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Mutational Analysis of the *GAL4*-Encoded Transcriptional Activator Protein of *Saccharomyces cerevisiae*

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ABSTRACT

The GAL4 protein of *Saccharomyces cerevisiae* binds to DNA upstream of each of six genes and stimulates their transcription. To locate regions of the protein responsible for these processes, we identified and characterized 88 *gal4* mutations selected *in vivo* to reduce the ability to GAL4 protein to activate transcription. These mutations alter two regions of GAL4 protein: the DNA binding domain, and the transcription activation domain. Some mutations in the DNA binding domain that abolish the ability of GAL4 protein to bind to DNA *in vitro* change amino acid residues proposed to form a zinc finger, confirming that this structure is indeed involved in DNA binding. Four different amino acid changes in the zinc finger appear to reduce (but not abolish) the affinity of GAL4 protein for zinc ions, thereby identifying some of the amino acids involved in forming the zinc-binding structure. Several other mutations that abolish the DNA binding activity of the protein alter the 20 amino acids adjacent to the zinc finger, suggesting that these residues are part of the DNA binding domain. Two amino acid changes in the region adjacent to the zinc finger also appear to affect the ability of GAL4 protein to bind zinc ions, suggesting that this region of the protein can influence the structure of the zinc binding domain. The transcription activation domain of GAL4 protein is remarkably resistant to single amino acid changes: only 4 of the 42 mutations that alter this region of the protein are of the missense type. This observation is consistent with other lines of evidence that GAL4 protein possesses multiple transcription activation domains with unusual sequence flexibility.

THE genes responsible for galactose utilization in the yeast *Saccharomyces cerevisiae* provide a model system for studying the regulation of gene expression in this simple eukaryotic organism (reviewed in JOHNSTON 1987b). The three genes that encode the enzymes of galactose utilization, *GAL1*, GAL7 and *GAL10*, as well as *GAL2*, which encodes the galactose permease, are stringently co-regulated: their expression is induced at least 1000-fold during growth on galactose and repressed during growth on glucose (ST. JOHN and DAVIS 1981; TSCHOPP *et al.* 1986). Two genes are responsible for this stringent regulation: *GAL4*, which encodes a protein that activates transcription of the *GAL* genes (HOPPER, BROACH and ROWE 1978), and *GAL80*, which encodes a protein that antagonizes GAL4 protein function (DOUGLAS and PELROY 1963; JOHNSTON, SALMERON and DINCHER 1987; MA and PTASHNE 1987b).

Knowledge of the nature of the GAL4 protein and the way in which it activates transcription is of central importance to understanding the mechanism of *GAL* gene regulation. The GAL4 protein binds to multiple sites in the DNA located 100–400 bp upstream of the transcription initiation sites in each of six genes (BRAM and KORNBERG 1985; GINIGER, VARNUM and PTASHNE 1985; KEEGAN, GILL and PTASHNE 1986). Binding of GAL4 protein to at least one of these sites is essential for any expression of *GAL1, GAL2, GAL7, GAL10* and *MEL1* (WEST, YOCUM and PTASHNE 1984; BRAM, LUE and KORNBERG 1986; TAJIMA, NOGI and Fukasawa 1986; POST-BEITTENMILLER, HAMILTON and HOPPER 1984), and increases the basal level of expression of *GAL80* (SHIMADA and FUKASAWA 1985). The events that occur subsequent to DNA binding that lead to transcription activation are unknown.

The regions of the GAL4 protein responsible for DNA binding and transcription activation are functionally separable: the first 74 amino acids are sufficient for DNA binding activity (KEEGAN, GILL and PTASHNE 1986); the carboxyl-terminal 95% of the protein provides the transcription activation function (BRENT and PTASHNE 1985). There appear to be two transcription activation domains within this carboxyl-terminal region of the protein, one near the DNA binding domain, the other at the extreme carboxyl terminus of the protein (MA and PTASHNE 1987a). To locate and characterize more precisely functional domains of the GAL4 protein, we identified a large number of *gal4* mutations. We have previously described the effects many of these mutations have on the DNA binding activity of GAL4 protein (JOHNSTON and DOVER 1987). We describe here the isolation, characterization and DNA sequence changes of these mutations.

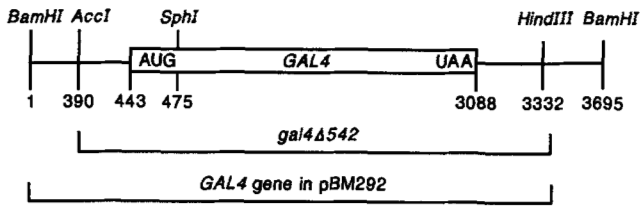

FIGURE 1.—Partial restriction map of the *Bam*HI fragment containing *GAL4* originally isolated by LAUGHON and GESTELAND (1984a, b). Only restriction sites relevant to this work are shown. The box denotes *GAL4* coding sequence. Indicated below the map of *GAL4* is the extent of the *GAL4* DNA fragment carried by the plasmid (pBM292) present in all of our mutants, and the extent of the chromosomal *gal4* deletion present in all our strains. The numbers below the map are numbers of nucleotides from the leftmost *Bam*HI site.

MATERIALS AND METHODS

Plasmids: Unless otherwise noted, all strains harbor plasmid pBM292, which carries the *Bam*HI-*Hin*dIII *GAL4* DNA fragment shown in Figure 1 inserted between the *Hin*dIII and *Bam*HI sites of pTC3 (both sites in the pBR322 portion of this plasmid). Plasmid pTC3, which carries *TRP1*, *ARS1*, and *CEN3*, was obtained from KIM NASMYTH via MAYNARD OLSON, and is described by SHAW and OLSON (1984). Some strains also carry pBM252, which contains the *HIS3* gene fused to the *GAL10* promoter in YCp50, a *URA3*, *ARS1*, CEN4 plasmid (JOHNSTON and DAVIS 1984).

The Bal31-generated deletions of *GAL4* used for genetic mapping were made in plasmid pBM452, which consists of the *Eco*RI-*Bam*HI fragment from pBM292 that includes *TRP1*, *ARS1*, and the *GAL4* fragment shown in Figure 1 inserted between the *Eco*RI and *Bam*HI sites of pBR322. The *Eco*RI site to the left of *TRP1* (*i.e.*, distal to *GAL4*), and the *Sal*I and *Sph*I sites in pBR322 sequences in this plasmid were then abolished, yielding pBM452. The Bal31-generated *gal4* deletions were subcloned (as *Bam*HI-*Eco*RI fragments) into pBM453, which is pTC3 lacking the EcoRI site to the left of *TRP1*. Plasmids were introduced into yeast cells as described by ITO *et al.* (1983).

