GAL4 IS REGULATED BY A GLUCOSE-RESPONSIVE FUNCTIONAL DOMAIN

by

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Abstract

Gene regulation in eukaryotes has been a major focus of molecular biological research, aiming to elucidate the genetic processes involved in cell development and disease. To better understand the fundamentals of gene regulation, simple cellular systems such as lower eukaryotes are being studied. These systems are easier to analyze through genetic mutation, providing an overall picture of a particular genetic pathway. One such system is the galactose utilization (GAL) pathway of the yeast *Saccharomyces cerevisiae.*

Expression of the *GAL* genes is determined by the availability of carbon sources in the media. This regulation is exercised through GAL4, a transcriptional activator of the *GAL* genes. The availability of galactose induces GAL4 activity by inhibiting the GAL4 negative regulator, GAL80. Glucose, the preferred carbon source, inhibits GAL4 activity by several mechanisms. I demonstrate that one mechanism of glucose repression is mediated by a large, previously uncharacterized, central region of GAL4. This region directly inhibits GAL4 activity in the presence of glucose; deletion of the central region eliminates glucose repression. Fusion of the central region to a heterologous transcriptional activator (LexA-VP16) confers glucose repression. Inhibitory domains in the central region constitutively inhibit activity when a region called the glucose response domain, also present in the central region, is deleted. Inhibition of LexA-VP16 by the central region is accompanied by loss of DNA binding.

I suggest that direct inhibition of GAL4 activity in glucose is mediated by interaction of the central region inhibitory domains with the DNA binding and dimerization domain, an interaction which prevents DNA binding. In the absence of glucose, DNA binding is restored by interaction of the glucose response domain with the inhibitory domains.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Bp	Base pair
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetate
GTP	Guanosine triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-Ethanesulfonic acid
O.D.	Optical density
ONPG	O-Nitrophenyl-ß-D-galactopyranoside
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl Fluoride
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate (0.15M NaCl, 0.015M sodium citrate)
TEMED	N',N',N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UTP	Uridine triphosphate
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
WT	WildType

UNITS OF MEASUREMENTS

Ci	curie
Da	dalton
dpm	disintegrations/minute
g	gram
k	kilo
l	liter
M	molar
m	milli
μ	micro
n	nano
p	pico

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Introduction

1. Eukaryotic Gene Regulation

The eukaryotic genome is a complex of expressed and unexpressed genes, the majority of which are repressed at the level of transcription (Struhl, 1989). The methods by which cells control gene expression have been a major focus of molecular biological research. Researchers have attempted to understand the functions of the eukaryotic cell, the development of the cancer cell, and the processes of viral infection. Such research can then be applied to the understanding of a variety of diseases and the development of novel treatments for these diseases, or can be used for genetic engineering in eukaryotes.

The turning on or off of genes, however, is often the end result of a complicated series of positive and negative control pathways. Phosphorylation and metabolic precursor pathways provide a set of biochemical equilibria within the cell. These equilibria coordinate precise adjustments of gene expression during changes to the cell environment. Such pathways must also be taken into account when studying the regulation of gene expression.

Gene regulation in eukaryotes is performed by a set of proteins termed transcriptional activators and transcriptional repressors. Activator proteins recognize specific DNA sequences on a gene's promoter and interact with TATA box associated proteins to turn on gene transcription by RNA polymerase II, while repressors appear to bind DNA at the promoter and block activation by a yet uncharacterized mechanism. The TATA box has the consensus sequence TATAA, a site found upstream of most eukaryotic genes. This site is thought to bind a complex of proteins (general initiation factors, GIFs) involved in the activation of RNA polymerase II. RNA polymerase II, another large complex, transcribes the chromosomal DNA into RNA. The interaction of transcriptional activators, repressors, general initiation factors, and RNA polymerase II is pictured in Figure 1.

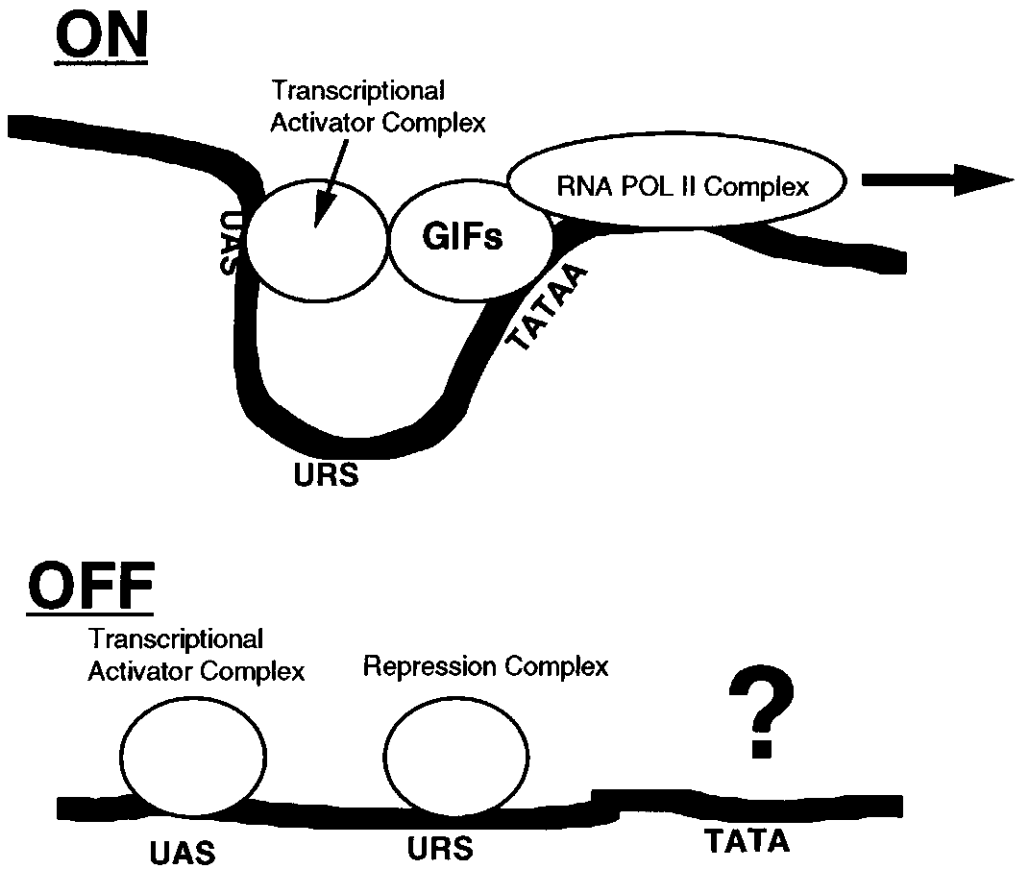


Figure 1. Schematic of Molecular Interactions at Active and Inactive Gene Promoters.

Shown is a model of interactions involved in the expression or repression of genes in eukaryotic systems. For active genes (on), the transcriptional activator complex appears to form direct interactions with general initiation factors (GIFs) associated with the TATA box. The GIFs then form interactions which induce activity of the RNA polymerase II complex. In repressed genes (off), a repressor complex binds DNA in the vicinity of the upstream activating sequence, blocking interaction of the activator complex and the GIFs.

We do not completely understand this interaction of activators, repressors, and the general initiation factors. Work summarized by Ptashne (1986) and Pugh and Tijan (1992), suggest that transcriptional activators and particular general initiation factors may interact directly. The TATA box Binding Protein (TBP), one of the general initiation factors, is thought to interact directly with transcriptional activators, but TBP Associated Proteins, called TAF's, may also play a role in this interaction, at least in the case of higher eukaryotes. In yeast TBP is a 27 kDa monomer.

Chromatin and the superstructure of DNA in the nucleus may also be targets of transcriptional activators. Activators may act by disrupting the chromatin structure, or could aid TBP directly in binding to DNA blocked by chromatin (Pugh and Tijan, 1992).

As some DNA binding sites for transcriptional activators are up to 200 base pairs from the TATA box (Ptashne, 1986), a number of models have been proposed as to how the activator and general initiation factors could interact. Of these models, the most logical is that the intervening DNA could loop out, allowing the DNA binding site and TATA box to interact (Ptashne, 1986).

2. Regulation of Galactose Catabolism in S. cerevisiae

Galactose catabolism in *Saccharomyces cerevisiae* has been a focus of genetic research since the mid 1950's, when mutant yeast unable to utilize galactose were isolated (De Robichon-Szulmajster, 1958). The genetics of the galactose metabolism pathway was studied because its relatively simple regulation would provide a framework for more complex mammalian gene regulation. Since yeast are easier to manipulate through genetic mutation, all the genes involved in galactose catabolism can be isolated and characterized. The galactose metabolism pathway therefore provides a model of gene regulation that is relatively simple to study.

To study galactose catabolism, genes encoding the enzymes which convert melibiose (a galactose-glucose disaccharide) to glucose-6-phosphate were characterized. These include GAL1 (kinase), GAL7 (transferase), GAL10 (epimerase), MEL1 (alpha-galactosidase), and GAL5 (mutase) (Figure 2). These genes are expressed at levels determined by the amount of galactose and glucose in the cell. When galactose is available to the cell, but glucose is scarce, these genes are transcribed at high levels. In the presence of glucose, the preferred carbon source, the cell represses expression of the *GAL* genes, as is the case when galactose is absent.

3. GAL4 and Galactose/Glucose Regulation

Regulation of the *GAL* genes occurs through GAL4, their transcriptional activator. GAL4 binds to the upstream activation sequence for galactose (UASg), an area of DNA containing one or more binding sites for a dimer of GAL4 molecules. GAL4 and its activation function are themselves regulated by the available sugars in the growth media. The presence of galactose results in high levels of GAL4 activation, while the absence of galactose or the presence of glucose, the preferred carbon source, prevents GAL4 activation. When galactose is absent (glycerol as carbon source), GAL4 is inactivated by protein-protein interaction with GAL80, a GAL4 regulating protein. When galactose is added to the medium an inducer molecule, produced by the GAL3 protein, causes a change in the GAL4-GAL80 interaction, resulting in transcriptional activation by GAL4 (Oshima, 1982). I discuss the interaction of GAL4 and GAL80 in more detail below.

Glucose repression of *GAL* gene transcription occurs through several mechanisms (Flick and Johnston, 1990, 1992; Griggs and Johnston, 1991; Nehlin et al., 1991). One mechanism involves a set of upstream repression sequences for galactose (URSg), located on various *GAL* gene promoters. These sites bind glucose regulated repressors which inhibit GAL4 from activating transcription. Studies of

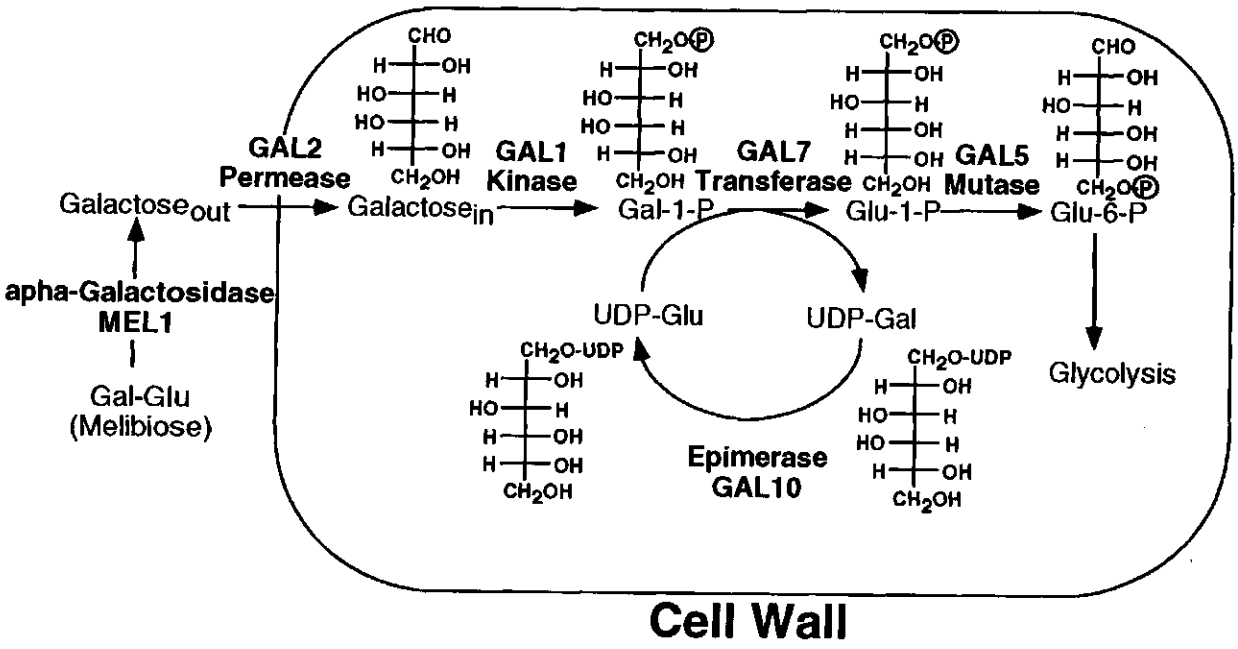


Figure 2. The Products of the *GAL* Genes Convert Galactose to Glucose-1-Phosphate.

