the *ppr1*-defective strain YM709::PPR104 by using the same plasmids as for the UAS_G assays (Table 1). The β-galactosidase activity data were in agreement with the DNA-binding and cell growth data with one possible exception: the LAC9(PPR1 34-75) chimera gave about threefold (230 versus 73 U) higher β-galactosidase activity than did wild-type PPR1. The reason for this result is not clear, but it could be due to differences in intracellular protein levels or to differences in the activities of one or more domains in the chimera versus the wild-type PPR1 protein.

The affinity of the chimeric LAC9 proteins for UAS_L was determined in the *leu3*-defective strain PFY423 (Table 1). *LEU3* was carried on the multicopy vector YEp24, and *LAC9* and chimeric genes were carried on the single-copy vector YCp50, which gives high levels of LAC9 activity in *S. cerevisiae* (36). In this strain, only the wild-type LEU3 protein showed β-galactosidase activity above the background level, indicating that the chimeric LAC9 proteins did not recognize UAS_L in vivo. These data are in complete agreement with the DNA-binding and cell growth data.

DISCUSSION

The data presented in this report demonstrate that the DNA-binding specificity of PPR1 is determined by a region including amino acids 34 to 75. By inference, the corresponding region of LAC9, amino acids 95 to 136, performs the same function. This conclusion is drawn from experiments with chimeric LAC9 proteins in which the C6 zinc finger, amino acids adjacent to the carboxyl side of the zinc finger, or both were replaced with the corresponding region from PPR1 or LEU3. Another purpose of the replacements was to determine whether the C6 zinc finger or the adjacent region or both made sequence-specifying contacts to DNA. The most significant data were obtained by comparing the DNA-binding specificity of LAC9(PPR1 34-61), containing only the PPR1 zinc finger, with that of LAC9(PPR1 34-75), carrying both the C6 zinc finger and the 14 carboxyl-adjacent amino acids of PPR1. LAC9(PPR1 34-61) retained the binding specificity of LAC9, because it had 20-fold greater affinity for UAS_G than for UAS_U and showed no gain in affinity for UAS_U compared with wild-type LAC9 (Table 1). In contrast, LAC9(PPR1 34-75) had 100-fold lower affinity for UAS_G than did LAC9(PPR1 34-61), but it gained affinity for UAS_U, since it bound UAS_U 14-fold better than it bound UAS_G (Table 1). The β-galactosidase assays (Table 1) shosed the same relative trends, with LAC9(PPR1 34-75) giving much greater enzyme activity than any other protein for the UAS_U assay. We conclude from these data that at

least some amino acids necessary for specific recognition of DNA are located within the 14 amino acids adjacent to the carboxyl side of the C6 zinc finger. On this point our results agree with those of Corton and Johnston (5), who found that the 14 amino acids adjacent to the PPR1 zinc finger were important determinants of DNA-binding specificity by a GAL4(PPR1 25-75) chimeric protein carrying a single amino acid change in the C6 zinc finger region.

We also examined the possibility that C6 zinc fingers perform a similar function(s). This possibility is suggested by the high conservation of amino acids in C6 zinc fingers, including the six cysteines and positively charged amino acids. Chimeric LAC9 peptides carrying the PPR1 or the LEU3 zinc finger had the same binding specificity as LAC9 and bound UAS_G preferentially (Fig. 3 and Table 1). In addition, the same chimeras, expressed as full-length LAC9 proteins, functioned like LAC9 in the cell growth and β-galactosidase assays (Table 1). Data for the LAC9(LEU3 37-68) chimera are particularly significant because the LEU3 C6 zinc finger has three more amino acids, including an extra proline, than the LAC9 zinc finger. These experiments argue strongly for functional similarity of C6 zinc fingers. These data differ from those of Corton and Johnston (5), who did not detect binding of a GAL4(PPR1 34-54) or a GAL4(PPR1 34-61) chimera to UAS_G. We think the difference is partly due to the sensitivity of the DNA mobility shift assays used; our assay was sensitive because of a high concentration of protein due to overproduction of the chimeras in *E. coli*, whereas Corton and Johnston's was less sensitive because chimeric proteins were produced at low levels in yeast cells. Also, our assay was quantitative because we could directly measure the amount of chimera used in the assay.

What function(s) might the C6 zinc finger play? One or more of the highly conserved positively charged residues might contact phosphates in the DNA and thus increase DNA-binding affinity. Also, such a phosphate contact(s) might be necessary for proper alignment of the protein to the DNA and in effect allow base-specific recognition. An example of this possibility has recently been proposed for proteins in which a helix-turn-helix motif is used to contact DNA (28). In addition, one or more amino acids in the zinc finger could make sequence-specific contact with the UAS. Alternatively, the zinc finger might not contact DNA directly and might only serve to maintain the conformation of the DNA-binding domain so that amino acids in the carboxyl-adjacent region contacted the UAS. The fact that the LAC9(PPR1) and LAC9(LEU3) chimeras had less affinity for UAS_G than LAC9 could be accounted for by any one of these explanations. Further replacements of the DNA-binding domain and studies of the domain structure will be necessary to differentiate between these functions.