Strains: The parental strain for the *gal4* mutants is YM725 = *ura3*-52 *his3*-200 *ade2*-101 (ochre) *lys2*-801 (amber) *trp1*-901 *tyr1*-501 *met*$^-$ *can1 gal4*-542 *gal80*-538 carrying plasmids pBM292 (*TRP1, ARS1, CEN3, GAL4*) and pBM252 (*URA3, ARS1, CEN4, GAL10-HIS3*). The strain carrying the deletions for mapping *gal4* genetically (on *TRP1-CEN3* plasmids) is YM850 = *a ura3*-52 *his3*-200 *ade2*-101 (ochre) *lys2*-801 (amber) *trp1*-901 *met*$^-$ *gal4*-542 *gal80*-538. The strain used to assay *GAL* gene expression (YM2271) contains the *GAL1-lacZ* fusion in pRY181 (YOCUM *et al.* 1984) integrated at the *LEU2* locus, and has the genotype *ura3*-52 *his3*-200 *ade2*-101 (ochre) *lys2*-801 (amber) *trp1*-901 *met*$^-$ *gal4*-542 *gal80*-538. All strains are congenic to strain S288C. The *his3*-200 mutation (constructed by M. FASULLO) deletes all *HIS3* coding sequences (STRUHL 1985); *trp1*-901 is a deletion of the 1.4-kb *Eco*RI fragment encompassing *TRP1* constructed by P. HIETER; *gal80*-538 deletes all *GAL80* coding sequences (YOCUM and JOHNSTON 1984); *gal4*-542 deletes all *GAL4* coding sequences, as shown in Figure 1 (its construction is described below).

Deletion of chromosomal GAL4 sequences: To map *gal4* mutations genetically by recombination between plasmid-borne *GAL4* genes, it was necessary to prevent recombination with the chromosomal *GAL4* gene. This was accomplished by deleting chromosomal *GAL4* sequences homologous to the *GAL4*-containing plasmid. We deleted *in vitro* *GAL4* sequences between the *Acc*I site (located just upstream of the transcription initiation site) and the *Hin*dIII site (located just downstream of the *GAL4* coding region) (see Figure 1) on a plasmid (YRp14). Since this *gal4* deletion spans the entire plasmid-borne *GAL4* gene (the *Bam*HI-*Hin*dIII fragment shown in Figure 1), there should be no recombination between *GAL4* on the plasmid and its chromosomal locus. The deletion was constructed by subcloning the *Hin*dIII-*Bam*HI fragment that lies downstream of *GAL4* (Figure 1) next to the *Bam*HI-*Acc*I fragment lying upstream of *GAL4* (with *Hin*dIII linkers on the *Acc*I site) in the proper orientation in the *Bam*HI site of YRp14. This deletion was introduced into the chromosome by transplacement (SCHERER and DAVIS 1979), as previously described (JOHNSTON and DAVIS 1984). The structure of the deleted *GAL4* region in the resultant strain was confirmed by Southern blot analysis.

Construction of gal4 mapping deletions: Two sets of *gal4* deletions were constructed using Bal31 nuclease for the purpose of mapping *gal4* point mutations, as previously described (JOHNSTON and DAVIS 1984). One set, which removes sequences from the 5′ end of *GAL4*, was constructed by (1) cleaving a *GAL4*-containing plasmid (pBM452) at the unique BamHI site upstream of the gene (Figure 1), (2) digesting with Bal31 nuclease for varying amounts of time, (3) filling in any "ragged" ends with the large (Klenow) fragment of *Escherichia coli* DNA polymerase I, (4) adding *Bam*HI linkers, (5) cleaving with *Bam*HI and *Eco*RI, (6) purifying the shortened *GAL4* DNA fragments from agarose gels, and (7) cloning these fragments between the *Eco*RI and *Bam*HI sites of pBM453. The other set of deletions, which removes sequences from the 3′ end of *GAL4*, were made in a similar way except that the initial cleavage was at the unique *Eco*RI site at the 3′ end of the gene (in vector sequences, 29 bp downstream of the *Hin*dIII site shown in Figure 1), and *Eco*RI linkers were added after Bal31 digestion. The extent of each deletion was estimated from the size of the resultant *GAL4 Eco*RI-*Bam*HI fragment, and the precise end points of selected deletions were determined by sequencing the appropriate end of *GAL4* using the universal primer (YANISCH-PERRON, VIEIRA and MESSING 1985), after subcloning the fragment in M13mp18. The extents of these deletions are diagrammed in Figure 2.

Selection of gal4 mutants and revertants: Mutants of *gal4* were selected as colonies resistant to 0.1% 2-deoxygalactose on minimal plates containing 5% glycerol as carbon source, essentially as previously described (PLATT 1984). 0.2 ml of a saturated culture (about 2×10^7 cells) was plated and incubated at 30° until visible colonies appeared (about 1 week). Some plates were mutagenized with UV light by inverting them on a UV transilluminator (302 nm) for 20 sec, resulting in about 30% killing of the cells. A few mutants were induced near a drop of ethyl methanesulfonate (EMS) placed on the plate. Mutant colonies were picked to a master plate and scored for their His phenotype (the parent strain is *his3*$^-$ and carries a *GAL10-HIS3* fusion) by replica plating to minimal plates lacking histidine with raffinose or glycerol as carbon source. Presumptive *gal4* mutants (*i.e.*, His$^-$ mutants) were confirmed by a complementation test with a known *gal4* mutant. A total of 1179 mutants resistant to 2-deoxygalactose were isolated (in 214 independent groups); 380 of these (32%) were His$^-$.

Mutants selected specifically for their zinc-remedial Gal$^-$ phenotype were isolated in a similar manner and printed to minimal plates containing 2% galactose, with or without 10 mM $ZnCl_2$ (the optimal concentration for correcting the Gal$^-$ phenotype of the zinc-remedial Pro26>Leu mutation). We found 294 putative *gal4* mutants among 1615 2-deox-

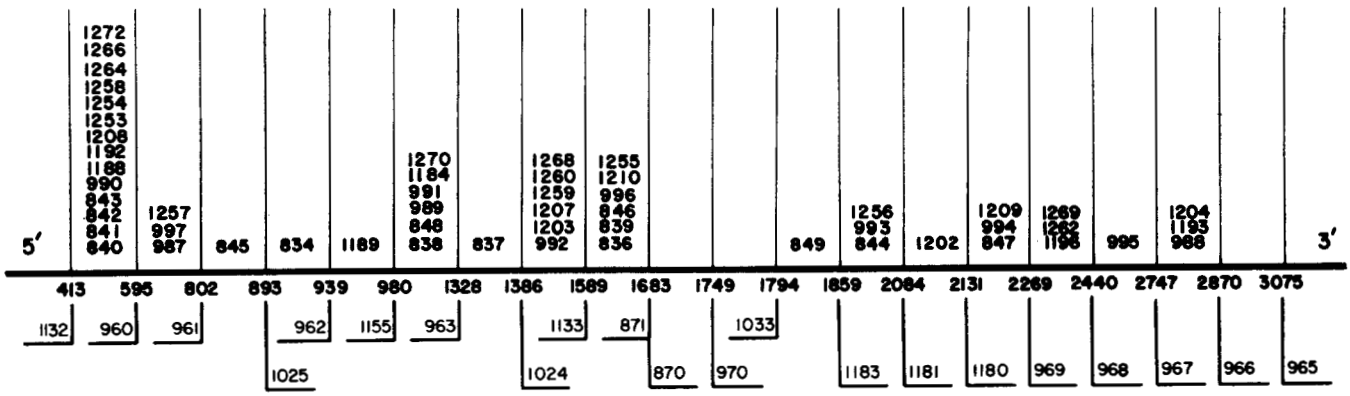

FIGURE 2.—Genetic map of *GAL4*. The horizontal line represents *GAL4* sequences in the 5′ to 3′ direction left to right. Below the line is shown the extent of each mapping deletion, the number of the yeast strain carrying each deletion, and, just below the line, the last nucleotide removed by the deletion (nucleotide numbering according to LAUGHON and GESTELAND 1984b). The allele numbers of *gal4* mutations are shown above the line in the deletion interval to which they map. Note that the map is *not* drawn to scale. The first codon of *GAL4* begins at nucleotide 443; the last codon begins with nucleotide 3085. Deletion 965 removes the last 4 amino acids of *GAL4* and does not cause a Gal$^-$ phenotype.

ygalactose-resistant mutants; the growth of 11 of these on galactose is corrected by $ZnCl_2$ (see below).