Gene products involved in the conversion of the galactose-glucose sugar melibiose to glucose-1-phosphate are pictured. All genes except *GAL5* are known to be regulated by the transcriptional activator GAL4. The molecular structure of the sugars, in their reduced form, is also shown

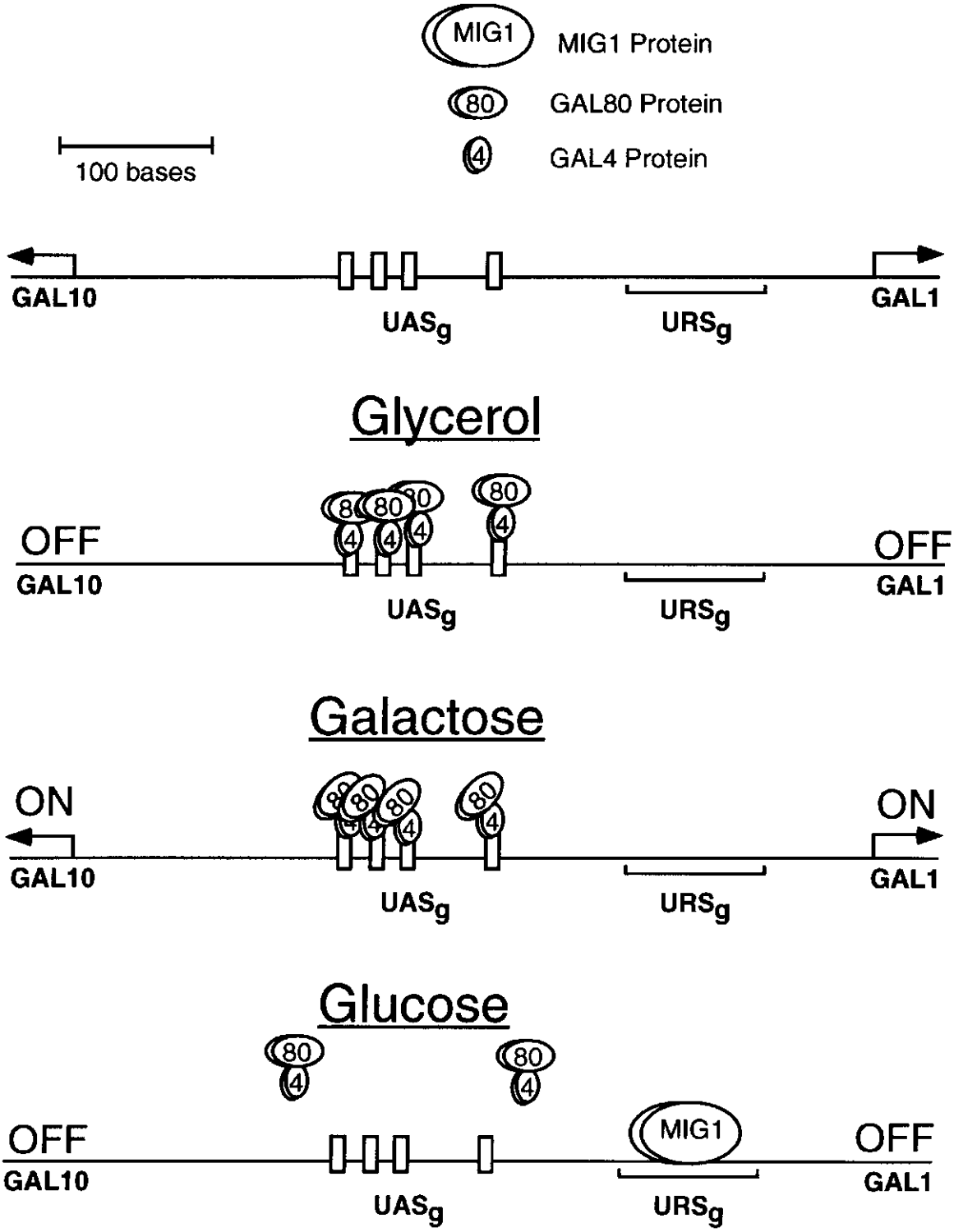
URSg repression (Nehlin et al., 1991; Flick and Johnston, 1990, 1992) provide evidence for a number of genes mediating the URSg response including: *MIG1*, *URR1*, *URR3*, *URR4*, *SSN6*, and *TUP1*. MIG1, the best characterized of the gene products, binds to the URSg, repressing activation of the *GAL1* and *GAL4* genes (Nehlin et al., 1991). Knockout mutants of *MIG1* show five times higher *GAL1* transcription in the presence of glucose than does wild type. MIG1 is thought to bind DNA via a zinc coordinated DNA binding motif but its mechanism of GAL4 inhibition is not clear. In addition to cis-acting elements, glucose inhibits GAL4 activation via GAL80. This is accomplished by glucose repression of *GAL2*, the galactose permease gene, which allows entry of galactose into the cell (Matern and Holzer, 1977). GAL80 continues to repress GAL4 activity since the GAL80 inducer cannot be produced. Knockouts of *GAL80* result in a six fold higher *GAL1* transcription in the presence of glucose than does wild type. However the combined effect of disrupting both *GAL80* and *MIG1* results in fifty fold de-repression in glucose. This is thought to be the result of a synergism between MIG1 and GAL80 repression, though there are as yet no theories of how this synergism takes place. A summary of these molecular interactions is shown in Figure 3.

4. The *GAL1-10* Divergent Promoter

Gene activation by GAL4 normally involves multiple GAL4 binding sites, where GAL4 dimers interact cooperatively (Kang and Sadowski, unpublished). The GAL4 dimer binds 17mer sites through its DNA binding domain, while a yet to be characterized cooperativity domain allows interaction of multiple dimers on a UASg. The *GAL1-10* UASg (Figure 3) contains four 17mer binding sites which, at the appropriate GAL4 concentration, bind GAL4 which then contacts GIF proteins bound at the TATA box of both *GAL1* and *GAL10* genes. The method by which the GAL4 activation region contacts general initiation factors is currently under investigation by a number of labs (Pugh and Tijan, 1992). For the *GAL1-10* UASg, presumably the activation regions of four GAL4 dimers are able to initiate

Figure 3. The *GAL1-10* UASg Allows the Bi-Directional Activation of the *GAL1* and *GAL10* Genes.

A 600 base pair segment of DNA between the *GAL1* and *GAL10* TATA boxes contain four GAL4 binding sites (UASg) and a region binding proteins involved in the repression of GAL4 activation (URSg). A model describing interaction of GAL4 and MIG1 with the UASg in various carbon sources is summarized.


MIG1
MIG1 Protein
80
GAL80 Protein
4
GAL4 Protein
100 bases
GAL10
GAL1
UASg
URSg
Glycerol
OFF
OFF
Galactose
ON
ON
Glucose

transcription from both the *GAL1* and *GAL10* general initiation factors. *GAL7* and *GAL2* contain two GAL4 binding sites, and *GAL80* and *MEL1* each containing a single site.

As well, URSg sites play a role in the expression of GAL1 and possibly GAL10. Analysis of the *GAL1-10* promoter reveals three URSg elements between UASg and *GAL1* (see Figure 3). Flick and Johnston (1992) have shown that URSg is capable of mediating glucose inhibition of a *HIS3* gene activated by a *LEU2* UAS (both of which are not themselves glucose regulated). Because this inhibition is present when the URSg is placed either in front of or behind UAS*leu2*, Flick and Johnston surmise that *GAL10* may also be inhibited *in vivo* (Flick and Johnson, 1992). As well, the *GAL4* and *GAL2* genes are regulated by MIG1, and likely contain at least one URSg site. Other *GAL* genes have not been shown to contain URSg sites.

5. GAL4 Structure and Function

a. DNA Binding and Dimerization

The GAL4 protein contains a number of structural motifs, some previously characterized and some of which are the basis of my investigations. The N-terminal region of GAL4 contains a DNA binding and dimerization domain (Keegan et al., 1986; Ma and Ptashne, 1987b; Carey et al., 1989; Marmorstein et al., 1992). DNA binding takes place through residues 1-65, which contain four cysteines that coordinate a Zinc atom. This structure sits in the major groove of the GAL4 binding site, a site with the consensus sequence CGGAGGACACAGGAGGC (Johnson, 1988). Dimerization takes place through residues 50 to 94 and involves direct interaction of residues 50 to 64, which form an amphipathic α-helical structure. Experiments by Johnston, 1987, show that the presence of a Zinc atom is necessary for DNA binding, and that some weak DNA binding mutants can regain their ability to activate transcription when grown in high concentrations of Zinc ion.

The GAL4 DNA binding domain contains homology to a variety of other yeast transcriptional activator proteins involved in regulating expression of genes for metabolic pathways. All contain the four zinc coordinating cysteines in addition to a number of other conserved regions. It is likely that these DNA binding domains evolved from a common ancestral protein.

There is weak evidence that glucose inhibition of GAL4 may correspond with loss of DNA binding ability. Lohr and Hopper (1985) looked at DNAse I hypersensitive sites on the *GAL1-10* UASg with a variety of GAL4 and GAL80 genotypes. Wild type GAL4 strains were found to contain hypersensitive sites corresponding to DNA binding of GAL4, but only when grown in glycerol as opposed to glucose. This DNA binding evidence unfortunately is complicated by the discovery that GAL4 mRNA levels are reduced five fold in the presence of glucose. As such, loss of DNA binding cannot conclusively be deemed a result of direct glucose inhibition of GAL4 as opposed to being the result of less GAL4 protein in the cell.

It is not known whether other proteins interact with GAL4 at the DNA binding site. X-ray crystallographic data suggests that there is space at this site for another protein to coordinate with GAL4, and studies of the protein GAL11 make it a likely candidate for such interaction (Himmelfarb et al., 1990). A potentiating mutant of GAL11, GAL11P, can increase transcriptional activation of a weak GAL4 mutant. As GAL11 has been shown to be capable of activating transcription independently, and depends on the GAL4 DNA binding domain to interact with GAL4, it has been suggested that GAL11 may coordinate with GAL4, acting as a secondary transcriptional activator. However, there is no conclusive evidence of direct interaction between the two proteins.

b. Transcriptional Activation

GAL4's transcriptional activation function is performed by a pair of acidic regions located internally and at the C-terminus of the protein (Gill and Ptashne, 1987). Activation region 1 (AR1, residues 149-238), and activation region 2 (AR2, residues 768-881) activate transcription when they are fused to the DNA binding and dimerization domain of GAL4 (Ma and Ptashne, 1987b). Characterization of these activation domains involved a series of C-terminal deletions of GAL4. Deletion to amino acid 763 resulted in loss of activity, while a protein consisting of the DNA binding domain and residues 768-881 was able to activate transcription. Similarly, amino acids 1-229 were able to activate transcription at moderate levels, while 1-147 was inactive.

