The Transcription Factor LAC9 from Kluyveromyces lactis-like GAL4 from Saccharomyces cerevisiae Forms a Zn(II)$_2$Cys$_6$ Binuclear Cluster*

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The DNA binding domain of the transcription factor LAC9 contains 6 cysteine residues with spacing in the primary peptide sequence identical to that found in the DNA binding domain of the GAL4 transcription factor. In GAL4, the Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$ motif has been shown to form a Zn(II)$_2$Cys$_6$ binuclear cluster (Pan, T. and Coleman, J. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2077–2081), representing a new structure for a Zn(II)-containing transcription factor which differs from the zinc finger motif first described for TFIIIA. LAC9 has been shown to bind two Zn(II) ions (Halvorsen, Y. C., Nandabalan, K., and Dickson, R. D. (1990) J. Biol. Chem. 265, 13283–13289). The similarity of the amino acid sequence and the Cys spacing within the DNA binding domain suggest that LAC9 should also be capable of forming the Zn(II)$_2$Cys$_6$ cluster found in GAL4. A fragment of LAC9 consisting of 144 amino acid residues spanning the DNA binding domain has been prepared with $^{113}$Cd NMR of this fragment (denoted LAC9(85–228*)) has been carried out in an attempt to test the hypothesis that LAC9, like GAL4, forms a binuclear cluster.

The chemical shifts of the two bound $^{113}$Cd(II) ions, 705 and 692 ppm respectively, are consistent with ligation of each $^{113}$Cd(II) ion to 4 sulfur atoms. The best model for such ligation is that of two of the cysteine S$^\text{−}$ form bridges between the two Cd(II) ions. Formation of a Zn(II)-Cd(II) hybrid form of LAC9(85–228*) has also been observed. We conclude that LAC9 contains a Zn(II)$_2$Cys$_6$ binuclear cluster as previously reported for GAL4.

LAC9 is a transcription factor of 865 amino acid residues which is required for the expression of the genes coding for galactose and lactose metabolizing enzymes of Kluyveromyces lactis (1, 2). LAC9 can function in place of the GAL4 transcription factor in Saccharomyces cerevisiae (3, 4), a similar protein required for the transcription of the genes coding for galactose metabolizing enzymes in the latter organism. This result implies that LAC9 can specifically bind to the DNA sequences recognized by GAL4, known as the UAS$_G$ sequences. Comparison of the amino acid sequence of LAC9 with that of GAL4 has revealed extensive homology in three regions (3, 4). One of them, encompassing residues 87–165 of LAC9 (corresponding to residues 5–81 of GAL4), forms the DNA binding domain of LAC9 (4, 5). This DNA binding domain contains 6 cysteine residues whose spacing is highly conserved among 10 other fungal transcription factors (6). In GAL4, this Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$ motif, Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$Cys$_X$, has been shown to bind two Zn(II) or Cd(II) ions. $^{113}$Cd NMR and phase-sensitive $^1$H COSY have established that this motif forms a Zn(II)$_2$Cys$_6$ binuclear cluster with two bridging cysteine ligands (7, 8). Conservation of all 6 cysteine residues suggests that the binuclear cluster may form in all eleven fungal transcription factors.

To test experimentally the hypothesis of binuclear cluster formation in the transcription factors other than GAL4, we carried out $^{113}$Cd NMR on the $^{113}$Cd(II)-derivative of a cloned fragment of LAC9 containing the entire DNA binding domain as well as the dimerization domain (denoted LAC9(85–228*)).

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of LAC9(85–228*)—To obtain large quantities of the LAC9 DNA binding domain, LAC9(85–228*), which contains residues 85 to 228 of the LAC9 protein plus 4 extra amino acids (Pro, Ser, Leu, and Asp) at the carboxyl terminus, was expressed in Escherichia coli and the proteinisolated and purified as described in Halvorsen et al. (5). This fragment of LAC9 includes residues 85–161, determined to be the minimal domain needed for DNA binding activity. For the $^{113}$Cd(II)-exchange experiments, the LAC9(85–228*) was further purified by chromatography on a fast protein liquid chromatography mono S column. The peptide was eluted in 20 mM HEPES, 7.5 mM β-mercaptoethanol, pH 7.5, 25 °C, with a 50–600 mM NaCl gradient.