Revertants of *gal4* mutants were selected on minimal plates containing 2% galactose. All mutants are of independent origin. The *gal4*-containing plasmids were recovered from crude yeast lysates and amplified in *E. coli* as previously described (DAVIS *et al.* 1980). Yeast media are described by SHERMAN, FINK and LAWRENCE (1974).

Genetic mapping of gal4 mutations: The *gal4* mutations were mapped by scoring Gal$^+$ recombinants in strains containing the *gal4* mutations on a *TRP1-ARS1-CEN3* plasmid (pBM292), and the series of *gal4* deletions diagrammed in Figure 2 on a nearly identical plasmid (pBM453). Only those strains carrying *gal4* mutations that lie outside the deletion yield Gal$^+$ recombinants above background levels. The background number of Gal$^+$ colonies (due to reversion) was determined for each *gal4* mutant in a strain whose *gal4* mapping deletion removes all *GAL4* sequences. Reproducible detection of Gal$^+$ colonies above background levels was taken as evidence that the mutation lies outside the deletion. We estimate that we can detect a recombination frequency that is reduced approximately 10^3-fold below that of our least restricted cross (between mutations and deletions that are furthest apart).

The mapping was done as follows. Strains carrying the deletion plasmids were mated to strains carrying the *gal4* mutations on YPD plates. After overnight incubation at 30°, diploids were selected *en masse* by prototrophic selection. Immediately after patching the cells on petri plates to select diploids, mitotic recombination was stimulated by inverting the plates over a UV transilluminator (302 nm) for 20 sec (approximately 30% killing). The diploids that grew were then scored for Gal$^+$ recombinants by printing these plates to minimal medium containing 2% galactose.

DNA sequencing: The DNA sequence change caused by each mutation was determined, after subcloning each altered gene between the *Eco*RI and *Bam*HI sites of M13mp18 (YANISCH-PERRON, VIEIRA and MESSING 1985), by the dideoxynucleotide chain termination method (SANGER, NICKLEN and COULSON 1977), using 8 oligonucleotide primers (16–21 nucleotides long) spaced uniformly throughout the gene. The sequence of the entire interval to which the mutation maps was determined for nearly all mutations.

DNA binding assays: Altered GAL4 proteins were synthesized in *E. coli* from the *tac* promoter, as previously described (JOHNSTON and DOVER 1987). The *gal4* mutations were inserted into the *E. coli* expression vector (pBM1123) by replacing the wild type *Sph*I-*Hin*dIII fragment (Figure 1) in this plasmid with the same fragment from each mutant. Preparation of crude *E. coli* extracts, and the assay of GAL4 protein DNA binding activity by a nitrocellulose filter binding assay has also been described (JOHNSTON 1987a).

RESULTS

Selection of *gal4* mutants: The selection method we used to isolate *gal4* mutants is based on the toxicity of 2-deoxygalactose to cells that express the *GAL1*-encoded protein, galactokinase (PLATT 1984). This enzyme catalyzes the production of 2-deoxygalactose-1-phosphate, which is not metabolized further. The resultant accumulation of this metabolite is toxic to yeast cells; mutants unable to express *GAL1* survive in the presence of 2-deoxygalactose. The majority of 2-deoxygalactose-resistant mutants carry mutations in *GAL1* or *GAL4* (PLATT 1984). To identify the *gal4* mutants we used a yeast strain, deleted for chromosomal *HIS3* sequences, that carries the *HIS3* gene under control of the *GAL10* promoter on a plasmid (pBM252, see MATERIALS AND METHODS). Mutants of *gal4* are unable to express the *HIS3* gene fused to *GAL10*, and are therefore His$^-$; *gal1* mutants are His$^+$. To facilitate further manipulation of the mutated *gal4* gene, the *gal4* mutants were selected using a strain whose only *GAL4* gene is carried on another yeast plasmid, (pBM292, see MATERIALS AND METHODS).

Spontaneous and UV light-induced mutants were selected on plates containing 2-deoxygalactose and scored for their His phenotype. Thirty-two percent of these were His$^-$ and therefore likely to carry a *gal4* mutation. This was confirmed by a complementation test: all of these mutations fail to complement a *gal4* deletion mutation. Several *gal4* mutants of independent origin were selected for further study. The plas-

mid carrying the mutated *gal4* gene was transferred from each yeast mutant to *E. coli*, and the size of the *gal4* DNA fragment determined. A small fraction of the mutants (about 5%) carried rearrangements of the *GAL4*-containing plasmid. Only those mutants with a *gal4* DNA fragment of normal size were analyzed further.

Genetic map of *GAL4*: To determine the DNA sequence changes caused by these mutations, and to verify that the sequenced changes are the cause of the Gal^- phenotype, we needed to locate them within the *GAL4* gene. This was accomplished by mapping each mutation by UV light-stimulated mitotic recombination, using a set of deletions of *GAL4* constructed *in vitro* (see MATERIALS AND METHODS). Each mutant was mated to the set of *GAL4* deletion strains. The resultant diploids cannot utilize galactose; Gal^+ recombinants arise only if the mutation lies outside of the deleted sequences.

The mutants were isolated in two groups. The first group of mutations map throughout the gene, but a disproportionate number (14 of 54, or 26%) map to the most 5′-proximal deletion interval of *GAL4* (under deletion 960), which includes only about 5% of the length of the gene (Figure 2). This region of *GAL4* encodes the DNA binding domain (KEEGAN, GILL and PTASHNE 1986), and these mutations abolish the DNA binding activity of the protein (JOHNSTON and DOVER 1987).

To isolate many more mutations that alter this DNA binding domain, a second group of *gal4* mutations was mapped against a more limited set of deletions, and only those mutations mapping to the most 5′-proximal deletion interval (deletion 960) were saved for further analysis. These mutations are not included in Figure 2. All of the mutations we identified lie within *GAL4* coding sequences.

The genetic mapping proved to be very sensitive: we could detect recombination between *gal4*-836 and deletion 1133, an interval of 17 bp; we were unable to detect recombination between *gal4*-1254 and deletion 960, an interval of only 7 bp.

DNA sequence changes of *gal4* mutations: These mutations were located on the DNA sequence by dideoxynucleotide sequencing, using eight different oligonucleotide primers located throughout the *GAL4* gene. For nearly all the mutations, the sequence of the entire deletion interval in which each mutation maps was determined. In each case only one or a few tightly clustered nucleotide changes were found in the appropriate deletion interval. These changes are listed in Table 1, and the amino acid changes some of them cause are shown in Figure 4.