Interestingly, both LAC9(PPR1 62-75) and LAC9(PPR1 34-75) maintained the ability to specifically recognize UAS_G (as measured by the sensitive in vitro DNA-binding assay [Fig. 7] but not by the less sensitive in vivo β-galactosidase assay [Table 1]), although their affinity was reduced 500-fold compared with wild-type LAC9. One interpretation of these results is that amino acids contributing to DNA sequence recognition are located outside of or may overlap the 14 amino acids adjacent to the carboxyl side of the LAC9 zinc finger. Data supporting this hypothesis were obtained previously by showing that some but not all amino acid changes in a potential α-helix-forming region located 11 to 22 amino acid residues on the carboxyl side of the C6 zinc finger reduced the affinity of LAC9 for UAS_G (35). Similar mutations in GAL4 also reduced DNA-binding affinity (18, 19). The LAC9(PPR1 62-75) and the LAC9(PPR1 34-75) chimeras maintained 9 of 12 amino acids in the LAC9 α-helix region, including the arginine located 13 amino acids (residue 135, Fig. 2) from the zinc finger, which has been shown to be necessary for normal LAC9 DNA-binding activity (35). The reduced affinity of the chimeric proteins for UAS_G may result from changing amino acids in the potential α-helix region that contact DNA. However, our data do not rule out the possibility that reduced affinity is due to indirect effects resulting from conformational change. Our proposed role for the potential α-helix region of LAC9 is analogous to that of Mader et al. (26), who showed that three amino acids important in determining the DNA-binding specificity of the glucocorticoid and estrogen receptors are located in a potential α-helix region on the carboxyl side of the C4 zinc finger. The use of α-helices to recognize DNA sequences has been well documented in bacterial regulatory proteins (28 and references therein).

Unexpectedly, wild-type LAC9 and chimeric peptides bound specifically to both UAS_G and UAS_U, although with different affinities (Table 1). The affinity of wild-type LAC9 for UAS_G was 100 times greater than for UAS_U, while the LAC9(PPR1 34-54) and LAC9(PPR1 34-61) peptides had 20 to 100 times greater affinity for UAS_G than for UAS_U. In contrast, LAC9(PPR1 34-75), containing both the C6 finger and the carboxyl-adjacent 14 amino acids of PPR1, had 14 times greater affinity for UAS_U than for UAS_G. This dual specificity may be due to the presence of the 5′-CGG . . .CCG-3′ dyad in UAS_G (CGG N_{11}CCG) and UAS_U (CGGN_6CCG), where N_n is a stretch of the indicated number of any nucleotides. LAC9 is known to bind UAS_G as a dimer, with each subunit contacting half of the UAS (Y. Halvorsen, K. Nandabalan, and R. Dickson, Biochemistry, in press). One explanation for dual DNA-binding specificity is that the relative orientation of the subunits can adjust to compensate for differences in spacing between the 5′ CGG and the 3′ CCG ends of the UAS. Alternatively, only one subunit in the dimer might interact with half of the UAS, specifically with the three bases at the end of the half-site. In either case, binding affinity would be reduced. Another example of a member of the C6 zinc finger proteins with dual DNA-binding specificity is HAP1 (30), which has approximately equal affinity for its two DNA recognition sites.

Data obtained with the three different assays revealed interesting comparisons (Table 1). For example, in the DNA-binding assay, wild-type LAC9 had 100-fold greater affinity for UAS_G than did LAC9(LEU3 37-68), while in the β-galactosidase assay the two proteins only gave a 9-fold difference. The same trend was seen for the LAC9(PPR1 34-54) and LAC9(PPR1 34-61) chimeras. These effects may simply be due to different intracellular protein levels in the β-galactosidase assay. On the other hand, the differences may be revealing significant biological functions that are not seen in the in vitro DNA-binding assay. For instance, the LAC9(LEU3 37-68), LAC9(PPR1 34-54), and LAC9(PPR1 34-61) proteins might act more like LAC9 in the β-galactosidase assay because of cooperative binding (12) resulting from the use of full-length proteins and four UAS_G in front of *lacZ*; only one UAS_G and less than full-length proteins were used in the DNA-binding assay. Alternatively, the enhanced relative performance of the chimeras might reflect another function of the zinc finger besides DNA-binding, such as transcriptional activation (21) or interaction with the negative regulator GAL80.

Our results suggest that the DNA-binding domain of LAC9 and related proteins having a C6 zinc finger should not be thought of simply as a two-component domain in which the zinc finger performs a general, perhaps structural, function and amino acids adjacent to the carboxyl side of the zinc finger determine DNA-binding specificity. Further experiments are needed to determine whether amino acids in the C6 zinc finger or other regions of the DNA-binding domain directly contact DNA and thus contribute to DNA-binding affinity and specificity.

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