Formation of the Cd(II) Derivative of LAC9(85–228*)—The purified LAC9(85–228*) contains 1.5–2.0 mol of Zn(II) per protein molecule, confirming the earlier data of Halvorsen et al. (5). Upon addition of a 2-fold molar excess of $^{113}$Cd(II) in the presence of β-mercaptoethanol, followed by incubation for 15 h at room temperature and dialysis against 40 mM Tris, pH 8.0, 250 mM NaCl, a Cd$_4$Zn$_{14}$LAC9(85–228*) hybrid protein was obtained. The $^{113}$Cd-LAC9(85–228*) was formed by addition of $^{113}$Cd(II) to the hybrid protein and subsequent removal of the free Zn(II) by a size-exclusion column. Complete replacement of the native Zn(II) by $^{113}$Cd(II) was obtained under the conditions described for the hybrid employing a longer exchange time, or most effectively by following the first $^{113}$Cd(II) treatment with a second exchange after removal of the Zn(II) initially displaced.

$^{113}$Cd NMR—$^{113}$Cd NMR was performed on a Bruker AM-500 spectrometer (110.9 MHz for $^{113}$Cd) with a 10-mm broad band probe at 25 °C. Samples of 1.9 ml containing ~0.45 mM LAC9(85–228*) protein were kept under nitrogen to prevent oxidation of sulfhydryl groups. The chemical shifts are referred to that of 0.1 M $^{113}$Cd(ClO$_4$)$_2$.

Metal Analysis—Zinc and cadmium analyses were performed by atomic absorption spectroscopy using an Instrumentation Laboratories (Lexington, MA) IL157 spectrometer.

1 Y. C. Halvorsen and R. C. Dickson, unpublished results.
2 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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RESULTS AND DISCUSSION

We have used $^{113}$Cd NMR previously as a probe to determine the number of sulfurs involved in Cd(II) ligation (Refs. 7 and 9, and for a review see Ref. 10). The $^{113}$Cd(II) form of the GAL4 DNA binding domain as well as a cloned construct containing the DNA binding and the dimerization domain of GAL4 (GAL4(149*)) (11) binds a UAS$_G$ DNA sequence as efficiently as the Zn(II) derivative and shows two $^{113}$Cd NMR signals at 707 and 669 ppm (Fig. 1A). These chemical shifts originally suggested that each Cd(II) ion was ligated at 3 or 4 sulfur atoms at each site (7, 11). Extensive two-dimensional NMR studies have now shown that GAL4 actually forms a Cd(II)$_2$Cys$_4$ binuclear cluster in which each Cd(II) is ligated to 4 cysteine sulfurs via a complex in which two of the $\equiv$S ligands bridge between the two Cd(II) ions (8).

LAC9(85–228*) is analogous to the form of GAL4 known as GAL4(149*), a peptide containing both the DNA binding and the dimerization domains. As in the case of GAL4(149*),

![Image of NMR spectra](image)

**FIG. 1.** $^{113}$Cd NMR of Cd$_2$GAL4(149*) (taken from Ref. 11) (A) and Cd$_2$LAC9(85–228*) (B). Relaxation delays were 2.5 s. The number of transients was 12,000 for B. A 50-Hz line broadening was applied for spectral enhancement.

we have found that limited tryptic proteolysis of LAC9(65–228*) results in the formation of a relatively stable smaller fragment (data not shown). This fragment appears to be due to cleavage at Arg$^{144}$ which corresponds to Arg$^{60}$ in the GAL4(149*) sequence (see Ref. 7). The observation of such subdomain cleavage sites are of importance in the attempted cloning of the soluble forms of small subdomains of proteins. For example, in the cloning of the DNA binding domain of GAL4, a 74 residue fragment was insoluble, while the large 149-residue fragment was soluble. On the other hand, GAL4(62*) which corresponds approximately to the natural subdomain produced by tryptic cleavage at Arg$^{63}$ was highly soluble (8, 11).