Mutation types: Most of the 88 mutations listed in Table 1 are changes of a single nucleotide. Nine are changes of two adjacent nucleotides; three are single base changes with an associated insertion or deletion of one nearby nucleotide; one mutation is a deletion of 20 nucleotides. The types of base changes in the mutant collection is summarized in Table 2. The predominant change is the C:G→T:A transition. In *E. coli*, 5-methylcytosine residues are hotspots for C→T transition mutations, because deamination of this modified base produces thymine (COULONDRE *et al.* 1978). However, since *S. cerevisiae* apparently does not possess methylated cytosines (PROFFITT *et al.* 1984), the preponderance of C→T transitions we observe must be due to some other mechanism. All seven frameshift mutations involve A-T base pairs.

Location of mutations: The GAL4 protein consists of at least two functionally separable domains: the DNA binding domain near the amino terminus (KEEGAN, GILL and PTASHNE 1986), and the domain responsible for activating transcription, which resides in the carboxyl-terminal 95% of the protein (Figure 3) (BRENT and PTASHNE 1985). There is a significant dearth of missense mutations among those that lie in the 3′ 95% of the gene: 38 of the 42 mutations in this region are of the nonsense or frameshift type (Figure 3). Similar results were obtained by GILL and PTASHNE (1987), who found that all five *gal4* mutants they isolated by a different selection method carry frameshift mutations. Two possibilities that could account for this result are (1) the GAL4 protein has multiple domains for activating transcription, or (2) there is some flexibility in the sequence of this region of the protein. In either case, single amino acid changes would be expected to have little effect on function of the protein. Evidence has recently been obtained that supports both of these possibilities (see DISCUSSION).

We did identify four missense mutations that lie in the 3′ 95% of the gene (Ser322→Phe, Leu331→Pro, Ser352→Phe, and Ser511>Pro). Because these mutations alter an apparently nonessential region of the protein between the two transcription activation domains, (it can be deleted with little loss of the transcription activation function (MA and PTASHNE 1987a)), it is unlikely that they directly affect a transcription activation domain (see DISCUSSION). They do not affect the DNA binding activity of the protein (JOHNSTON and DOVER 1987).

The DNA binding domain of GAL4 protein is sensitive to single amino acid changes: 37 of the 46 mutations that alter this domain are of the missense type (Figure 3). This region of the protein contains a "cysteine-zinc finger," a sequence motif found in a large number of other DNA binding and transcriptional regulatory proteins (KLUG and RHODES 1987; EVANS and HOLLENBERG 1988, see Figure 4). All of the mutations that alter this sequence motif abolish the DNA binding activity of GAL4 protein, confirming its role in this function of the protein (JOHNSTON

TABLE 1

***gal4* mutations**

Allele	Nucleotide affected	Nucleotide change	Amino acid change[a]	No. of individual isolates[b]	Expression level[c]
1817	**483**	**G→A**	**Cys14→Tyr**		<0.3
1272#	484–5	CC→TT	Arg15→UGA		
1208#	485	C→T	Arg15→UGA	4	
2259@	**485**	**G→A**	**Arg15→Gln**		<0.3
1253*	491	A→T	Lys17→UAA		
1192*	**491**	**A→G**	**Lys17→Glu**	3	<0.3
1840*	**498**	**T→C**	**Leu19→Pro**		<0.3
1258*	**507**	**C→T**	**Ser22→Phe**	5	
1815*	**507–8**	**CC→TT**	**Ser22→Phe**	3	
2257@	**518**	**C→T**	**Pro26→Ser**	3	<0.3
841#	**519**	**C→T**	**Pro26→Leu**	3	0.35
1266*	**519–20**	**CC→TT**	**Pro26→Leu**		
1188*	**537**	**C→T**	**Leu32→Pro**		<0.3
1819	**554**	**T→G**	**Cys38→Gly**		<0.3
842	**564**	**C→T**	**Ser41→Phe**		<0.3
1834*	**566**	**C→T**	**Pro42→Ser**		<0.3
1836*	**567**	**C→T**	**Pro42→Phe**		<0.3
840#	**582**	**C→T**	**Ser47→Phe**	3	<0.3
843	**584**	**C→A**	**Pro48→Thr**		
990#	**585**	**C→T**	**Pro48→Leu**	3	<0.3
1264*	**591**	**C→T**	**Thr50→Ile**	2	<0.3
1850	**595**	**G→C**	**Arg51→Ser**		<0.3
1254*	602–3	CT→TA	Leu54→UAG		<0.3
2266@	**611**	**G→A**	**Val57→Met**		1.2
987#	662	C→T	Arg74→UGA	2	
997	752	A→T	Lys104→UAA		
845	863	C→T	Gln141–UAA		
834	889–90	+T	Ser150→frshft		
1189*	963	G→A	Trp174→UGA		<0.3
991	1170	T→A	Leu243→UAA		<0.3
989	1278	G→A	Trp279→UAG		
838	1283,1287–91	A→T, −T	Ile281→Phe;Phe283→frshft		
1184*	1283–4,1286	+T, C→T	Asn284→frshft		
848	1317	G→A	Trp292→UAG		
837	1350–5	+T	Tyr305→frshft		
1259	**1407**	**C→T**	**Ser322→Phe**		<0.3
1260	**1434**	**T→C**	**Leu331→Pro**		<0.3
1268	1460	C→T	Gln340→UAG		
1854*	1481,1483–6	A→T, +T	Asn347→Tyr;Phe348→frshft		
1821	1493–6	−T	Ser352→frshft		
1203*	**1497**	**C→T**	**Ser352→Phe**		<0.3
1207	1571	C→T	Gln377→UAA		
839	1579–80	CC→AT	Arg380→UGA		
1852*	1590	G→A	Trp383→UAG		
992	1591	G→A	Trp383→UGA		
836#	1606	G→A	Trp388→UGA	4	
996	1642–3	CC→TT	Gln401→UAG		
835	1750–1	AC→TT	Leu436→Phe;Gln437→UAA		
1256*	**1973**	**T→C**	**Ser511→Pro**		<0.3
844	2021–5	−A	Lys528→frshft		
993	2045	C→T	Gln535→UAA		

The mutations are listed in the order of their location in the gene, from 5′ to 3′. The nucleotide numbering is according to LAUGHON and GESTELAND (1984b). Missense mutations are in bold-face type. Alleles designated with an asterisk (*) were isolated following ultraviolet light mutagenesis; mutations designated with a (#) were independently isolated more than once both spontaneously and after UV light mutagenesis. Mutations designated with (@) were induced by EMS and and selected for their zinc-remedial phenotype (see text). All other mutations originated spontaneously.

[a] In the case of frameshift mutations (designated frshft), the first amino acid altered by the mutation is listed.

[b] Unless otherwise noted, each mutation was isolated only once.

[c] Expression level denotes the level of β-galactosidase produced from a *GAL1-lacZ* fusion (pRY181) in strains whose only *gal4* gene carries the designated mutation. Presented is the percent of fully induced wild type activity. A strain carrying wild-type *GAL4* gave 355 units of β-galactosidase; a strain carrying no *GAL4* gave <1 unit.

