The GAL4 protein belongs to a large class of fungal transcriptional activator proteins encoding within their DNA-binding domains (DBD) six cysteines that coordinate two atoms of zinc (the Zn2Cys6 domain). In an effort to characterize the interactions between the Zn2Cys6 class transcriptional activator proteins and their DNA-binding sites, we have replaced in the full-length GAL4 protein small regions of the Zn2Cys6 domain with the analogous regions of another Zn2Cys6 protein called PPR1, an activator of pyrimidine biosynthetic genes. Alterations between the first and third cysteines abolished binding to GAL4 (upstream activation sequence of GAL (UASG)) or PPR1 (upstream activation sequence of UAS) DNA-binding sites and severely reduced transcriptional activation in yeast. In contrast, alterations between the third and fourth cysteines had only minor effects on binding to UASG but led to substantial decreases in activation in both yeast and a mammalian cell line. In the crystal structure of the GAL4 DBD-UASG complex (Marmorstein, R., Carey, M., Ptashne, M., and Harrison, S. C. (1992) Nature 356, 408–414), this region is facing away from the DNA, making it likely that there exists within the GAL4 DBD an accessible domain important in activation.

The GAL4 protein is an 881-amino acid transcriptional activator of the GAL and MEL1 genes in Saccharomyces cerevisiae. The mechanism by which GAL4 activates transcription is conserved because GAL4 also activates transcription in plant, Drosophila, and mammalian cells if GAL4 DNA-binding sites (UASG) are present in the promoter of appropriate reporter genes (reviewed in Ref. 1). GAL4 is a member of a large family of fungal transcriptional activators that contain within their DNA-binding domains a conserved cysteine-rich region encoding six cysteine residues that coordinate two atoms of zinc (the Zn2Cys6 domain). The DNA recognition sequences to which these activators bind have been identified and all have in common a CGG or related triplet in each of the two symmetrically opposed half-sites (2–4). The sequences between the triplets as well as the spacing of the triplet sequence are highly variable, indicating that these variables may in large part determine specific recognition. Based on the extent of homology between the Zn2Cys6 activators, it was hypothesized that the Cys-rich region performs a function common to all members of this class of proteins and the region immediately adjacent, which encodes divergent sequences, determines DNA binding specificity (5). We previously demonstrated that this model is essentially correct (6). All but one (Lys23) of the 28 amino acids in the Zn2Cys6 region of GAL4 can be replaced by the analogous sequences of PPR1, an activator of pyrimidine biosynthetic genes (7), without changing DNA binding specificity. In contrast, replacing the 14 amino acids immediately adjacent to the Zn2Cys6 region led to substantial decreases in activation in both yeast and a mammalian cell line. Within the crystal structure of the GAL4 DBD-UASG complex, this region is accessible to the environment and thus may make a contact crucial for activation.

**EXPERIMENTAL PROCEDURES**

**Construction of GAL4 DNA-binding Mutants**—Production of single-stranded DNA, site-directed mutagenesis of full-length GAL4, cloning into the multicopy plasmid YEps51, and sequencing were performed as detailed earlier (6). GAP71 and GAP72 were constructed from GAP1 and GAP2 using oligonucleotide 4PPR1-11, GAP100 and GAP110 were made from GAL4 and GAL4 Glu23, respectively, using oligonucleotide 4PPR1-13. GAL4 Glu23 was made using oligonucleotide 4PPR1-12. The GAL4, GAP100, GAP110, GAP71, and GAP72 amino acid sequences 1–10 were replaced with the analogous sequences from PPR1 by replacing the wild-type BamHI-SphI fragment with the BamHI-SphI fragment from GAP5N/pUC119 to give the corresponding N derivative. The GAP3N mutant was made from GAP3 (6) using the oligonucleotide 4PPR1-8. Plasmids used for transfection into the COS-1 cells were made by amplifying the DNA-binding domains with oligonucleotides PAL-30 and PAL-32, cutting with BamHI and XhoI, and cloning into the unique BglII and XhoI sites of the plasmid pSGVP (13). All constructions were confirmed by sequencing. The sequence of all oligonucleotides used in this study are available upon request.