Studies of GAL4 mutants with altered transcriptional activation ability (Gill and Ptashne, 1987) indicate that activation is related to the presence of acidic residues. Decrease in activation is the result of a decrease in acidity of the activation region. Research has also been performed to investigate whether acidity is the only requirement for yeast gene activation. In one series of experiments (Ma and Ptashne 1987c), random sequences of E. coli DNA were cloned to GAL4 1-147, creating a library of random C-terminal polypeptides. These were then screened for proteins capable of activating transcription. In all cases, the proteins which activate contain high proportions of acidic residues in what appeared to be an alpha helical structure. This suggests that acidic residues, arranged in some form of alpha helical structure, are the only requirement for transcriptional activation by GAL4. These acidic residues are assumed to contact one or more proteins among the general initiation factors, and may act to disrupt chromatin and the chromosomal superstructure in order to activate transcription. For GAL4, Fedor and Kornberg (1989) and Workman et al., (1991) have shown that the UASg, active GAL4, and galactose as inducer are the only requirements for converting the nucleosome array on the chromosome to the conformation of an induced state.

c. GAL80 Interaction

Deletion analysis has shown that GAL4 interacts with the GAL80 protein via its C-terminal thirty amino acids (Ma and Ptashne, 1987a). It is presumed that GAL80 interacts directly with GAL4, as GAL80 co-purifies with GAL4 on a GAL4 DNA binding column (Chasman and Kornberg, 1990). As the C-terminal 30 amino acids are part of AR2, it is also thought that GAL80 masks AR2 in order to repress GAL4 activity. It is known that GAL4 and GAL80 are in contact both when repression is taking place (ie. no galactose), and after galactose induction when GAL4 is actively inducing transcription (Leuther and Johnston, 1992). In addition, GAL4 is known to remain bound to the UASg *in vitro* during GAL80 repression (Ma and Ptashne, 1987a).

Serine 837 of GAL4, a site which is phosphorylated when GAL4 is transcriptionally active, may also play a role in GAL4-GAL80 interaction. GAL4 inhibited by GAL80 is not phosphorylated, but becomes rapidly phosphorylated after induction with galactose and is phosphorylated in the absence of galactose in *gal80-* cells (Mylin et al., 1989). In addition, recent results (Neidbala and Sadowski, unpublished) indicate that while mutation of Serine 837 to Alanine does not affect transcriptional activation levels over the long term, when this mutant is induced in galactose and then repressed by GAL80 (by removing galactose from the media), the Ser837 mutant is repressed more rapidly than wild type GAL4. Therefore the phosphorylation at Ser837 may prevent the immediate repression of GAL4 by GAL80.

d. Central Region

The central region (CR) of GAL4, an area comprising over 60% of the protein, has as of yet not been assigned a function (residues 239-767). Deletion analysis of GAL4 by Ma and Ptashne (1987b) demonstrated that complete deletion of CR was required to see activation by AR1. This was thought to be a consequence

of steric hindrance by the mutant protein. However, a GAL4 deletion mutant missing AR1 (Sadowski et al. 1991) also shows inhibition by the CR, as subsequent deletion of CR increases activity. These data suggest that the CR may play a role in regulating GAL4 activity.

The central region also contains a stretch of around 100 amino acids (residues 320-412, a region I call ID1) that bear homology to a number of yeast transcriptional activator proteins. This includes LAC9, a lactose and galactose regulator in the yeast strain *Kluyveromyces lactis*, as well as the activators PUT3, PPR1, ARGRII, LEU3, PET111, QUTA and PDR1. All are involved in the transcriptional regulation of proteins involved in specific metabolic pathways. The region of homology between GAL4 and these activators has not been studied extensively, and no definitive function for this region has been established. Johnson and Dover (1988) provided evidence that this region may be involved in regulating GAL4 activity. In a series of random GAL4 point mutants, they found that the only mutants able to severely decrease GAL4 activity were contained either within the DNA binding domain, at amino acids 322, 331, and 351 (all contained within ID1), or at amino acid 511. The three mutations found within ID1 suggest that ID1 is capable of inhibiting GAL4 activity. My studies have shown ID1 to in fact be an inhibitory domain.

The aim of my research has been to determine the role of the GAL4 central region in GAL4 function. My research has shown that the central region is involved in regulating GAL4 in response to glucose. The central region contains two functional regions: a series of inhibitory domains (residues 238-585) and a glucose response domain (GRD, residues 600-767). The inhibitory domains appear to constitutively inhibit GAL4, possibly through the DNA binding-dimerization domain, while the GRD down regulates the inhibitory domains in the absence of glucose. A similar method of regulation may occur with other yeast transcriptional activator proteins that contain regions of homology to the GAL4 central region.

Materials and Methods

1. Yeast Strains and ß-Galactosidase Assays

All experiments were performed with the yeast strain YT6 (Himmelfarb et al., 1990) (MATα, *gal4, gal80, ura3, his3, ade2, ade1, lys2, trp1, ara1, leu2, met*), bearing reporter genes integrated at *URA3*. Strain YT6::171 has a wild-type *GAL1-lacZ* reporter gene bearing both the UASg and URSg (Yocum et al., 1984). The *GAL1-lacZ* reporters in YT6::SV15 and YT6::6LexOp are deleted for both UASg and URSg, and instead contain a single consensus GAL4 "17-mer" (Giniger et al., 1985), or 6 LexA operator sites (Ruden et al., 1991) at -130, respectively.

Transcriptional activation by GAL4 and LexA derivatives was measured by assaying ß-galactosidase activity in cells grown to mid-log phase in minimal selective media, as described by Himmelfarb et al. (1990). Triplicate cultures of cells containing derivatives were grown in trp- ura- dropout media to an $O.D._{600}$ of 1.0 to 1.5. In all experiments, glycerol grown cultures contained 2% v/v glycerol, 2% v/v lactic acid, 2% v/v ethanol, while glucose grown cultures contained 2% w/v glucose, 2% v/v glycerol, 2% v/v lactic acid, 2% v/v ethanol. Cells were then pelleted and re-suspended in 1 ml Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 0.7% (v/v) ß-Mercaptoethanol, 1mM DTT, 0.02% SDS (w/v)). Cells were again pelleted and the supernatant aspirated. Cells were resuspended in 100µl Z-buffer per 5 ml yeast culture, and 100µl of glass beads (600 micron and 212 micron) were added. The tubes were then frozen at -70°. Tubes were vortexed at 4° for 30 minutes to lyse cells, and spun at 4° for 5 minutes. Supernatant, containing the total cell protein extract, was removed to a clean tube. **protein assay:** 5 µl of extract was added to 800µl dH_2O and 200µl concentrated BioRad protein assay reagent. After mixing and incubation for 5 minutes at room temperature, the O.D. at 595nm was read. This absorbance was then correlated to a standard curve of protein concentration for Bovine Serum Albumin. **ß-Galactosidase Assay:** In a test tube,

from 5 to 40 µl of extract was added to 500µl Z buffer. Tubes were placed at 30° for 2 minutes, and then 100µl of 0.4 percent ONPG was added to start the reaction. Reactions were stopped with 500µl of 1M Sodium Carbonate (pH11) when tubes turned a visible yellow color. Absorbance at 420nm was read against a blank (no protein extract). Tubes which became deep yellow before five minutes, or did not turn yellow, were re-assayed at a more suitable concentration of protein. Activity (in units) was calculated as the pmol of ONPG hydrolyzed per minute per milligram protein. The three independent cultures assayed gave activities with a standard deviation of less than 20 percent.

2. Plasmids

Most plasmids for expression of GAL4 and LexA derivatives were derived from pMH76 (Sadowski et al., 1992), a single copy vector in which GAL4 (wild type) is expressed from the *ADH1* promoter, with transcripts terminated within the *ADH1* terminator (See Figure 4 for diagrams of select clones). The precise residues present in the GAL4 and LexA derivatives (Figures 1, 2, and 4), and in the fusion junctions, are shown in Table 1. All DNA manipulations followed the standard protocols (Sambrook et al., 1989).

GAL4 Deletion Derivatives: Plasmids expressing GAL4 derivatives 5-1, 5-2, 5-3, 5-4, and 9-9 have XhoI-HindIII backbones from pMH76, with XhoI-HindIII inserts from pKW1 (Sadowski et al., 1991), pCD21XX, pMA236, pMA246, and pMA242 (Ma and Ptashne, 1987b), respectively. Derivative 6-2 was constructed by digesting 5-1 with EcoRI, which cleaves between the coding sequences for GAL4 (1-147) and CR, and with SalI, which cleaves at codon 547; the plasmid ends were religated using an oligonucleotide adapter (5'-AATTCTCGAG-3'). Derivative 5-10 was constructed by digesting 5-1 with MluI and SalI, followed with blunt ending and ligation of the vector. Plasmids encoding derivatives 6-1, 6-3, 6-4, and 6-5 were generated by cutting derivative 5-1 with EcoRI, followed by digestion with BAL31,

and ligation to an EcoRI linker (5'-GGAATTCC-3'). In-frame fusions of these deletions, encoding C-terminal portions of GAL4, were then created with GAL4 (1-147) by cloning EcoRI-HindIII fragments into the vectors pY1, pY2, and pY3 (Sadowski et al., 1992). To facilitate fusion of GAL4 AR2 to several deletion derivatives, a BamHI site was introduced at GAL4 codons 767-768 by oligonucleotide directed mutagenesis; this mutation has no apparent effect on GAL4 activity (not shown), and was subsequently introduced into derivatives 5-1 and 6-1 to form 5-1^{Bam} and 6-1^{Bam}. Derivatives 6-6 and 6-7 were then created by digesting 5-1^{Bam} and 6-1^{Bam} with SalI-BamHI, and the vector ends ligated together using an adapter consisting of two oligonucleotides (5'-TCGAGGTGG-3', 5'-GATCCCACC-3'). To construct derivatives 9-6, 9-7, and 9-8, XhoI-EcoRI inserts from pCD56X, pCD38XX, and pCD52XX (Ma and Ptashne, 1987b) were first ligated to an XhoI-EcoRI backbone of derivative 9-9. A self annealing oligonucleotide adapter (5'-AATTGGATCC-3') was ligated into the EcoRI sites of these intermediates to generate in-frame fusions of CR with AR2. Derivatives 6-8, 6-9, and 6-10 have a SalI-HindIII backbone from 6-2, and SalI-HindIII inserts from derivatives 9-6, 9-7, and 9-8, respectively.

PHO5 promoter were derived from pML315 (contributed by Mark Lamphier), a derivative of pMA448 (Ma and Ptashne, 1987a) in which the *GAL4* promoter has been replaced with that of *PHO5*. YCpPHO5-GAL4 (Table 3) has a BamHI-HindIII fragment from pML315, containing the *PHO5* promoter and GAL4 coding sequence, cloned into YCplac22 (Gietz and Sugino, 1988). YCpPHO5-GAL4ΔCR (see Tables 1 and 3) was constructed by subcloning an XhoI-MluI fragment from pMA242 (Ma and Ptashne, 1987b) into YCpPHO5-GAL4.

GAL4-VP16 Fusion Derivatives: DNA encoding the VP16 activating domain (an acidic activating region produced by the Herpes simplex virus) was derived from pSGVPΔ488, which contains a SalI-BamHI fragment (Triezenberg et

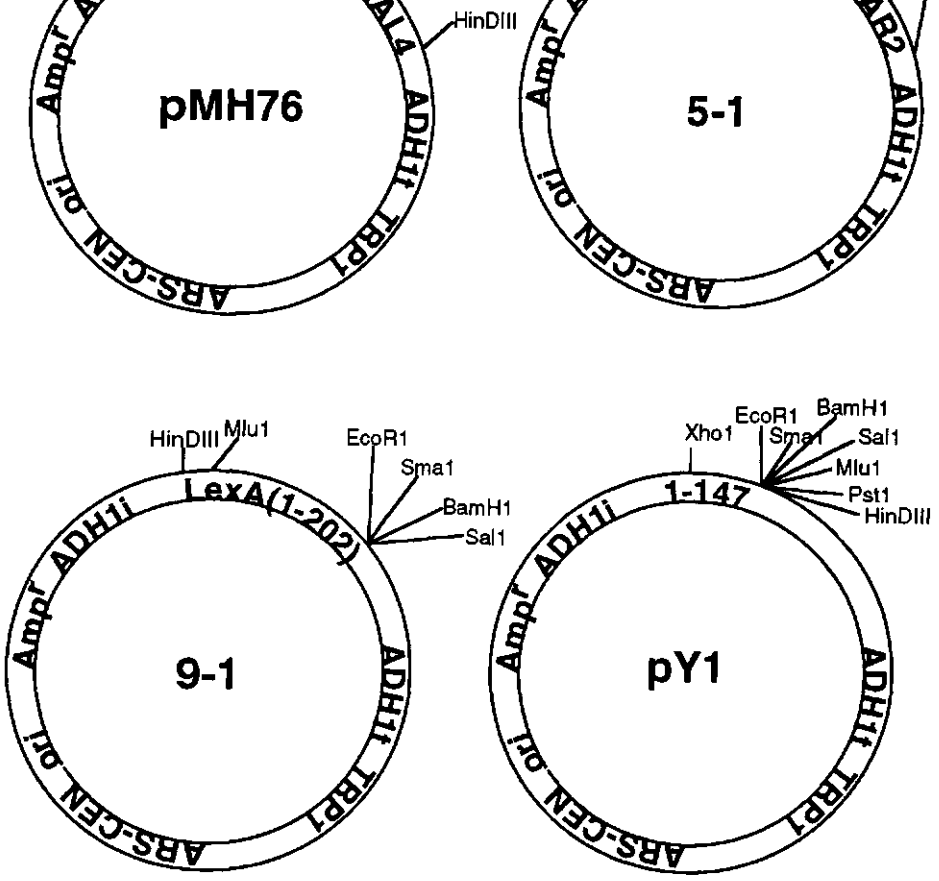


Figure 4. Select Plasmids Used for the Expression of GAL4 and LexA Derived Mutants and Fusion Proteins

Shown are cartoons of four plasmids used in the construction of most derivatives shown in this thesis. pMH76 expresses wildtype GAL4; 5-1 expresses GAL4 (1-147 + 238-881); 9-1 expresses LexA (1-202); and pY1 expresses GAL4 (1-147) followed by a multiple cloning site. All plasmids contain an ampicillin resistance gene and origin of replication for bacterial cloning, as well as an ARS-CEN sequence, a TRP1 gene, and an ADH1 promoter for yeast expression.