(Table 1 continued on p. 68)

TABLE 1—*Continued*

Allele	Nucleotide affected	Nucleotide change	Amino acid change[a]	No. of individual isolates[b]	Expression level[c]
1202*	2066	G→T	Glu542→UAA		
1209	2069–88	deletion	Val543→frshft		
847	2182	T→A	Tyr580→UAA		
994	2226	C→A	Ser595→UAA		
1262*	2264	C→T	Gln608→UAA		<0.3
1261*	2301	T→A	Leu620→UAA		
1196*	2348	C→T	Gln636→UAA		
1269	2385	C→G	Ser648→UGA		
995	2492	G→T	Glu684→UAG		<0.3
1193*	2756	C→T	Gln772→UAA		<0.3
1204*	2781	C→G	Ser780→UGA		<0.3
988#	2834	C→T	Gln798→UAA		<0.3

TABLE 2

Mutation types

Base change	No. of occurrences: Spontaneous	UV-induced	EMS-induced	Total
C:G→T:A	24 (57%)	34 (72%)	5	62
A:T→G:C	1 (2%)	5 (11%)		6
A:T→T:A	5 (12%)	4 (9%)		9
A:T→C:G	1 (2%)	0		1
C:G→A:T	4 (10%)	1 (2%)		5
C:G→G:C	2 (5%)	1 (2%)		3
+A:T	3 (7%)	2[a] (4%)		5
−A:T	2 (5%)	0		2

[a] Both UV-induced frameshift mutations were associated with a single base change of a nearby nucleotide.

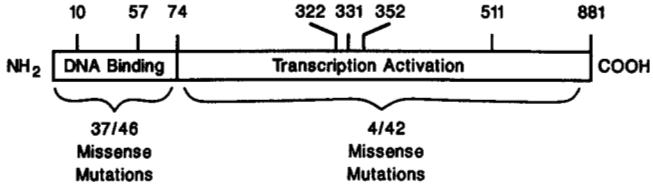

FIGURE 3.—Distribution of *gal4* mutation types. The location and types of the mutations listed in Table 1 is summarized. The numbers above the diagram refer to landmark GAL4 protein amino acids referred to in the text. All mutations altering the DNA binding domain affect residues 10 through 57; the DNA binding domain was localized by KEEGAN, GILL and PTASHNE (1986) to the first 74 amino acids; the location of the transcription activation domain was located by BRENT and PTASHNE (1985) to the region of the protein between amino acids 74 and 881. The four other numbers are positions of the four missense mutations that lie in the 3′ 95% of the gene.

and DOVER 1987). In addition, the 20 amino acids adjacent to the cysteine-zinc finger (Leu32-Arg51) are also likely to be part of the DNA binding domain, because mutations that affect these amino acids abolish or severely reduce the ability of GAL4 protein to bind to DNA *in vitro* (JOHNSTON and DOVER 1987).

GAL gene expression in the mutants: The ability of several of the altered GAL4 proteins to activate GAL gene expression was determined by measuring β-galactosidase levels produced from a *GAL1-lacZ* fusion (see the last column of Table 1). The only mutant with significant activity is Val57→Met, our "leakiest" Gal⁻ mutant.

Revertants of missense mutations: The fact that three of the four missense mutations that map outside the DNA binding domain cluster in a small region of the gene raised the possibility that they alter some specific domain of the protein. Alternatively, they could indirectly affect GAL4 protein function by simply destabilizing it. If these mutations do alter a specific function, we might expect them to revert to Gal⁺ by insertion of an amino acid identical or very similar to the wild-type amino acid. In addition, since three of these four missense mutations affect serine, a phosphorylatable amino acid, we entertained the hypothesis that they affect essential phosphorylation sites of the protein. This idea seemed reasonable, since loss of the *SNF1*-encoded protein kinase prevents GAL gene expression (CARLSON, OSMOND and BOTSTEIN 1981; CELENZA and CARLSON 1986).

Gal⁺ revertants were sought for the four missense mutations that lie outside the DNA binding domain (Ser322→Phe, Leu331→Pro, Ser352>Phe, Ser511→Pro). One mutant (Ser322→Phe) is too "leaky" to obtain revertants; another (Ser352→Phe) gave small Gal⁺ revertant colonies at a low frequency (about 1 per 10^8 cells plated) that was not substantially increased by UV light or EMS, and only one of the six revertants analyzed is due to a mutation in *GAL4*. The two other mutants gave rise to *GAL4* revertants at a similar frequency (about 1 per 10^8 cells plated) that was increased by both UV light and EMS. All of the revertants we analyzed proved to alter the same amino acid affected in the parent mutant; their sequence changes are listed in Table 3.

The fact that these three mutants regain function by insertion of amino acids very different from the normal residue suggests to us that Leu331, Ser352, and Ser511 are not absolutely essential for protein function. Also, since the Ser511 and Ser352 mutants revert to Gal⁺ by acquiring non-phosphorylatable residues, these positions appear not to be sites of phosphorylation essential for protein activity. These con-

TABLE 3

***GAL4* mutations and *GAL* gene expression in Gal$^+$ revertants**

Allele	Wild-type codon	Mutant codon	Revertant codon	Independent isolates	Expression level[a]
2209	CTT Leu331	CCT Pro	CAT His	3	10.9
2519	TCC Ser352	TTC Phe	GTC Val	1	2.2
2255	TCT Ser511	CCT Pro	CTT Leu	2	25.4
2208	TCT Ser511	CCT Pro	CGT Arg	1	12.6

[a] Percent of fully induced wild-type expression level.

TABLE 4

Phenotypic correction of *gal4* mutants by $ZnCl_2$

Strain	Doubling time in hours on galactose		β-Galactosidase activity[a] after growth on:		
			Raf[b]	Raf[b]	Gal[b]
	$-ZnCl_2$	$+ZnCl_2$	$-ZnCl_2$	$+ZnCl_2$	$+ZnCl_2$
Wild type	4.4	6.9 ± 0.6	355	114	283
Pro26→Leu	>48	6.7 ± 0.2	<1	5.5	21.2

[a] For β-galactosidase assays, cells were grown to log phase (OD_{600} = 1–2) and assayed as described by YOCUM *et al.* (1984). Numbers presented are units of activity.

[b] Raf is minimal media containing 2% raffinose; Gal is minimal media containing 2% galactose. When added, $ZnCl_2$ is at a concentration of 10 mM.