**Synthesis of Proteins**—Fragments of DNA encoding amino acids 1–147 of the DNA-binding domains of GAL4 or GAPs were amplified by the polymerase chain reaction using Tag DNA polymerase and the GAL4 or GAP genes on the plasmid YEps51 as starting templates for...
polymerase chain reaction. The DNAs were amplified in the presence of oligonucleotide PAL-4, which defines the 3' end, and oligonucleotides that incorporate the T3 RNA polymerase recognition site into the 5' end (PAL-1 for GAL4 N-terminal amino acids or PAL-2 for hybrids containing PPR1 amino acids at the N terminus). The PPR1 coding region amino acids 25–171 was amplified with PAL-2 and PAL-6. Transcription reactions were performed using T3 RNA polymerase, and translation reactions were performed in rabbit reticulocyte lysates according to the supplier's instructions (Promega). The same RNA was used to program synthesis in two parallel reactions, one containing [35S]methionine (Amersham Pharmacia Biotech) and the other containing cold methionine. The [35S]labeled proteins were analyzed on denaturing SDS 15% polyacrylamide gels (19:1, acrylamide:bis-acrylamide). Prestained low molecular weight protein standards were from Stratagene.

**Electrophoretic Mobility Shift Assays—Assays of protein-DNA interactions** were performed as described earlier (6). All experiments were repeated at least twice with similar results.

**Yeast Culturing and o-Galactosidase Assays—**Yeast culture conditions and o-galactosidase assays were performed as described (14). Protein concentrations of the yeast extracts were determined using the BCA protein assay reagent (Pierce) and bovine serum albumin to generate the standard curve. All assays were performed with at least three independent transformants with standard deviations less than 20%.

For illustrative purposes, the activities of the GAL4-PPR1 hybrids are presented as percentages of the activity of wild-type GAL4.

**Determination of Protein Levels in Yeast—**All plasmids were tested for level of production of GAP proteins in the B80-1 strain (a GAL4 GAL80-100 ura3-52 leu2-3 112 MEL1) by assaying growth on galactose (6).

**Culturing and Transfection of COS-1 Cells and CAT Assay—**Cells were transfected using the calcium-phosphate precipitation method, extracts were prepared, and CAT activity was measured using standard methods (15).

**RESULTS**

To gain insight into the sequences important in determining DNA binding specificity as well as structure of the Zn2Cys6 domain, mutants of full-length GAL4 were made by replacing different regions of the GAL4 Zn2Cys6 domain and N-terminal flanking region with the analogous sequences from PPR1. The sequence changes in the Zn2Cys6 region of GAL4 were grouped into two subregions (Fig. 1). Subregion A is highly conserved in the Zn2Cys6 class of activator proteins (Fig. 1A, bottom). There are three amino acid differences between GAL4 and PPR1 within this region. Lys17 and Lys18 in GAL4 (10) or Lys40 and Lys41 at the equivalent positions in PPR1 (11) make specific contacts with the CGG motif within the UASG or UASu, respectively. Subregion B is less well conserved and includes three amino acid differences clustered between the third and fourth cysteines.

Replacement of the two subregions had pronounced but contrasting effects on transcriptional activation in yeast and DNA binding *in vitro*. Replacing the three amino acids in subregion A of GAL4 with those of PPR1 produced the GAP100 protein, which had severely decreased ability to activate MEL1 (Fig. 1A). Using *in vitro* transcribed and translated proteins in electrophoretic mobility shift experiments, binding of GAP100 to UASG was not detected (Fig. 1B). In our DNA binding experiments with GAL4 and mutants that exhibited UASG binding, two DNA-protein complexes were observed: a major complex (bottom arrowhead) and a slower-migrating, more labile complex that was not consistently observed (top arrowhead). Both complexes contained GAL4 DBD, as shown by supershift experiments using anti-GAL4 DBD antibodies (data not shown). Compared with earlier studies of interactions between GAL4 and UASg (12), the lower complex is most likely a dimer of the GAL4 DBD bound to 1 UASg. The upper complex may then be a GAL4 DBD dimer complexed to an additional protein. In addition a minor faster migrating GAL4-UASg complex may represent a degradation product of GAL4 DBD. Replacement of subregion A and an additional amino acid in subregion B (Gln23) to give GAP110 abolished the ability to activate MEL1.
the structures of subregion A of GAL4 and PPR1 are not interchangeable.