Table 1. Amino Acid Residues Present in GAL4, LAC9, and LexA Deletion and Fusion Derivatives

Deriv.	GAL4, LexA, LAC9, and VP16 Amino Acid Residues
WT	GAL4(1-881)
5-1	GAL4(1-147)+RNSA+GAL4(237-881)
5-2	GAL4(1-763)+PEFRRVWNHYRDV
5-3	GAL4(1-147)+PEF+GAL4(768-881)
5-4	GAL4(1-238)+PEF
5-5	GAL4(1-147)+PEFP+VP16(411-488)+ANSA+GAL4(237-881)
5-6	GAL4(1-763)+PEFP+VP16(411-488)+GSIRRL
5-7	GAL4(1-147)+PEFP+VP16(411-488)+ANSA+GAL4(237-763)+PEFRRVWNHYRDV
5-8	GAL4(1-147)+RNSA+GAL4(237-763)+PEFP+VP16(411-488)+GSIRRL
5-9	GAL4(1-147)+PEFP+VP16(411-488)+GSIRRL
5-10	GAL4(1-147)+ RNSA+GAL4(237-763)+PEFRRYWNHVRDV
6-1	GAL4(1-147)+RNS+GAL4(320-881)
6-2	GAL4(1-147)+RNSR+GAL4(412-881)
6-3	GAL4(1-147)+PEFP+GAL4(554-881)
6-4	GAL4(1-147)+GIP+GAL4(585-881)
6-5	GAL4(1-147)+RNS+GAL4(600-881)
6-6	GAL4(1-147)+RNSA+GAL4(237-412)+EV+GAL4(768-881)
6-7	GAL4(1-147)+RNS+GAL4(320-412)+EV+GAL4(768-881)
6-8	GAL4(1-147)+RNSR+GAL4(412-614)+PEF+GAL4(768-881)
6-9	GAL4(1-147)+RNSR+GAL4(412-593)+RNWINF+GAL4(767-881)
6-10	GAL4(1-147)+RNSR+GAL4(412-478)+RNWINF+GAL4(767-881)
6-11	LexA(1-202)+PGIRRPAAKLIPGEFLMIYDFYY
6-12	LexA(1-202)+EFPGIW+VP16(411-488)+GSIRRRYPGEFEL
6-13	LexA(1-202)+EFL+GAL4(320-412)+GSA+VP16(411-488)+GSIRRRYPGEFEL
6-14	LexA(1-202)+EFL+LAC9(776-850)+ GSA + VP16(411-488)+ GSIRRRVPGEFEL
9-1	LexA(1-202)+EFP+VP16(411-488)+ANSA+GAL4(237-763)+PEFRRVWNHYRDV
9-2	LexA(1-202)+EFP+VP16(411-488)+ANSA+GAL4(237-412)+VDLQPS
9-3	LexA(1-202)+EFP+VP16(411-488)+ANSA+GAL4(237-614)+ELPRPAAKLNSRRISYDL
9-4	LexA(1-202)+EFP+VP16(411-488)+P+GAL4(602-763)+PEFRRVWNHYRDV
9-5	LexA(1-202) +EFL+ GAL4(602-763)
9-6	GAL4(1-614)+PEF+GAL4(767-881)
9-7	GAL4(1-593)+RNWINF+GAL4(767-881)
9-8	GAL4(1-478)+RNWINF+GAL4(767-881)

al., 1988) encoding VP16 amino acids 411-488, cloned blunt into the SmaI site of pSG424 (Sadowski and Ptashne, 1989). To insert the VP16 activating domain in place of GAL4 AR1, an oligonucleotide linker containing an XhoI site (5'-AATTGGCCTCGAGGCC-3') was first inserted into the EcoRI site of derivative 5-1. An XhoI fragment, encoding GAL4 residues 74-147, from this intermediate was then replaced with an XhoI-SalI fragment from pSGVPΔ488 to form derivative 5-5. Several GAL4-VP16 derivatives were derived from pKW21 (provided by Kristen Wood), which has the same backbone as pY1 (Sadowski et al., 1992), but the polylinker following the coding sequence for GAL4(1-147) contains restriction sites for EcoRI, PstI, SalI, and XbaI. Derivative 5-9 was constructed by cloning an EcoRI-SalI fragment, encoding VP16 amino acids 411-488, from pSGVPΔ488 into pKW21; to facilitate subsequent cloning steps, the SalI site was destroyed by digestion and end-filling with Klenow. Derivative 5-6 contains an XhoI-EcoRI backbone from derivative 5-9, and XhoI-EcoRI insert from 5-2. Derivative 5-7 contains a SalI-HindIII backbone from 5-5, and SalI-HindIII insert from 5-2. To construct 5-8, the XhoI-SalI fragment of derivative 5-6, spanning GAL4 codons 74-478, were replaced with that of derivative 5-1.

LexA-VP16 Fusion Derivatives: The LexA coding sequence (encoding a bacterial DNA binding protein) was derived from plasmid pLexA(1-202)+PL (Ruden et al., 1991) which contains on a 2 micron yeast vector, the *ADH1* promoter directing the expression of LexA (amino acids 1-202), the coding sequence for which is followed by a polylinker and *ADH1* terminator. An SphI fragment from this plasmid was inserted into pMH76, replacing the *GAL4* coding sequence with LexA and the polylinker. Derivative 6-12 was constructed by inserting a BglII-BamHI fragment encoding VP16 amino acids (411-490), from pCRF2 (Triezenberg et al., 1988) into the BamHI site of derivative 6-11. A fragment encoding GAL4 ID1 was generated by PCR from WT *GAL4* DNA using the oligonucleotide primers 5'-GGAATTCCTCGAGTCAGGTTC-3', and 5'-CGGGATCCGAAGAAGGGAAG-

3'; this fragment was digested with EcoRI-BamHI, and cloned into derivative 6-11 following LexA. Derivative 6-13 was then constructed from this intermediate by inserting a BglII-BamHI fragment encoding VP16 amino acids (411-490), from pCRF1 (Triezenberg et al., 1988) into the BamHI site following ID1. Derivative 6-14 was generated by digesting a PCR product from *LAC9* DNA (using the primers 5'-GGAATTCCCTGAGAATAACG-3' and 5'-CGGGATCCCTGAAGATTGGG-3') with EcoRI and BamHI and cloning this fragment into 6-11. This construct was then digested with BamHI and a BglII-BamHI fragment encoding VP16 amino acids (411-490) from pCRF1 was inserted to form 6-14. Derivative 9-1 consists of an EcoRI backbone from 6-11, and EcoRI insert from 5-7. DNA encoding the C-terminal portion of the GAL4 central region was removed by cutting with SalI and religating the backbone to generate derivative 9-2, and by cutting with SalI and ligating in a SalI-XhoI insert from 9-6, to generate 9-3. A fragment encoding the glucose response domain was generated using derivative 5-1^{Bam} as a template for PCR with oligonucleotides 5'-GGAATTCCCTGAGAATAACG-3' and , 5'-TATCAGCAATATTCCCAC-3'; the PCR fragment was digested with EcoRI-BamHI, and cloned into derivative 6-11, creating 9-5. An EcoRI fragment from pSGVPΔ488, encoding VP16 amino acids 411-488, was then cloned into this intermediate, to form 9-4.

Other Plasmids: Hybridization templates for measuring nuclear run-on transcription were cloned into pSP72 as follows: *HIS3*, 1.4 kb BamHI fragment from pYF92 (Storms et al., 1979); *GAL10*, 1.6 kb SalI-EcoRI fragment from pNN77 (St. John et al., 1981); *lacZ*, 3 kb EcoRI-KpnI fragment from pCH110 (Hall et al., 1983). The LexA operator DNA fragment for gel-mobility shift assays was generated by digestion of pUC18(LexA-Op) (provided by D. Ruden), containing a single LexA binding site, with EcoRI-HindIII.

3. Metabolic Labeling and Immunoprecipitations

Labeling of cells expressing GAL4 derivatives was as described previously (Sadowski et al., 1991). Five or ten ml cultures were grown to mid-log phase in minimal selective medium containing glycerol; two hours prior to labeling, glucose was added to 2%. The cells were harvested, washed three times with 1ml methionine free media (2% glucose) , resuspended in 100µl met- glucose media and labeled with 400µCi of ^{35}S-methionine for 90 minutes at 30°. 1ml ice cold Yeast Lysis Buffer (YLB) (50mM Tris pH8, 5mM $MgCl_2$, 150mM NaCl, 5mM NaF, 2mM $ZnCl_2$, 10µg/ml Aprotinin, 1µM Leupeptin, 10mM PMSF) was added, cells were pelleted and washed twice with cold YLB, discarding all supernatants in the radioactive waste. Cells were resuspended in 400µl of cold YLB in a flat bottom eppendorf tube, and 100µl of 212 and 600 micron glass beads were added. Cell suspensions were then vortexed for 30 minutes at 4°. 400µl 2X RIPA buffer (10mM Tris pH8, 100mM NaCl, 1mM EDTA, 1% NP40, 0.5% Sodium Deoxycholate, 0.1% SDS, protease inhibitors as for YLB) was added and tubes vortexed a further 20 seconds. Cell debris was pelleted by centrifuging at 12K for 30 minutes at 4°. The supernatant was carefully removed to a clean tube on ice, and used for immunoprecipitation.

For immunoprecipitation, 5µl of preimmune rabbit serum and 50µl of heat inactivated Staphylococcus aurius (S.A.) cells were added to the extracts, and tubes were placed on a rocker platform at 4° for 30 minutes. S.A. was precipitated by 20 second centrifugation and the supernatant was removed to a clean cold eppendorf tube. Rabbit polyclonal antibodies against GAL4 (5µl) or LexA (1µl) were added to the extract, mixed and incubated on ice for 60 minutes. 50µl of 10% S.A. was added to the tubes and the tubes were placed on a rocker for 60 minutes. S.A. was pelleted, the supernatant aspirated, and the pellet resuspended in 1ml Buffer 1 (10mM Tris pH8, 1M NaCl, 0.1% NP40, protease inhibitors as for YLB). S.A. was then pelleted and washed in Buffer #2 (10mM Tris pH8, 0.1M NaCl, 0.1% NP40, 0.1% SDS,

protease inhibitors), Buffer #3 (10mM Tris pH8, 0.1% NP40), and finally RIPA buffer. S.A. was again pelleted and half the sample treated with phosphatase. S.A. was resuspended in 1ml of phosphatase buffer, and 500µl transferred to a new tube. The S.A. was pelleted and the samples to be phosphatased were resuspended in 90µl phosphatase buffer. Potato Acid Phosphatase was added to the samples, mixed and incubated at 37° for 30 minutes. After incubation, 1ml RIPA buffer was added to the phosphatased samples, the Staphylococcus aurius were pelleted, and resuspended in 30µl 1X SDS sample buffer. Meanwhile, the other half of the samples was washed once more with 1ml RIPA buffer, pelleted for 30 seconds, and resuspended in 30 µl 1X SDS sample buffer. Most samples were then resuspended in 1ml RIPA buffer (no SDS) and re-immunoprecipitated prior to analysis.