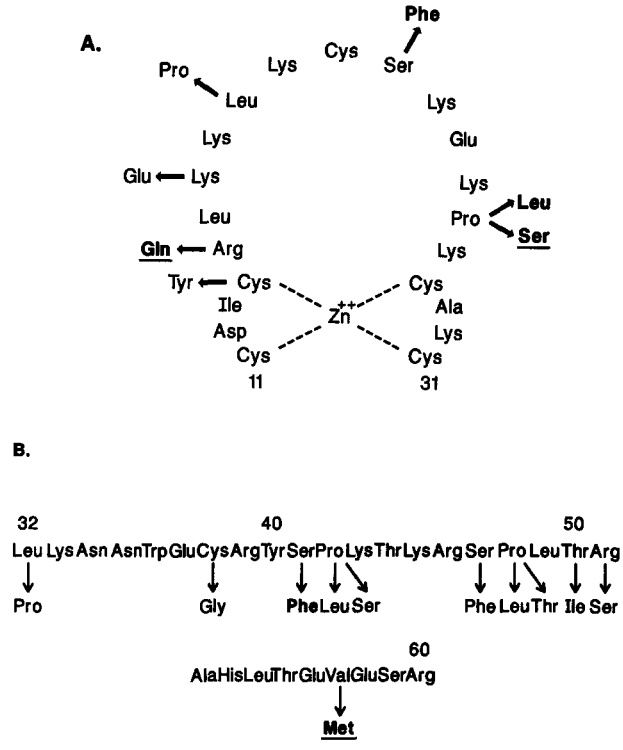

FIGURE 4.—(A) Sequence and proposed structure of the *GAL4* zinc finger, (B) sequence of the 29 amino acids adjacent to the zinc finger, and (A and B) amino acid changes caused by mutations that alter the *GAL4* DNA binding domain. This figure summarizes the amino acid changes caused by all *gal4* missense mutations except the four missense mutations that lie downstream of the DNA binding domain. The Gal$^-$ phenotype caused by the amino acid changes in bold is corrected (to varying degrees) by high concentrations of zinc (see text). The underlined amino acid changes are those found in *gal4* mutants specifically selected for their zinc-remedial Gal$^+$ phenotype (see text).

clusions are qualified, however, because the revertants only regain 2% to 25% of the wild-type levels of GAL gene expression (Table 3).

Mutations that reduce the affinity of GAL4 protein for zinc ions: *Zinc-remedial mutant*: The *gal4* Pro26→Leu mutation apparently reduces the affinity of GAL4 protein for zinc ions. This conclusion is based on the fact that the Gal$^-$ phenotype of cells carrying this mutation is reversed by high concentrations of zinc ions in the growth medium: the growth of this mutant on galactose-containing plates is stimulated near crystals of $ZnCl_2$ (JOHNSTON 1987a, see also Figure 7A). This result is confirmed quantitatively by the results in Table 4. Increased levels of $ZnCl_2$ in the growth media partially restore the ability of the altered protein to activate *GAL* gene expression, and completely correct the growth defect of this mutant. Apparently, these levels of *GAL* gene expression are sufficient to support normal rates of growth. Furthermore, the DNA binding defect caused by the Pro26→Leu mutation, measured *in vitro* using protein produced in *E. coli* cells, is corrected by high zinc ion concentrations (JOHNSTON 1987a; also, see below). These results strongly support the idea that the proposed zinc finger of GAL4 protein indeed contains zinc ions that are essential for its function.

Isolation of additional zinc-remedial *gal4* mutants: To identify other amino acid residues in the DNA binding domain of GAL4 protein responsible for tight binding of zinc ions, we sought additional zinc-remedial *gal4* mutants. We tested 294 newly isolated putative *gal4* mutants for a zinc-remedial Gal$^-$ phenotype. The growth of 11 of these mutants on galactose plates is stimulated to varying extents by $ZnCl_2$. All of these mutants grow normally on glucose without added $ZnCl_2$.

Six of the mutants were found to have mutations in a different nuclear gene. The basis of the defect in these mutants is currently under investigation. Mutations in the five remaining mutants alter the DNA binding domain of GAL4 protein; their sequence changes are included in Table 1, and the three different amino acid changes they cause are underlined in Figure 4.

Three of them carry identical base changes that alter Pro26, the same residue we previously identified as essential for high affinity zinc binding. The phenotype caused by the Pro26→Ser mutation, shown in Figure 5A, is indistinguishable from that caused by the Pro26→Leu change (compare to Figure 7A). In

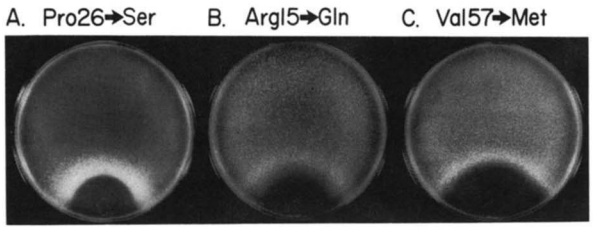

FIGURE 5.—Zinc-remedial Gal⁻ phenotype of *gal4* mutants, tested as described previously (JOHNSTON 1987a). Approximately 10^7 cells were spread in soft agar on the surface of a minimal plate with galactose as sole carbon source. A few crystals of $ZnCl_2$ were placed at the bottom of the plate, and the plates incubated for 2.5 days at 30°. Yeast growth is inhibited at high concentrations of $ZnCl_2$, but at intermediate concentrations, (approximately 10 mM) the growth of these mutants is stimulated. At a greater distance from the $ZnCl_2$ crystals, the zinc concentration is apparently too low to correct the mutant *gal4* defect. The mutants shown in B and C are "leaky," and therefore show substantial growth that is stimulated near the $ZnCl_2$. The growth of all other *gal4* mutants (except those shown here and in Figure 7) is not stimulated by $ZnCl_2$. Of several heavy metal salts tested, only zinc stimulates growth of these mutants; other zinc salts are also able to stimulate growth (JOHNSTON 1987a).

addition, the ability of the Pro26→Ser GAL4 protein to bind to DNA *in vitro* is restored in the presence of $ZnCl_2$ to an extent similar to that for the Pro26→Leu protein (Figure 6A).

Another of the newly isolated *gal4* mutants carries an Arg15>Gln change. The *gal4* defect of this mutant is only partially zinc-remedial: it exhibits a "leaky" Gal⁻ phenotype that is only modestly corrected by increased zinc concentrations (Figure 5B); the altered protein, produced in *E. coli*, has reduced but still substantial DNA binding activity *in vitro* that is slightly stimulated by $ZnCl_2$ (Figure 6B). These results suggest that Arg15 has a minor role in forming the structure that tightly chelates a zinc ion.

Finally, we found a Val57>Met change in the last zinc-remedial *gal4* mutant. This mutation causes a very "leaky" Gal⁻ phenotype that is only weakly corrected by zinc (Figure 5C). This change also results in reduced but still substantial *in vitro* DNA binding activity of the protein that is only modestly increased by zinc (Figure 6B). This mutation alters an amino acid well outside the proposed zinc finger (Figure 4). Perhaps this region of the protein interacts with the zinc finger and influences its structure (see DISCUSSION).