In contrast to the subregion A changes, replacement of the three amino acids in subregion B (GAP71) or subregion B plus four adjacent C-terminal amino acids (GAP72) had less drastic or no effects on UASG binding, respectively (Fig. 1B). Despite significant binding to UASG, GAP71 and GAP72 were severely compromised in their ability to activate MEL1. The defects in activation by the GAL4-PPR1 hybrids were not unique to the MEL1 gene, which is under control of one UASG, but were also observed when a reporter gene under control of two UASG's from the GAL1-GAL10 promoter region was assayed (data not shown). Large differences in the ability of the different GAL4-PPR1 hybrids to bind UASG could not be attributed to differences in protein expression because expression of all of the hybrids examined in this study varied by no more than 4-fold (Fig. 1C). Differences in in vivo expression and stability of the hybrid proteins were tested in a GAL80\(^5\) mutant strain that cannot grow on galactose because of constitutive repressive effects of a mutant of GAL80. Titration of the GAL80\(^5\) protein by either wild-type GAL4 or any of the GAL4-PPR1 hybrid proteins allowed equal growth on galactose, indicating that expression of the proteins in vivo was indistinguishable. The empty vector or the vector expressing PPR1, which does not bind GAL80, did not allow growth (data not shown). Taken together, these results show that alterations within subregion A drastically affect DNA binding, whereas alterations within subregion B preferentially affect activation.

The region N-terminal to the Zn\(_2\)Cys\(_6\) region is highly variable in both length and sequence between the Zn\(_2\)Cys\(_6\) family members. Only the amino acids immediately adjacent to the Zn\(_2\)Cys\(_6\) region have been conserved to an appreciable extent (Fig. 1A, bottom). Very little is known about the role of this region in supporting the structure of the Zn\(_2\)Cys\(_6\) domain or in DNA binding. Replacing the GAL4 N-terminal sequences with those from PPR1 had different effects on DNA binding and transcriptional activation depending on which GAL4 subregions contained PPR1 sequences. Replacement of the GAL4 N-terminal region with the analogous sequences from PPR1 in wild-type GAL4 (GAL4N) had no detectable effect on DNA binding, as shown earlier (6), but did have a small negative effect on activation of MEL1 (Fig. 1A). Replacing the N-terminal sequences of GAP100 with that of PPR1 to give GAP100N did not appreciably change the ability of the protein to activate or bind DNA. In contrast, addition of the PPR1 N-terminal sequence enabled the hybrids containing one (GAP110N) or three (GAP71N and GAP72N) PPR1 amino acids in subregion B to activate MEL1 better (2.7–51-fold). The ability of the hybrids containing PPR1 N-terminal sequences to bind DNA was unaffected. Minor differences in binding could be explained by differences in the amount of expressed protein. These data indicate that PPR1 amino acids in the N-terminal region were able to enhance the activation activities of hybrids containing PPR1 amino acids in subregion B but not subregion A. Despite this enhancement, all of the hybrid proteins containing changes in subregion B were moderately to severely defective in transcription activation.