For analysis, samples were heated to 100° for 2-5 minutes and loaded on 7.5% SDS-PAGE gels along with protein molecular weight markers. After a suitable running time, gels were stained for 1 hour, destained overnight , washed with water and soaked in Enhance (DuPont Chemicals) for 30 minutes, washed 10 minutes in water, and dried at 65° under vacuum for 3 hours. Gels were marked with radioactive liquid paper, and exposed to pre-flashed film for from 2 to 60 days at -70°.

4. Nuclear Run-on Transcription

Cells for nuclear run-on transcription assays were grown to mid-log phase in 10 ml minimal selective medium containing glycerol, at which time 1 ml of 20% glucose was added to appropriate samples. 30 minutes and 4 hours later, cultures were poured into Corex centrifuge tubes containing crushed ice. The cells were collected by centrifugation, and nuclear run-on transcription for each sample performed as described by Warner (1991). Cells were resuspended in 950µl cold dH_2O. 50µl of 10% (w/w) N-Laurosarcosin (Sodium Salt) was added, cells were mixed gently and left on ice for 15 minutes. Cells were then spun in a microfuge for

2 minutes at 4° and the supernatant completely aspirated. Cells were resuspended in 120µl of Reaction Mix (50mM Tris pH7.9, 100mM KCl, 5mM $MgCl_2$, 1mM $MnCl_2$, 2mM DTT, 0.5mM ATP, 0.25mM GTP and CTP, 10mM Creatine Phosphate, 12ng/µl Creatine Kinase, and 100µCi of ^{32}P-UTP) and incubated at room temperature for 10 minutes. One ml of cold TMN (10mM Tris pH7.4, 100mM NaCl, 5mM $MgCl_2$, and 50µM UTP) was added, the reaction mixed, and the cells pelleted 20 seconds at 4°. The radioactive supernatant was discarded. Labeled RNA was isolated as described by Ma and Ptashne (1987b). Cells were resuspended in 200µl of RNAse free LETS buffer (0.1M LiCl, 10mM EDTA, 10mM Tris pH7.4, 0.2% SDS, 0.1% Diethylpyrocarbonate) and placed in flat bottom eppendorf tubes. 100µl of 212 and 600 micron glass beads and 200µl of LETS equilibrated Phenol were then added and the tubes vortexed for 20 minutes at 4°. Another 200 µl of LETS buffer was added and the solution vortexed a further 5 minutes. Tubes were spun 3 minutes at 4° and the aqueous phase was transferred to a clean eppendorf. The solution was extracted twice more with 400µl LETS equilibrated Phenol Chloroform (1:1) with a 2 minute vortex, 2 minute spin at 4°. 5µg of Yeast tRNA and 40µl 5M LiCl were added and the RNA left to precipitate overnight at -20°. RNA was pelleted with a 5 minute spin at 4°, washed with 1ml 70% ethanol, and briefly dried under vacuum.

RNA was dissolved in 100µl hybridization fluid (2XSSC, 50% formamide, 0.2% SDS, 1 X Denhardt's) and 1µl was counted in scintillation fluid. Equal counts were then diluted to 2mls in hybridization fluid, added to nitrocellulose dot-blots containing 5 µg denatured hybridization template DNA (see above), and incubated at 42°C for 72 hours, with gentle shaking. The filters were washed with excess 2XSSC-40% formamide for one hour, and excess 2XSSC-0.2% SDS 5 minutes (repeated 10 times). Dot blots were dried at 80° under vacuum and exposed to pre-flashed X-ray film overnight. Labeled hybridized RNA was quantitated by scanning densitometry.

5. Gel Mobility-Shift Assays

Yeast expressing LexA derivatives were grown to mid-log phase in minimal selective medium containing glycerol or glucose. The cells were collected by centrifugation, washed once with ice cold water, and resuspended in buffer A (25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% Glycerol, 1 mM PMSF). Glass beads were then added and the cells broken by vortexing. The extracts were clarified by centrifugation in a microfuge for 5 minutes, and used for binding reactions immediately, or frozen at -20°. Binding reactions contained 15 μg crude extract protein, 0.5 pmole ^{32}P end-labeled LexA operator fragment, 0.75 μg sonicated salmon sperm DNA, 0.1 μg poly-dIdC, in buffer A. Binding reactions were performed on ice for 40 min, prior to analysis on non-denaturing gels as described by Carey et al. (1989). Binding reactions with cold competitor were performed identically, but included 10-fold excess unlabeled LexA operator. Binding reactions with LexA antibody were performed as above with the addition of a suitable dilution (1:100) of polyclonal rabbit antibody to LexA amino acids 1-202. The gel contained 4.5 percent 29:1 acrylamide in 0.5 times TBE (45mM Tris, 0.5mM EDTA, 45 mM Boric acid) and 1 percent glycerol, and was run in the same solution. Gels were pre-run for 90 minutes at 10 volts/cm and soaked in a change of buffer for 2 to 3 more hours before loading. Binding reactions were slowly layered in wells and the gel run at 5 volts/cm.

Results

1. The Central Region of GAL4 Inhibits Transcriptional Activation

As noted in the introduction, results from Ma and Ptashne (1987b) and Sadowski et al. (1991) indicate that, while either GAL4 activating region can activate when fused to the GAL4 DNA binding domain, their activity is severely reduced when present in chimera with the central region (CR). These results imply that CR may have an inhibitory effect that is unmasked upon deletion of either GAL4 activating region.

I began characterizing this effect by creating a series of GAL4 deletion mutants. These mutants are expressed from the relatively strong ADH1 promoter on a single copy ARS-CEN vector. The mutants (Figure 5), were assayed in a *gal4-*, *gal80-* yeast strain with a reporter system containing the *GAL1* promoter fused to the *LacZ* gene (Figure 5, bottom). I found that, in agreement with earlier studies, deletion of AR1 or AR2 resulted in a marked decrease in activity when assayed in glucose (5-1 and 5-2) , while subsequent deletion of CR gave mutants with higher levels of activation (5-3 and 5-4). When comparing activation levels, the central region appears to severely affect the activation strength of the mutant. When AR2 is deleted, activation falls to less than one unit, but increases to 12 units when the central region is also deleted. For the AR1 deletion, activation goes from 12 to 403 units with the subsequent deletion of CR.

To confirm these results, and to ascertain whether CR inhibition is specific to the GAL4 activation domains, I created a series of heterologous activators containing GAL4 domains and the acidic activating region of the protein VP16 (Sadowski et al., 1988; Triezenberg et al., 1988). VP16, a Herpes simplex viral protein, contains a strong acidic activation domain within a region of 77 amino acids (VP16 residues 411-488). This region was used to replace either AR1 or AR2 in the GAL4 protein. My results (Figure 5) indicate that VP16 responds in a similar

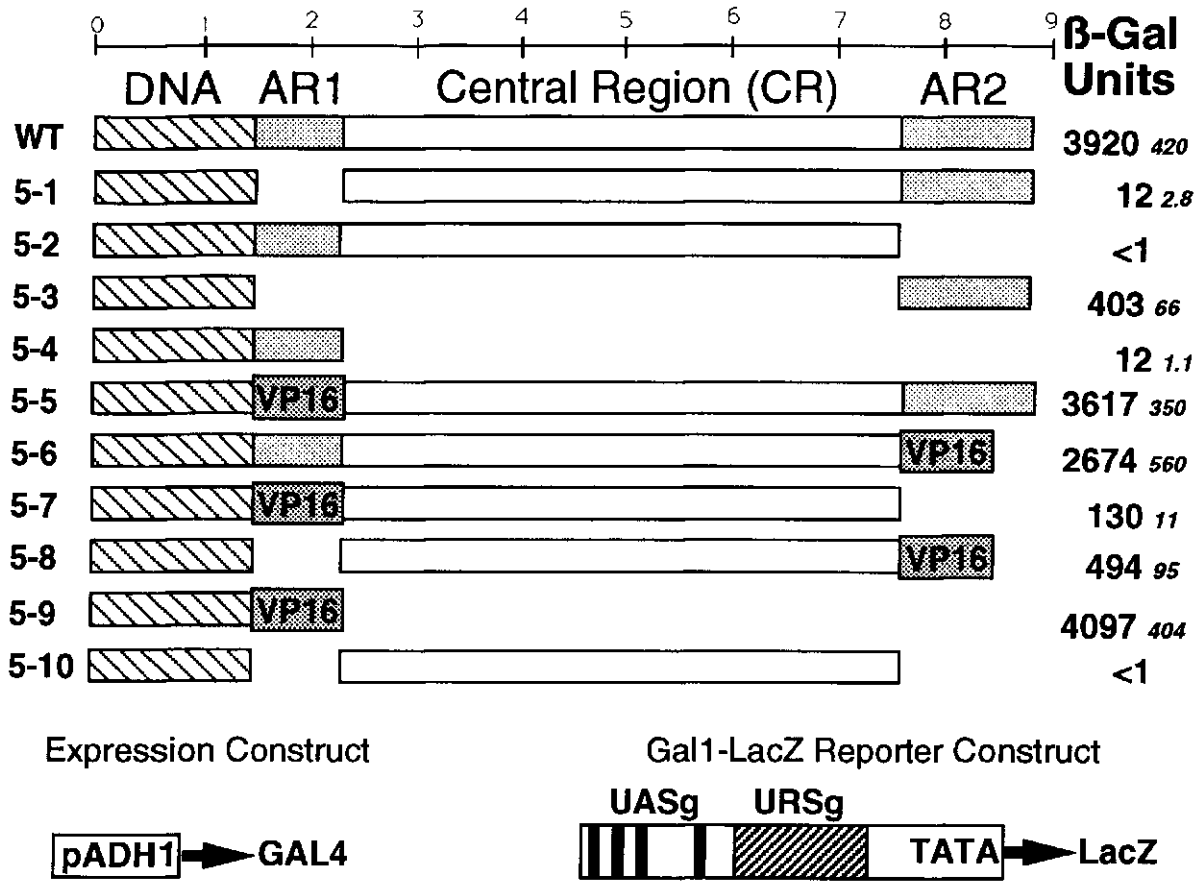


Figure 5. The Presence of the Central Region Inhibits Transcriptional Activation by GAL4 Derivatives

Activity of GAL4 deletion derivatives was determined by measuring ß-galactosidase produced from a *GAL1-lacZ* reporter gene (bottom, right) in yeast grown in 2% glucose. GAL4 derivatives were expressed from the *ADH1* promoter (bottom, left) on a single copy plasmid vector. The *GAL1-lacZ* reporter gene contains both the UAS$_G$, containing four GAL4 binding sites, and URS$_G$. The indicated values represent an average of at least three samples, and with the exception of derivative 5-9, did not vary more than 20% with standard deviation given in italics.

manner to the presence of CR. Comparing activities, replacement of AR1 or AR2 by VP16 and subsequent deletion of the other GAL4 activation region results in severe loss of activation function in glucose (compare derivatives 5-5 with 5-7 and 5-6 with 5-8). The subsequent removal of CR allows full activation of transcription (5-9). To control for the possibility that CR activates transcription, a construct containing the DNA binding domain and CR was also assayed (5-10). It gave no activity, consistent with the model that CR affects activation, but is not itself capable of transcriptional activation. GAL4 (1-147) was also inactive (not shown).