Weakly zinc-remedial mutants: When we initially tested all of our *gal4* DNA binding mutants, only the Pro26→Leu mutant exhibited growth on galactose near crystals of $ZnCl_2$ (JOHNSTON 1987a). However, upon longer incubation, two additional mutants (Ser22→Phe and Ser41→Phe) form Gal⁺ "revertant" colonies near the added zinc (Figure 7, B and C). The Ser22→Phe mutant forms Gal⁺ "revertants" only near the $ZnCl_2$ (Figure 7B); the Ser41>Phe mutant reverts at a higher frequency that is moderately stimulated

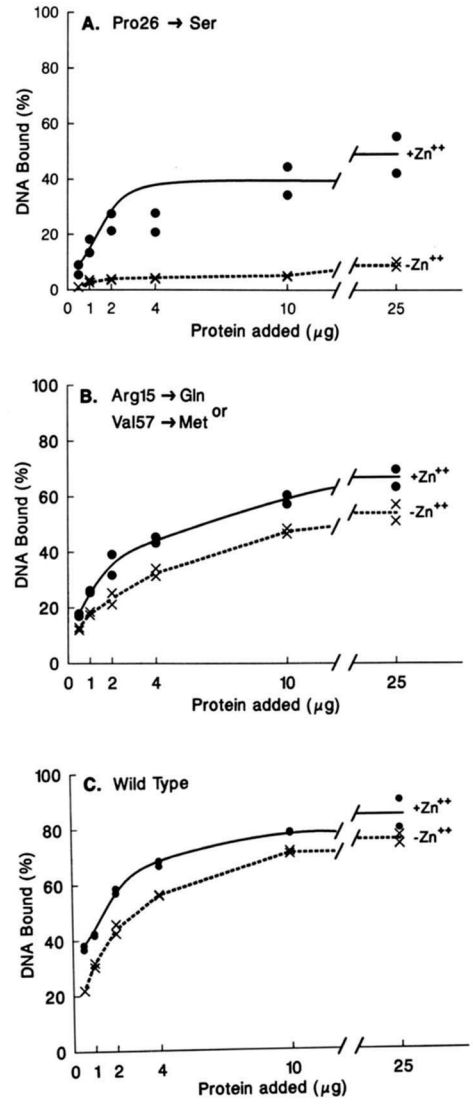

FIGURE 6.—*In vitro* DNA binding activity of mutant gal4 proteins that cause a zinc-remedial Gal⁻ phenotype. Each altered GAL4 protein was expressed in *E. coli*, and crude lysates made and assayed in the presence (solid lines) or absence (dotted lines) of 2 mM $ZnCl_2$ by a nitrocellulose filter binding assay, as previously described (JOHNSTON 1987a). The data reported are the percent DNA (pBM992, containing four binding sites for GAL4 protein) retained on nitrocellulose filters as a function of amount of protein added to the assay.

near crystals of $ZnCl_2$ (Figure 7C). These "revertants," when picked and retested for zinc-remediability, exhibit a phenotype similar to the Pro26→Leu mutant (Figure 7A).

We believe that these two mutations create nonfunctional proteins whose defects are weakly corrected by increased zinc ion concentrations, and that reversion to a zinc-remedial Gal⁺ phenotype is due to increased copy number of the *gal4*-containing plasmids in these two mutants. If the defects in GAL4 protein caused by the Ser22>Phe and Ser41>Phe mutations are only weakly suppressed by zinc, phenotypic correction by zinc might only be revealed in

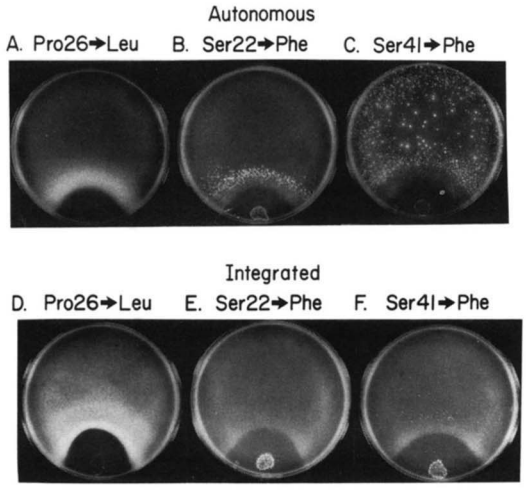

FIGURE 7.—Zinc-remedial Gal⁻ phenotype of partially corrected *gal4* mutants. The ability of $ZnCl_2$ to stimulate growth of *gal4* mutants on galactose plates was tested as described in the legend to Figure 5. The top row of plates (A–C) are spread with strains containing the designated *gal4* mutation present on an autonomously replicating *TRP1-ARS1-CEN3* plasmid (pTC3). Similar results were obtained with strains carrying the same mutations on the *URA3-ARS1-CEN4* plasmid YCp50. The bottom row of plates (D–F) contain strains with the designated *gal4* mutations present in YIp5 integrated in single copy at the chromosomal *URA3* locus. Lengths of incubation of the plates (at 30°) were: A and D, 2.5 days; C, 4.5 days; B, E, and F, 5.5 days. The slight stimulation of growth near $ZnCl_2$ in E and F does not require GAL4 protein, because it also occurs with other strains that carry no *GAL4* gene.

strains harboring multiple copies of the plasmid. [Even though the plasmids carry a centromere, a small fraction of the cells might be expected to contain several copies (HIETER *et al.* 1985).] The results described below are consistent with this hypothesis.

First, several of these Gal⁺ "revertants" were found to carry no additional mutations by the following criteria. (1) The sequence of the region of *GAL4* encoding the DNA binding domain in several "revertants" was determined and found to be identical to the parent mutant. (2) The plasmids in these "revertants," when recovered in *E. coli* and reintroduced into yeast, yielded strains with a phenotype identical to the parent mutant (same as in Figure 7, B and C). These two results demonstrate that the *gal4* gene in the "revertants" (present on an autonomous plasmid) does not carry an additional mutation causing the revertant phenotype. (3) The plasmids containing the *gal4* gene were segregated from several of the "revertants," and the resulting strains retransformed with the *gal4*-containing plasmids carrying the original mutations. All such transformants have the original mutant phenotype. This result demonstrates that the "revertants" harbor no chromosomal mutation responsible for their phenotypic correction by zinc. In addition, the zinc-remedial Gal⁺ phenotype of these "revertants" is unstable, being lost after several generations of growth in the absence of selection for the *GAL4*-containing plasmid.

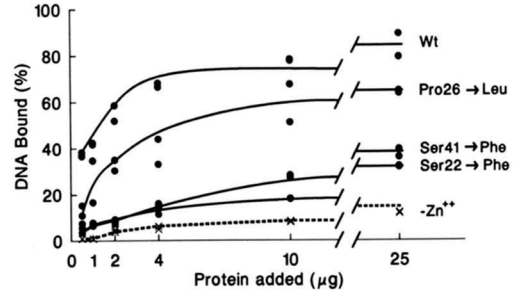

FIGURE 8.—*In vitro* DNA binding activity of mutant gal4 proteins that cause a partially zinc-remedial Gal⁻ phenotype. The altered proteins were expressed in *E. coli*, and extracts were made and assayed in the presence (solid lines) or absence (dashed line) of added $ZnCl_2$ (2 mM), as previously described (JOHNSTON 1987a). The dashed line represents data for the Pro26→Leu mutant, but the Ser22→Phe and Ser41→Phe mutant extracts gave almost identical results in the absence of added $ZnCl_2$. Other gal4 mutations tested whose DNA binding defect is not corrected by increased zinc concentrations are: Cys14→Tyr, Lys17→Glu, Leu19→Pro, Leu32→Pro, and Cys38→Gly.

The behavior of mutants carrying these *gal4* mutations in a chromosome in single copy is also consistent with our hypothesis: neither such mutant reverts to Gal⁺ near the $ZnCl_2$ (Figure 7, E and F). Growth of a mutant carrying the Pro26>Leu mutation in a single chromosomal copy, however, is still stimulated near $ZnCl_2$ (Figure 7D).