To determine whether mutations in subregion B also affect transcriptional activation in a mammalian context, the DBDs from the GAL4-PPR1 hybrids (Fig. 2A) were fused to the VP16 activation domain (13). The expression plasmids were cotransfected into a green monkey kidney cell line, COS-1, with a plasmid encoding the reporter gene CAT under control of one UASG (Fig. 2A). Compared with GAL4-VP16, the GAL4N-VP16 exhibited a minor decrease in ability to activate the reporter gene (Fig. 2B). In contrast, the GAP72-, GAP72N-, GAP71-, and GAP71N-VP16 proteins activated the CAT gene to only a fraction of that activated by GAL4-VP16. Notably, the pattern of activation in COS-1 cells paralleled the activation in yeast, i.e. GAP71 activated less than GAP72 and both mutants were partially corrected by the PPR1 N-terminal sequences. Thus, the structural defects in subregion B that affect activation in yeast also have parallel effects on activation in a mammalian cell line.

**DISCUSSION**

In an effort to define the amino acids responsible for conferring DNA binding specificity and protein stability on Zn\(_2\)Cys\(_6\) class proteins, we have identified a short sequence within the Zn\(_2\)Cys\(_6\) domain of GAL4 that, when altered, results in a preferential decrease of transcriptional activation in yeast and a mammalian cell line but only minor effects on DNA binding. All of the proteins that exhibited this phenotype possessed at least four PPR1 amino acids replacing the analogous GAL4 amino acids between the third and fourth cysteines. In addition, the N-terminal region containing PPR1 amino acids was shown to be important in restoring the ability of hybrids containing PPR1 amino acids in subregion B to activate transcription. This could be only partly attributed to increased UASG binding. The close proximity of the N terminus and subregion B in the GAL4-UASG co-structural model supports the idea that the compensatory effects of the N-terminal sequences are through direct interaction with subregion B. Although the structure of the Zn\(_2\)Cys\(_6\) modules from GAL4, PPR1, and PUT3 are similar (10–12) and can be exchanged between the proteins without altering DNA binding specificity (6, 8, 9), our work indicates that replacing amino acids within the Zn\(_2\)Cys\(_6\) module of GAL4

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**Fig. 2. Mutations in the DNA-binding domain of GAL4 affect transcriptional activation in a mammalian cell line.** A, structure of plasmids used in COS-1 cell transfections. The transcriptional activators consisted of the GAL4 or GAP DNA-binding domains (amino acids 1–147) fused to the transcriptional activation domain of the herpes simplex virus protein, VP16. Constitutive expression was under control of the SV40 promoter. The reporter gene CAT was under control of one UASG, B, activity of GAP-VP16 mutants in COS-1 cells. The plasmids encoding GAL4-VP16 or GAP-VP16 (1 μg) and the UASG-CAT gene (1 μg) were cotransfected with 3 μg of a plasmid expressing secreted alkaline phosphatase. CAT activity was normalized either to secreted alkaline phosphatase or to protein concentration with similar results. Results shown represent the means ± the S.D. calculated from at least three independent experiments.
Transcription Activation Mutants in GAL4 DNA-binding Domain

FIG. 3. Subregion B of GAL4 is directed away from the UASG.
The structure of the DNA-binding domain of GAL4 (residues 1–65) bound to DNA looking down the DNA helix axis. The DNA is shown in gray in stick representation, and the protein is shown as a ribbon diagram with one monomer depicted in blue and the other in purple. The zinc atoms are shown as yellow spheres, and the side chains of residues 22–25 belonging to subregion B of each monomer are shown in ball and stick form. The figure was drawn with the program MOLSCRIPT (32) and rendered with RASTER3D (33, 34).

with analogous amino acids from PPR1 can lead to two types of defects. Alterations of amino acids in proximity (e.g., GAP100) to Lys17 and Lys18 that make direct contact with the CCG triplets within the UASG can disrupt DNA binding, possibly by disrupting the intramolecular interactions within the module. Alterations within the amino acids between the third and fourth cysteines can preferentially disrupt transcriptional activation without adversely affecting DNA binding.