2. The Central Region Contains a Cluster of Inhibitory Domains, Capable of Inhibiting Activating Region 2.

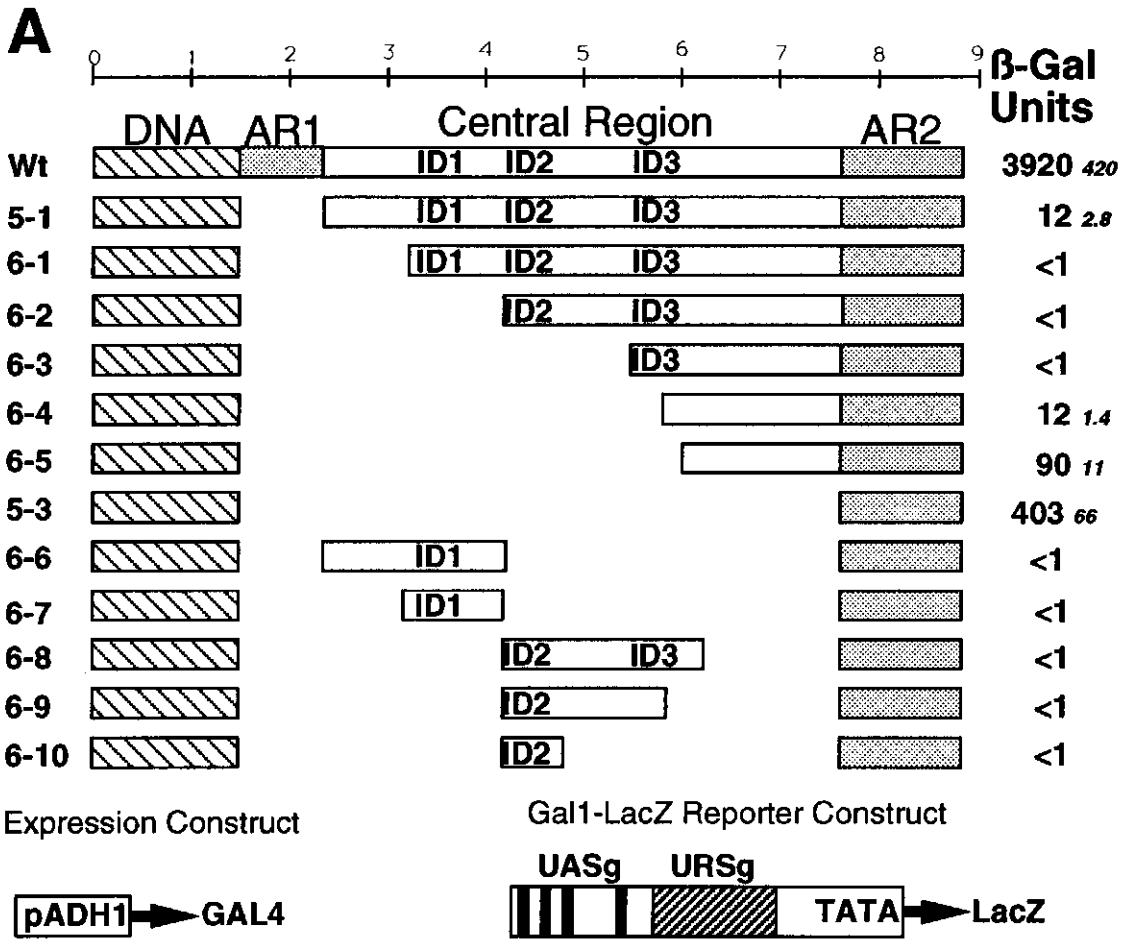
To further characterize the mechanism responsible for CR dependent inhibition of GAL4, I created a series of internal deletions of the CR (Figure 6a). These deletions complement the set of C-terminal deletions created by Ma and Ptashne (1987b). My results indicate that regions within CR residues 238 to 585 are capable of inhibiting activation by AR2. Internal deletions up to residue 554 gave no activity (6-3), while deletion to residue 585 gave a derivative with moderate activity (6-4). To define specific regions within this span involved in GAL4 inhibition, I created a set of derivatives containing short internal segments of CR. Derivatives bearing 320-412 (6-7) or 412-478 (6-10) were unable to activate transcription, implicating these domains in inhibition of GAL4. I denoted these segments Inhibitory domain 1 (ID1) and Inhibitory domain 2 (ID2). Computer generated homology searches indicated no significant areas of amino acid homology between these two regions. There are likely other inhibitory domains, one between residues 554 and 585 (ID3) and as well, Ma and Ptashne (1987b) noted that deletion of residues 276 to 238 resulted in an active protein, suggesting that an inhibitory domain may also be located in this area.

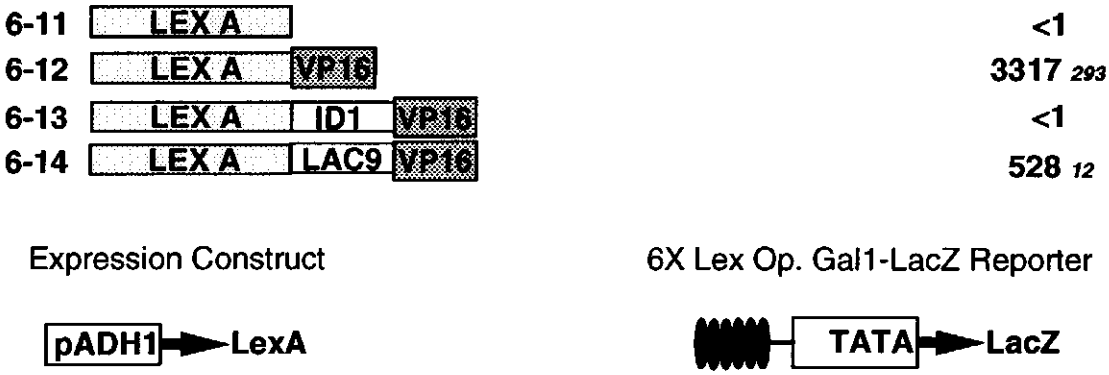
Figure 6. The Central Region Contains at Least Three Regions that can Inhibit Transcriptional Activation by GAL4 AR2

Activity of GAL4 deletion derivatives was determined by measuring ß-galactosidase produced from a *GAL1-lacZ* reporter gene in yeast cells grown in 2% glucose. Standard deviations are given in italics.

A) GAL4 derivatives were expressed from the *ADH1* promoter (bottom, left) on a single copy plasmid vector in yeast strain YT6::171, bearing a *GAL1-lacZ* reporter gene containing both UAS_G and URS_G (bottom, right).

B) LexA fusion derivatives were expressed from the *ADH1* promoter (bottom, left) on a single copy plasmid vector in yeast strain YT6::6LexOp, bearing a *GAL1-lacZ* fusion reporter gene in which both UAS_G and URS_G were deleted and replaced with 6 LexA operators (bottom, right).


A
0 1 2 3 4 5 6 7 8 9
ß-Gal Units
DNA AR1 Central Region AR2
Wt ID1 ID2 ID3 3920 420
5-1 ID1 ID2 ID3 12 2.8
6-1 ID1 ID2 ID3 <1
6-2 ID2 ID3 <1
6-3 ID3 <1
6-4 12 1.4
6-5 90 11
5-3 403 66
6-6 ID1 <1
6-7 ID1 <1
6-8 ID2 ID3 <1
6-9 ID2 <1
6-10 ID2 <1
Expression Construct
pADH1 GAL4
Gal1-LacZ Reporter Construct
UASg URSg
TATA LacZ


B
6-11 LEX A <1
6-12 LEX A VP16 3317 293
6-13 LEX A ID1 VP16 <1
6-14 LEX A LAC9 VP16 528 12
Expression Construct
pADH1 LexA
6X Lex Op. Gal1-LacZ Reporter
TATA LacZ

Residues 320-412 (ID1) contain homology to a variety of fungal transcriptional activator proteins, including LAC9 of *Kluveromyces lactis* (Wray et al., 1987; Figure 7). While it has been shown that this region is highly conserved, and that mutations in this region cause loss of activity for wild type GAL4, no function for ID1 has been proposed. I created a heterologous activator protein to see if ID1 is capable of inhibiting a transcriptional activator other than GAL4. LexA residues 1-202, a bacterial DNA binding and dimerization domain (Figure 6b, 6-11), was fused to the VP16 activating domain (6-12). This derivative was assayed on a reporter gene containing six LexA binding sites positioned upstream of a *GAL1-LacZ* fusion deleted for both UASg and URSg (Ruden et al., 1991; Figure 6b, bottom). Figure 6b shows that the LexA-VP16 derivative is a strong activator, but is inhibited with the introduction of ID1 into the protein (compare 6-12 and 6-13). In addition, I fused the ID1 homology region of LAC9 into the LexA-VP16 construct, and again observed inhibition (6-14).

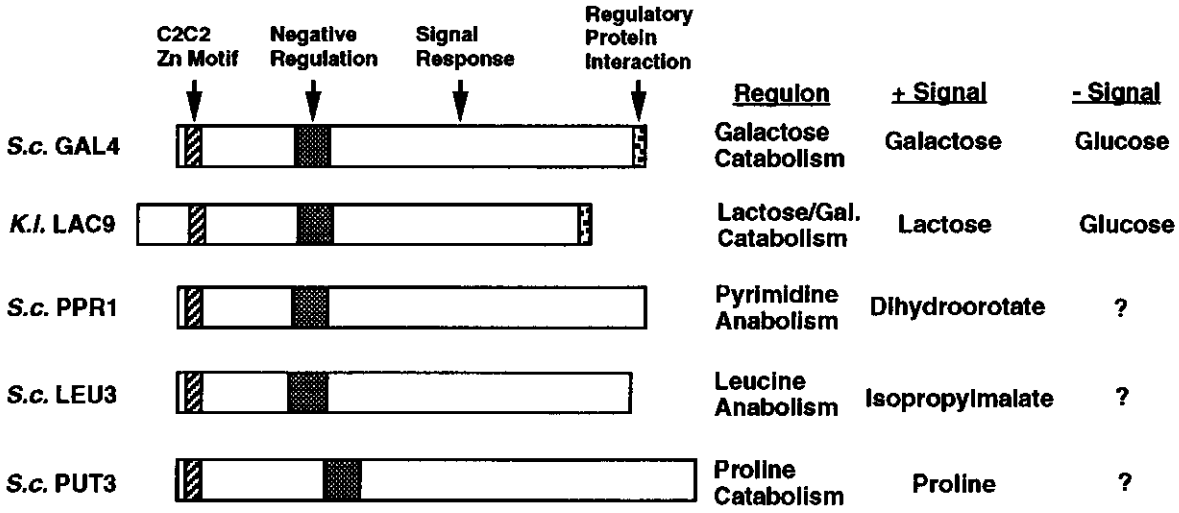
3. Immunoprecipitation of Derivatives and Phosphorylation Analysis

Immunoprecipitations of various derivatives were performed to determine if constructs were stable and to compare levels of expression (Figure 8). All derivatives are produced at similar levels, and molecular weight comparison indicates that the correct size protein is being produced. The absence of degradation products for most derivatives indicates that the derivatives are stable, even those derivatives with low activity. Derivatives 6-9 and 6-10 show degradation, but this is likely a consequence of the abnormally high expression of the protein.

Sadowski et al. (1991) have shown previously that GAL4 phosphorylations occur as a consequence of protein activity, and my results confirm this finding. Phosphorylated species, detectable through mobility shifting of the protein, are present as two bands on the wild type GAL4 protein. One of these sites is at residue 837 (Sadowski et al., 1991) and the other is yet to be characterized. These shifts,

Figure 7. A Family of Related Fungal Transcriptional Activator Proteins Share Homology Within the GAL4 Central Region

Shown are schematic representations of GAL4, and a group of GAL4-related proteins from *Saccharomyces cerevisiae* (*S.c.*), and *Kluyveromyces lactis* (*K.l.*), each of which share regions of homology with the zinc requiring DNA-binding motif (C2C2 Zn Motif) and ID1 of GAL4 (shaded). GAL4 and LAC9 also have homologous regions at their C-termini, which mediate interaction with a negative regulator (Regulatory Protein Interaction). Each of these proteins regulate transcription of a group of genes (Regulon), whose products catalyze a common anabolic or catabolic pathway. The metabolic precursors of these pathways are known to stimulate activity of the corresponding transcriptional activator proteins (+ Signal). Additionally, activity of GAL4 and LAC9 are inhibited by glucose (- Signal), a response that requires the central portion of GAL4 (Signal Response). Also shown is the amino acid sequence of the proteins within their region of negative regulation. All proteins show over 50 percent homology to a consensus sequence of the region. The consensus was derived from eight proteins, including those noted as well as PDR1, QUTA, and PET111.