Our hypothesis predicts that the DNA binding activity of the Ser22→Phe and Ser41→Phe mutant proteins, produced in *E. coli* and assayed *in vitro*, will be partially corrected by zinc. The results presented in Figure 8 confirm this prediction. These results, taken together, suggest that these two mutations cause the GAL4 protein to have a much reduced affinity for zinc ions, so that increased zinc ion concentrations only partially restore protein function. Because the Ser22→Phe mutation alters the proposed zinc finger, it is not surprising that it has this effect. The Ser41→Phe mutation, however, alters a region of the protein thought to lie outside the zinc finger. It is possible that the zinc finger includes more amino acids than are shown in Figure 4A, or that adjacent amino acids interact with it to influence its structure (see DISCUSSION).

DISCUSSION

The 88 *gal4* mutations we have characterized can be separated into two groups: those that alter the DNA binding domain, and those with changes in the transcription activation domain. Each of these groups are discussed separately below.

DNA binding domain: The DNA binding domain of GAL4 protein contains a zinc finger, a sequence motif common to a wide variety of eukaryotic DNA binding proteins (KLUG and RHODES 1987; EVANS and

HOLLENBERG 1988). Our genetic analysis confirms that the zinc finger is involved in DNA binding, because mutations that alter this structure abolish the ability of GAL4 protein to bind to DNA (JOHNSTON and DOVER 1987). The 20 amino acids adjacent to the zinc finger may also be directly involved in DNA binding, because all of the mutations that alter this region of the protein abolish or severely reduce its DNA binding activity.

Our results strongly support the proposal that zinc ions are indeed part of the zinc finger, and identify some of the amino acids required for forming the zinc binding structure. One residue critical for this function is the proline located two amino acids in front of the second pair of cysteines thought to be responsible for zinc chelation (Figure 4A). The fact that two different amino acids at this site cause apparently identical zinc-remedial phenotypes (both *in vivo* and *in vitro*) suggests that it is the loss of the proline, and not the insertion of a particular amino acid at this site, that reduces the affinity of GAL4 protein for zinc ions. We imagine that this proline causes a bend in the protein that brings the two pairs of cysteines close enough together to chelate tightly a zinc ion. Consistent with this idea is the fact that proline resides at an identical position in six of the nine fungal proteins that contain a zinc finger similar to that of GAL4 protein [see Figure 5 of JOHNSTON (1987b) for a comparison of some of these sequences]. [The other three proteins, encoded by *MAL63* (J. KIM and C. MICHELS, personal communication), *LEU3* (ZHOU *et al.* 1987; FRIDEN and SCHIMMEL 1987), and *PDR1* (BALZI *et al.* 1987) also have a proline, but located one amino acid closer to the second pair of cysteines.] The first arginine residue of the finger (Arg15) may have a minor role in forming the zinc binding structure. Interestingly, all nine fungal proteins that possess a zinc finger similar to that of GAL4 protein have an arginine residue at this same position. Finally, a mutation that alters the serine residue near the tip of the finger (Ser22) appears to have a major effect on the zinc binding structure, because it causes a Gal$^-$ phenotype that is only weakly corrected by zinc.

We were surprised to find two mutations causing a zinc-remedial phenotype that alter amino acids outside the proposed zinc finger (Val57→Met, Ser41→Phe). This suggests that the region of the protein adjacent to the zinc finger can influence its structure. It is worth noting that the locations of two additional cysteine residues are conserved in nine of the ten yeast proteins that contain a zinc finger: one is within the finger (GAL4 Cys21, see Figure 4A); the other is located 6 amino acids C-terminal to the zinc finger (GAL4 Cys38, see Figure 4B). Perhaps these two cysteines also have a role in chelating zinc ions. A mutation that alters the serine residue located three amino acids C-terminal to Cys38 appears to reduce the affinity of GAL4 protein for zinc ions. Since serine only differs from cysteine by an oxygen atom in place of sulfur, perhaps Ser41 is involved in chelating a zinc ion through this oxygen, together with Cys38. In any case, our results support a role for some of the conserved residues of the zinc finger in forming a structure that tightly binds zinc ions, and suggests that the region adjacent to the zinc finger may influence its structure.

Transcription activation domain: The marked paucity of missense mutations altering the transcription activation domain of GAL4 protein suggests that there are very few single amino acid changes that severely affect this function of the protein. Results recently obtained by others provide some explanations for this phenomenon. First, there may be two regions of the protein able to activate transcription, one close to the DNA binding domain, and the other at the extreme carboxyl terminus of the protein (MA and PTASHNE 1987a) (however, see further discussion below). More importantly, the feature of the sequence of these regions that appears to be most important for their function is the content of acidic amino acids (glutamic or aspartic acid) (GILL and PTASHNE 1987). In fact, several different sequences can provide the transcription activation function, provided they have an overall acidic character (MA and PTASHNE 1987c). Our inability to isolate a significant number of missense mutations altering the transcription activation function of GAL4 protein is consistent with the idea that there exist multiple, relatively sequence nonspecific regions of the protein for this function.

As noted above, it has been reported that GAL4 protein contains two domains that are each by themselves able to activate transcription: region I, near the amino terminus (amino acids 148–196), and region II, at the carboxyl terminus (amino acids 768–881) (MA and PTASHNE 1987a). Even though most of our nonsense mutations (all those C-terminal to Trp174) leave region I intact, they fail to allow transcription activation. Sequences in the middle of the protein, which would be present in many of our nonsense mutants, appear to mask the activity of activating region I (MA and PTASHNE 1987a), and this could be the reason that this domain is not active in our mutants. In addition, a major difference between our experiments and those that define activating region I is that we measure function of low levels of GAL4 protein produced from its own weak promoter; the experiments in which region I is active measure the function of high levels of GAL4 protein produced from the strong *ADH1* promoter. It is possible that the ability of region I to activate transcription is an artifact caused by abnormally high levels of GAL4

protein. Alternatively, *all* of our nonsense mutations could destabilize the protein in yeast.

The four missense mutations we did find outside the DNA binding domain are unlikely to affect directly the transcription activation function, because they lie in a nonessential region of the protein between the two activation domains that can be deleted with little loss of protein function (MA and PTASHNE 1987a). These mutations could simply destabilize GAL4 protein. However, the fact that three of them (except Ser511>Pro) cluster in one of the three regions of the protein that is similar to the distantly related LAC9 protein from *Kluyveromyces lactis* (SALMERON and JOHNSTON 1986; WRAY *et al.* 1987) raises the possibility that they may affect a specific function of GAL4 protein.

The GAL4 protein is thought to activate gene transcription by directly contacting some component(s) of the transcriptional apparatus (*e.g.*, RNA polymerase, TATA box binding factors, histones, etc.). With the ultimate goal of identifying these components, we had hoped to obtain a collection of missense mutations altering the transcription activation domain. Our results make it clear that such mutations will have only marginal effects on the function of GAL4 protein, severely limiting their use in genetic approaches aimed at identifying proteins that interact with GAL4 protein. Future progress toward this goal will require analysis of mutants with more subtle defects.

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