The subregion B mutants characterized in this study are similar to the positive control (pc) mutants first isolated in a repressor (17), which bind DNA with high affinity but are defective in transcriptional activation. pc-type mutants have been identified in other transcriptional activators that coordinate zinc in their DBDs including the yeast ADR1 protein (18, 19) and the human glucocorticoid receptor (20). The mutational changes in ADR1 and human glucocorticoid receptor map to the amino acids in the C-terminal half of the first or second zinc fingers, respectively, in regions analogous to subregion B in GAL4. Although these regions do not share any significant sequence or structural homologies with GAL4, a common pc-like mutation occurs in which Lys23 in GAL4, Arg115 in ADR1, or Arg488 in glucocorticoid receptor was changed to Gln. Examination of the environment of this region in crystal structures of Zf268 (21) structurally similar to ADR1, (22) and glucocorticoid receptor (23) reveals that at least some of the amino acids that were altered in the pc-like mutants are not making contact with the DNA and are accessible to the surrounding environment. In the GAL4-UASG co-crystal structure (10) subregion B is also exposed to the environment (Fig. 3). Three of the four amino acids within this region (Ser22, Glu24, and Lys25) are directed away from the DNA. Lys25 forms a hydrogen bond with a phosphate in the UASG (10). The amino acids between the third and fourth cysteines of PPR1 (11) and PUT3 (12) also do not make contact with the DNA and are directed out toward the surrounding environment based on the protein-UAS crystal structures. Taken together, these data indicate that a number of DNA-binding transcriptional activator proteins that coordinate zinc possess a region within the DNA-binding domains important in determining optimal gene activation.

How do mutations in GAL4 subregion B lead to defects in transcriptional activation? One possibility is that GAL4 pc mutants simply have an altered conformation that inhibits the activation domain. Examples of this mechanism comes from work on pc mutants of the glucocorticoid receptor (24) and another Zn2Cys6 protein, HAP1 (25), which indicated that appropriate transcription factor-UAS interactions are necessary for optimal transcriptional activation.

Another possibility is that subregion B or a region affected by changes in subregion B defines an interactive site with another protein important in transcriptional specificity. Physical interactions have been characterized between coactivators EIA (26) or SWI3 (27) and the DBDs of transcriptional activators. A number of proteins exhibit genetic or biochemical properties that make them candidates for interacting with the GAL4 DBD. A mutant of one protein GAL11, called GAL11P, potentiates transcriptional activation by proteins containing the GAL4 DNA-binding domain and weak transcriptional activation domains from other transcription factors (28). The positive effect of GAL11P is reversed by a GAL4 mutation (Lys20 to Glu) in close proximity to the subregion B amino acids. However, there is no evidence for a direct interaction between wild-type GAL11 and the GAL4 DBD. Secondly, subregion B may be interacting with components of the nucleosome. Mutations in histones H3 (29) and H4 (30) allow hyperactivation or reduced activation, respectively, of a number of GAL4-regulated genes. In this scenario, the GAL4 subregion B might operate like a histone plow, as proposed earlier (5). Lastly, other regions of GAL4 itself could be interacting with subregion B. The conserved region between the DBD and activation domains in Zn2Cys6 class protein (or middle homology region) has been proposed to bridge interactions between the Zn2Cys6 module and the activation domain and to enhance DNA binding specificity (2). In a recent study, however, DNA binding and transcriptional activities of GAL4 mutants that lack the middle homology region were not adversely affected (31).

Intensive investigation is presently focused on identifying the protein target(s) of the activation domains from different types of transcriptional activators. Our work demonstrates that the GAL4 DBD possesses a region that determines optimal transcriptional activation. Thus, to completely understand the mechanism of transcriptional activation by intact GAL4 in yeast or by GAL4 activation domain hybrids in mammalian cells, it will be necessary to determine the interactions made between the DBD and other proteins. Selection of suppressors of GAP71 or GAP71N activation defects in yeast should identify the protein(s) important in determining transcriptional specificity of GAL4 and allow for determination of conservation in higher eukaryotes.

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