Negative Regulation Region Homology

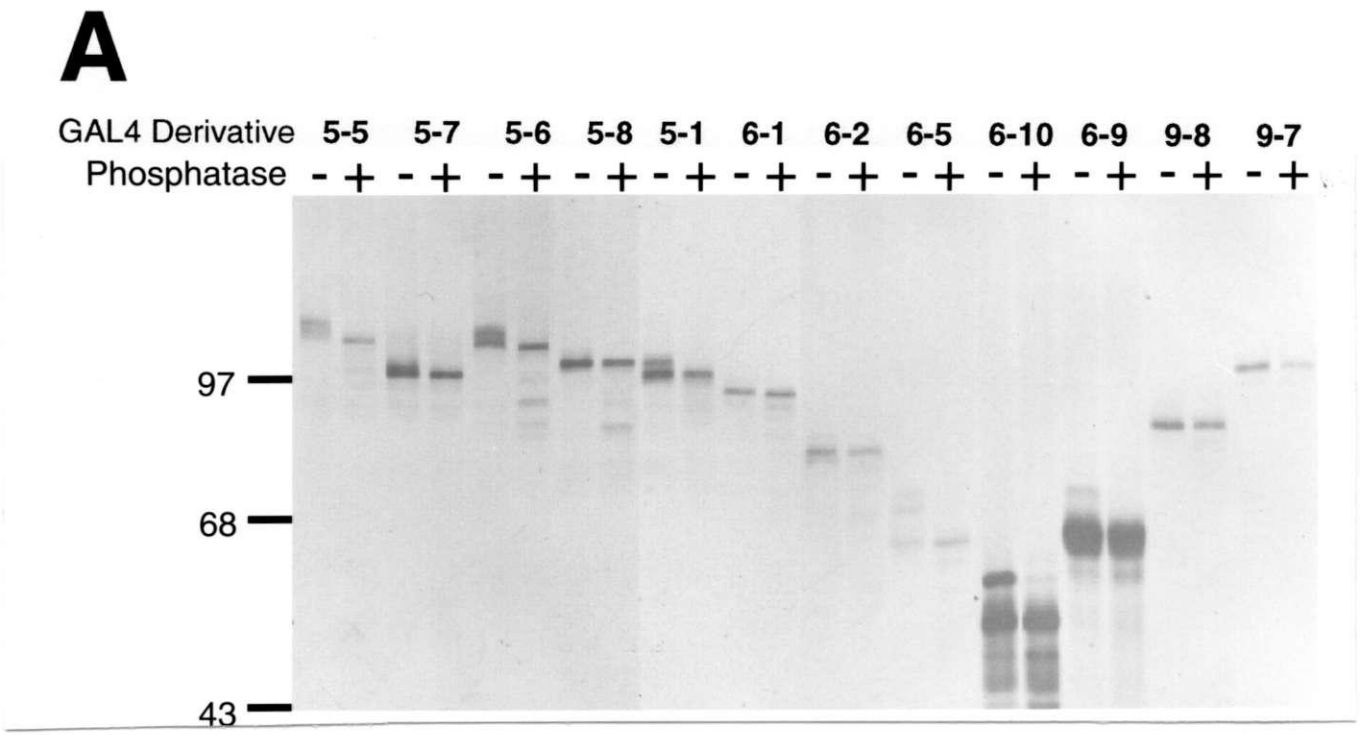
```
GAL4  ESGSIIL--V  TALHLLSRYT  QWRQKTN--T  SYNFHSFSIR  MAISLGLNRD  LPSSFSDSSI
LAC9  ETGSTDL--T  IALILLTHYV  QKMHKPN--T  AWSLIGLCSH  MAISLGLHRD  LPNSTIHD--
PPR1  FSSSDRLEAL  AGTLIMVIYS  IMRPNQP--G  VWYTMGSVLR  LTVDLGLHSE  KINKNYDAFT
LEU3  LNVA-SVYSV  QAFLLYTFWP  PLTSSLSADT  SWNTIGTAMF  QALRVGLNCA  GFSKEYASAN
PUT3  ENVTKKGGI-  EVLLLYAFFL  QVADYTL--A  SYFYFGQALR  TCLILGLHVD  SQSDTLSRYE
Cons. ESGS--L---  -ALLLL--Y-  Q--------T  SY---G-A-R  -AISLGLHRD  --S---D---

GAL4  LEQ---RRRI  WWSVYSWIEQ  LSLLYGRSIQ  L
LAC9  QQL---RRVL  WWTIYCTGCD  LSLETGRPSL  L
PPR1  REI---RRRL  FWCVYSLDRQ  ICSIFGRPFG  I
LEU3  SELVNEQIRT  WICCNVVSQT  VASSFGAPAY  V
PUT3  IEH---HRRL  WWTVYMFERM  LSSDAGLPLS  F
Cons. -E----RRRL  WW-VY--E--  LSSK-GRP--  -
```

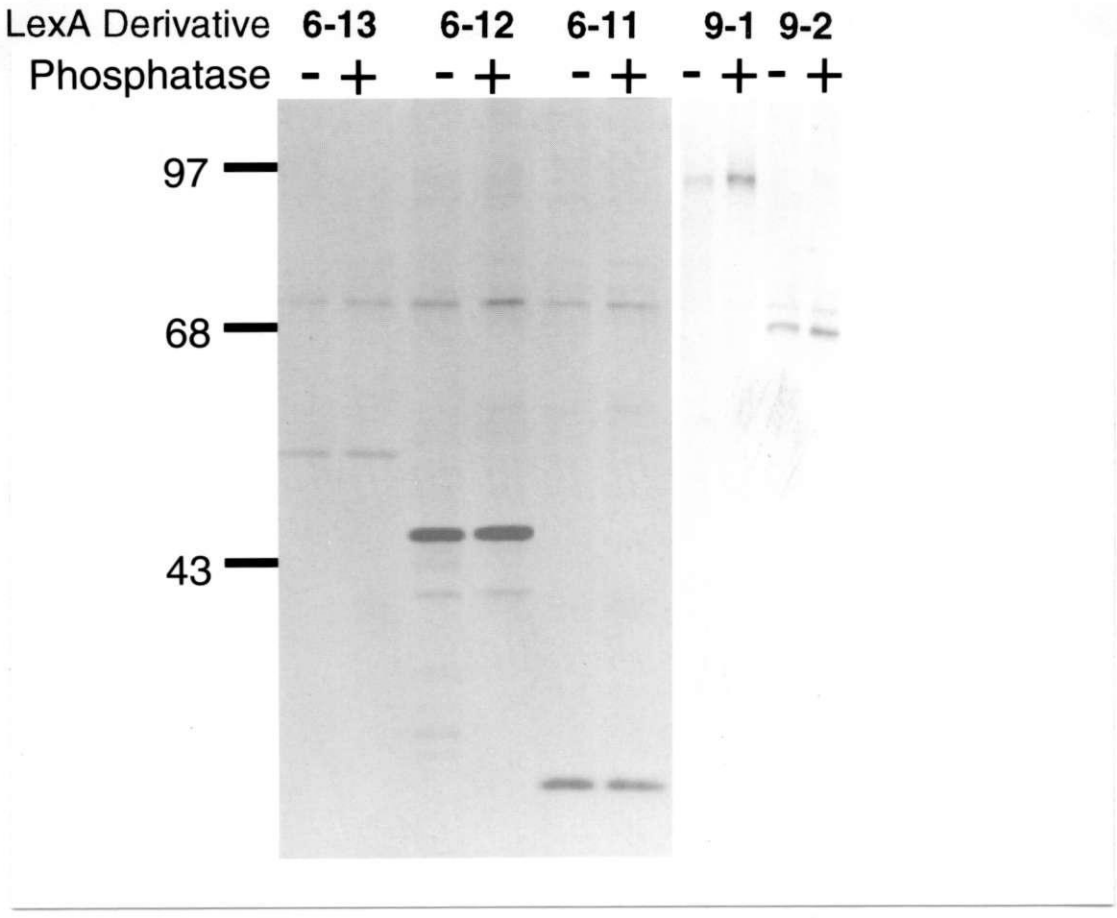
Figure 8. Immunoprecipitation of GAL4 and LexA Deletion/ Fusion Proteins

Yeast expressing the indicated derivatives, numbered as in Figures 5, 6, and 9, were grown to mid-log phase in medium containing glycerol, at which time glucose was added. Two hours later, the cells were harvested and labeled for 90 minutes with [^{35}S]methionine in met$^-$ minimal medium containing glucose. The yeast were lysed, and the proteins immunoprecipitated with specific antibodies. Proteins were either untreated (-), or treated with phosphatase (+), prior to analysis on 10% SDS-polyacrylamide gels.

A) GAL4 proteins produced in yeast strain YT6::171 were immunoprecipitated with rabbit polyclonal antibodies produced against GAL4(1-147).

B) LexA proteins produced in yeast strain YT6::6LexOp were immunoprecipitated with rabbit polyclonal antibodies raised against LexA.


A
GAL4 Derivative 5-5 5-7 5-6 5-8 5-1 6-1 6-2 6-5 6-10 6-9 9-8 9-7
Phosphatase - + - + - + - + - + - + - + - + - + - + - +
97
68
43


B
LexA Derivative 6-13 6-12 6-11 9-1 9-2
Phosphatase - + - + - + - + - +
97
68
43

however, are absent from my inactive derivatives (Figure 8a); Derivatives 6-1, 6-2 and 6-3 are inactive and show no phosphorylated species while derivatives 6-4 and 6-5, both containing the GAL4 residues present in 6-3, etc., are active and show two distinct phosphorylated species. 6-9 and 6-10, both inactive, do show low levels of phosphorylation, but this is likely a consequence of over expression.

4. The Central Region is Required for Direct Repression of GAL4 by Glucose

a. Glucose Repression of GAL4 Deletion Mutants

To determine if inhibition mediated by the central region is glucose dependent, derivatives in Figure 5 were also assayed in glycerol. Levels of activity in glycerol could then be compared to that of the construct in glucose to determine the fold glucose repression of activation (Table 2). In all cases, the presence of the central region increased the amount of glucose repression, when compared to derivatives lacking CR. For instance, AR1 fused to both the DNA binding domain and the central region is repressed at least 82 fold, while removal of the central region results in only 17 fold repression. Similarly, glucose repression for AR2 falls from 33 fold to 6 fold with the removal of CR. A similar result is seen for the VP16 containing derivatives, whether CR is fused to the C-terminus or N-terminus of VP16 (Derivatives 5-7 and 5-8; Table 2).

b. Inhibition of Wild Type GAL4 by Glucose Also Occurs in the Absence of Other Known Glucose Repression Mechanisms

While the results summarized in Table 2 support the theory that CR is involved in glucose repression, I wanted to distinguish CR mediated repression from glucose repression mediated by URSg, and to assay GAL4 at a physiologically relevant concentration. To accomplish this, GAL4 derivatives were assayed in a *gal4 -gal80-* strain containing a URSg-less reporter gene (SV15 *Gal1-LacZ*; as noted in Table 1) with expression of GAL4 from the repressible *PHO5* promoter. This

Table 2. The Central Region Potentiates Repression of GAL4 Derivatives in Glucose

	Fold Repression in Glucose[a]	
GAL4(1-147) fused to:	- CR (Derivative[b])	+ CR (Derivative[b])
GAL4 AR1	17 (5-4)	82 (5-2)
GAL4 AR2	6 (5-3)	33 (5-1)
VP16 (aa 411-488)	0.5 [c] (5-9)	15 (5-7)
VP16 (aa 411-488)	0.5 [c] (5-9)	5 (5-8)

Yeast strain YT6::171 bearing a GAL1-lacZ reporter gene and GAL4 expression plasmids (shown above) were grown in either glycerol or glucose. Cells were harvested and assayed for ß-galactosidase activity.

[a] Fold repression represents the ratio of ß-galactosidase activity obtained from the glycerol grown samples and the glucose grown samples.

[b] Schematic diagrams for each GAL4 derivative are shown in Figure 5.

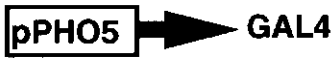
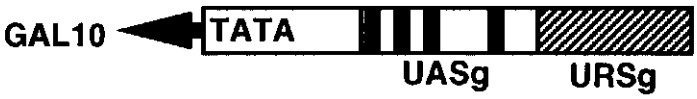
[c] The standard error for derivative 5-9 is higher (up to 100 percent) because production of this protein severely reduces the rate of cell growth. When assayed on minimal media plates containing X-Gal, activity in glucose and glycerol are equivalent.

system allowed me to look only at glucose repression from the GAL4 central region, with physiologically relevant levels of GAL4 (Lamphier and Ptashne, 1992). When wild type GAL4 is expressed in this system, glucose inhibition is 1.6 fold, while a derivative deleted for CR (GAL4ΔCR) is not inhibited by glucose (0.8 fold repression). Equivalent values have been obtained by other researchers (Lamphier, M., personal communication). This suggests that glucose inhibition of GAL4 requires the central region.