If LAC9 also forms the Cd(II)$_2$Cys$_4$ binuclear cluster as previously proposed, we should observe two signals at similar chemical shifts, i.e., in the range of 660–720 ppm. The $^{113}$Cd NMR spectrum of Cd$_2$LAC9(85–228*) shows two signals whose chemical shifts, 705 and 692 ppm, are consistent with a structure analogous to the binuclear cluster formed by GAL4 (Fig. 1B). The difference between the upfield signals of GAL4 and LAC9, 669 and 692 ppm, respectively, arise from a chemical shift dependence on the protein conformation. In the case of $^{133}$Cd$_2$-metallothionein where all the $^{133}$Cd(II) ions share one or two Cys ligands, the $^{113}$Cd chemical shift differences...
can be as large as 60 ppm, although each of the $^{113}\text{Cd}$(II) ions is coordinated by 4 cysteines (10). Therefore, almost certainly LAC9 forms a $\text{Cd}$(II)$_2\text{Cys}_8$ binuclear cluster with two bridging cysteine ligands.

GAL4(63) is capable of forming a transiently stable Cd-Zn hybrid as reported previously (7). The hybrid form gave some information on the $\text{Cd}$(II)-Zn(II) exchange properties of GAL4(63), since it showed one site to be more exchange labile than the other (7). The preparation of a rather stable $\text{Cd}_{0.5}\text{Zn}_{0.5}\text{LAC9}(85-228)^*\text{Cys}_8$ derivative provided the opportunity to investigate the hybrid form of LAC9(85-228*). As shown in Fig. 2, this sample actually contains two species, a mixture of the $\text{Cd}_2$- species and the $\text{Cd}_2\text{Zn}_0$- species. At the moment we cannot be certain whether the hybrid form retains the $^{113}\text{Cd}$(II) ion in the site giving the downfield or upfield signal in the $^{113}\text{Cd}_2$- species, although its chemical shift at 687 ppm is suggestive that the $^{113}\text{Cd}$(II) occupies the site giving the more upfield signal. With the discovery of the relatively stable $\text{Zn}_0\text{Cd}_{0.5}$ hybrid of LAC9(85-228*), we found that extensive dialysis of a mixture of $\text{Zn}_{1.5}$ and $^{113}\text{Cd}_0\text{GAL4}(62)^*$ resulted in a similar hybrid (Fig. 2B). As for the LAC9 hybrid, the $^{113}\text{Cd}$ signal of the GAL4 hybrid is shifted upward, suggesting a similar site occupancy in both hybrid proteins. In Cd$_{0.5}$Zn$_{0.5}$-metallothionein, $^{113}\text{Cd}$(II) ions in the mixed metal clusters show chemical shifts that differ from those shown by the homogenous $^{113}\text{Cd}$(II) clusters by up to 8 ppm (summarized in Ref. 10). The ability of LAC9(85-228*) to form a Zn(II)/Cd(II) hybrid whose chemical shifts differ from the Cd$_1$-species further indicates formation of a GAL4-like binuclear cluster in LAC9.

CONCLUSIONS

We have shown previously that the DNA binding domain of GAL4 forms a Zn(II)$_2\text{Cys}_8$ or Cd(II)$_2\text{Cys}_8$ binuclear cluster (7, 8). In addition to GAL4, 10 other fungal transcription factors have been found so far to contain this Cys$_8$ motif within their putative DNA binding domains. LAC9 is now the second member of this family of transcription factors which appears to form a binuclear metal cluster within its DNA binding domain. In the case of GAL4, formation of the metal complex is absolutely essential for specific DNA binding, a structure-function relationship that has recently been shown for LAC9 (5) and which is probably true of the other nine proteins in this group as well. $^{113}\text{Cd}$ NMR of LAC9(85–228*) unambiguously identifies two Cd(II) binding sites with coordination of 4 sulfurs at each site. The chemical shifts of both signals, when compared to GAL4, suggest that two shared cysteine ligands between the two bound Cd(II) ions explains the observed $^{113}\text{Cd}$ chemical shifts in the presence of only six potential -S- ligands.

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