5. Nuclear Run On Assay of GAL4 Inhibition by Glucose

While GAL4 is directly inhibited by glucose, this inhibition is weak, suggesting the central region may not have a strong effect on long term growth in glucose. To examine short term inhibition of GAL4 activity by glucose, I assayed mRNA levels in a *gal4 - gal80 -* yeast strain expressing derivatives from a *PHO5* promoter. Assays were performed on either an SV15 *Gal1-LacZ* reporter or the endogenous *GAL10* gene, grown in glycerol and after 30 minutes and 4 hours of exposure to glucose. Nuclear run-on experiments involve the [^{32}P] radio-labeling of total cellular RNA followed by hybridization of this RNA with gene specific probes spotted on nitrocellulose. Radiation levels for each spot indicate the relative amount of a particular RNA being produced in the cell. Table 3 shows that *LacZ* transcription is inhibited 7 fold following 30 minutes of exposure to glucose for yeast expressing wildtype GAL4, while GAL4ΔCR expressing yeast are not inhibited. Similarly, *GAL10* transcription is inhibited 45 fold after 30 minute exposure of cells to glucose when wild type GAL4 is present, but is inhibited only two fold when GAL4ΔCR is used as transcriptional activator. *LacZ* is inhibited four fold after four hour exposure to glucose when wild type GAL4 is present, but when GAL4ΔCR is present, *LacZ* is not inhibited. After four hour exposure to glucose, the *GAL10* gene is inhibited two fold when expressing wild type GAL4, but is uninhibited when expressing GAL4ΔCR. These results suggest that strong short term glucose regulation is taking place in the yeast cell, and it is dependent on CR.

Table 3. The Central Region Mediates Rapid Repression of GAL4 Activity in Response to Glucose Addition

Time Following Glucose Addition[a]	Fold Repression and Activity Levels of Nuclear Run-on Transcription Following Glucose Addition[b]			
				
	WT GAL4 (Glycerol: 177)		GAL4ΔCR (Glycerol: 39)	
SV15 GAL1-lacZ[c]	Repression	Activity	Repression	Activity
30 minutes	7.1	25	0.8	47
4 hours	4.3	41	1.2	33
				
GAL10[d]	WT GAL4 (Glycerol: 344)		GAL4ΔCR (Glycerol: 115)	
	Repression	Activity	Repression	Activity
30 minutes	45	7.6	2.0	57
4 hours	2.5	140	1.5	75

Yeast strain YT6::SV15 bearing a *GAL1-lacZ* fusion construct with a single consensus GAL4 DNA-binding site, lacking both the UASg and URSg (see above), were transformed with plasmids expressing GAL4 derivatives from the *PHO5* promoter (see above), and grown to mid-log phase in selective medium without glucose.

[a] One half of each culture received an addition of 2% glucose. Samples were harvested 30 minutes and 4 hours following glucose addition.

[b] Specific transcription was determined by hybridization of ^{32}P labeled nuclear run-on transcripts to DNA probes on nitrocellulose filters, and quantitated by scanning densitometry. Values represent a ratio of the result, normalized to *HIS3* transcription, for the sample without glucose and the sample to which glucose had been added.

[c] Nuclear run-on transcripts hybridized to a probe containing *lacZ* sequences (see diagram).

[d] Nuclear run-on transcripts hybridized to a probe containing *GAL10* sequences (see diagram).

6. The GAL4 Central Region can Confer Glucose Repression to a Heterologous Transcriptional Activator

A series of LexA-VP16 constructs fused to the central region were created to examine the glucose repression mechanism independently from the rest of the GAL4 protein. These constructs were assayed on a reporter gene (6LEX) where the UASg and URSg were removed and replaced by six LexA binding sites. All glucose repression for this system should be mediated by the GAL4 central region, as LexA and VP16 are not glucose regulated (see Figure 9a, 6-12). Figure 9a shows a variety of LexA-VP16 derivatives. Without CR, the LexA-VP16 derivative shows no glucose inhibition, rather a slight glucose induction likely a result of the ADH1 promoter (6-12). This confirms that our reporter system does not contain any latent glucose regulation mechanisms. The addition of CR, however, renders the system 39 fold inhibited in glucose (9-1). Removal of the C-terminus of CR and the retention of ID1, or of all three inhibitory domains, results in constitutive inhibition of the system (see 9-2 and 9-3). 9-2 shows moderate activation while derivative 9-3 shows very low levels of activation, occurring equally in glucose and glycerol. Amino acids 600 to 768 do not severely repress activation (9-4), though they are required (9-1) for the glucose repression mechanism. To ensure that the glucose regulation of 9-1 was not the result of a glucose repressible activation domain between residues 600 to 768, this region of GAL4 was fused to LexA in the absence of VP16. The derivative gave no significant levels of activation, suggesting it has no latent activation capabilities.

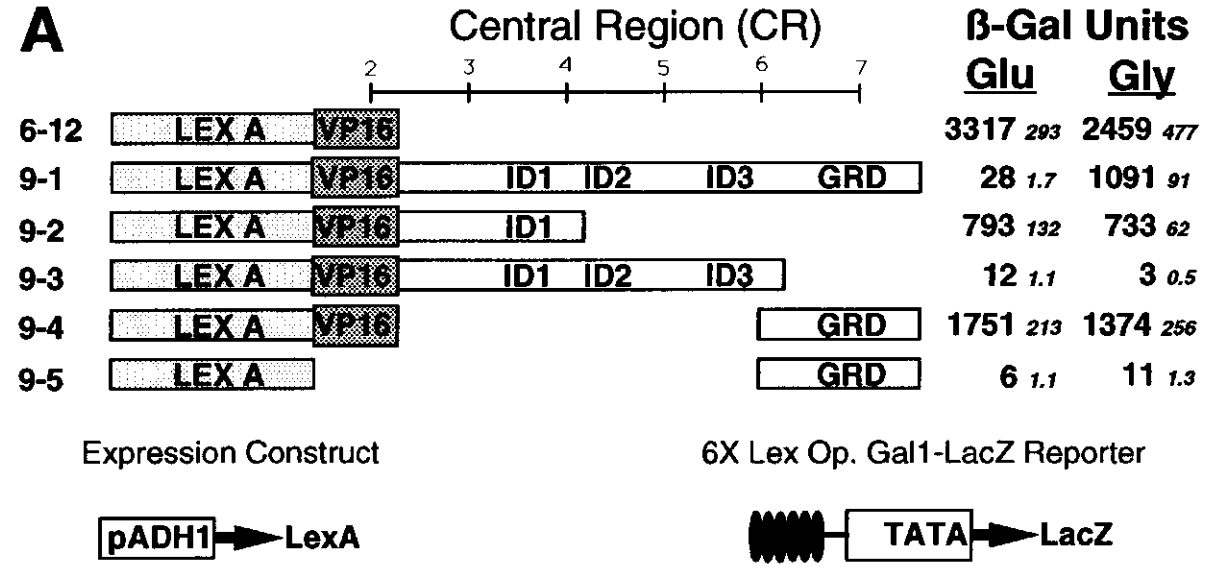
Overall, these experiments suggest that the GAL4 central region is capable of conferring glucose repression to a heterologous transcriptional activator. As both LexA and VP16 are not involved in any glucose response mechanisms, inhibition through the central region is the only available means of glucose repression. The absence of glucose repression for 6-12 indicates that there is no latent glucose repression occurring through the promoter of the reporter gene. I have termed

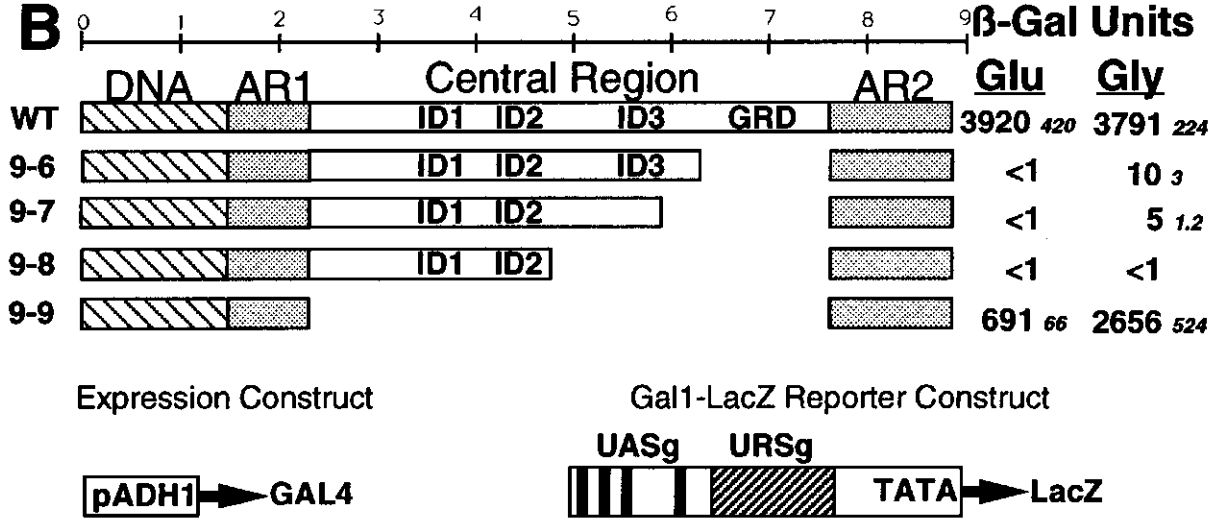
Figure 9. The GAL4 Central Region Mediates Glucose Repression

Activity of LexA and GAL4 fusion/ deletion derivatives was determined by measuring ß-galactosidase produced from a *GAL1-lacZ* reporter gene in yeast grown in either glucose (Glu) or glycerol (Gly). All standard deviations are below 20 percent (shown in italics).

A) The GAL4 central region can confer glucose repression on a heterologous transcriptional activator. LexA derivatives were expressed from the *ADH1* promoter on a single copy plasmid vector (bottom, left) in yeast strain YT6::6LexOp, which has a *GAL1-lacZ* reporter gene in which both UAS_G and URS_G were deleted and replaced with 6 LexA operators (bottom, right).

B) Deletion of the glucose response domain (GRD) constitutively inactivates GAL4. GAL4 derivatives were expressed from the *ADH1* promoter (bottom, left) on a single copy plasmid vector in yeast strain YT6::171, bearing a *GAL1-lacZ* reporter gene containing both UAS_G and URS_G (bottom, right).


A
Central Region (CR)
2 3 4 5 6 7
ß-Gal Units
Glu Gly
6-12 LEX A VP16 3317 293 2459 477
9-1 LEX A VP16 ID1 ID2 ID3 GRD 28 1.7 1091 91
9-2 LEX A VP16 ID1 793 132 733 62
9-3 LEX A VP16 ID1 ID2 ID3 12 1.1 3 0.5
9-4 LEX A VP16 GRD 1751 213 1374 256
9-5 LEX A GRD 6 1.1 11 1.3
Expression Construct
pADH1 LexA
6X Lex Op. Gal1-LacZ Reporter
TATA LacZ


B
0 1 2 3 4 5 6 7 8 9
ß-Gal Units
DNA AR1 Central Region AR2
Glu Gly
WT ID1 ID2 ID3 GRD 3920 420 3791 224
9-6 ID1 ID2 ID3 <1 10 3
9-7 ID1 ID2 <1 5 1.2
9-8 ID1 ID2 <1 <1
9-9 691 66 2656 524
Expression Construct
pADH1 GAL4
Gal1-LacZ Reporter Construct
UASg URSg
TATA LacZ

residues 600-768 a "glucose response domain" (GRD) because its presence is necessary for glucose response to occur.

7. The GRD is Essential for Activity of GAL4 when the Inhibitory Domains are Present.

Figure 9b indicates that the GRD must be present for GAL4 to activate transcription in the presence of the inhibitory domains. These internal deletion derivatives were expressed from the *ADH1* promoter and assayed on the *GAL1-LacZ* reporter gene containing the UASg and URSg (Figure 9b bottom). Derivative 9-6 shows that deletion of GRD results in almost complete loss of activity. Even in the presence of two activating regions, ID1 and ID2 are able to severely repress GAL4 activity, although high levels of activation are restored when the full central region is deleted (9-9). These results are consistent with the findings of Figure 9a: the glucose response domain is necessary for the repression of the inhibitory domains, an event which occurs only in the absence of glucose. As well, derivative 9-9 (GAL4ΔCR), shows glucose repression in this system, whereas glucose repression was absent when the derivative was assayed on a URSg-less reporter. These results indicate that repression by URSg is independent of the central region, and likely occurs through a distinct pathway.

8. Glucose Mediated Inhibition of LexA-VP16-CR is Accompanied by Loss of DNA Binding

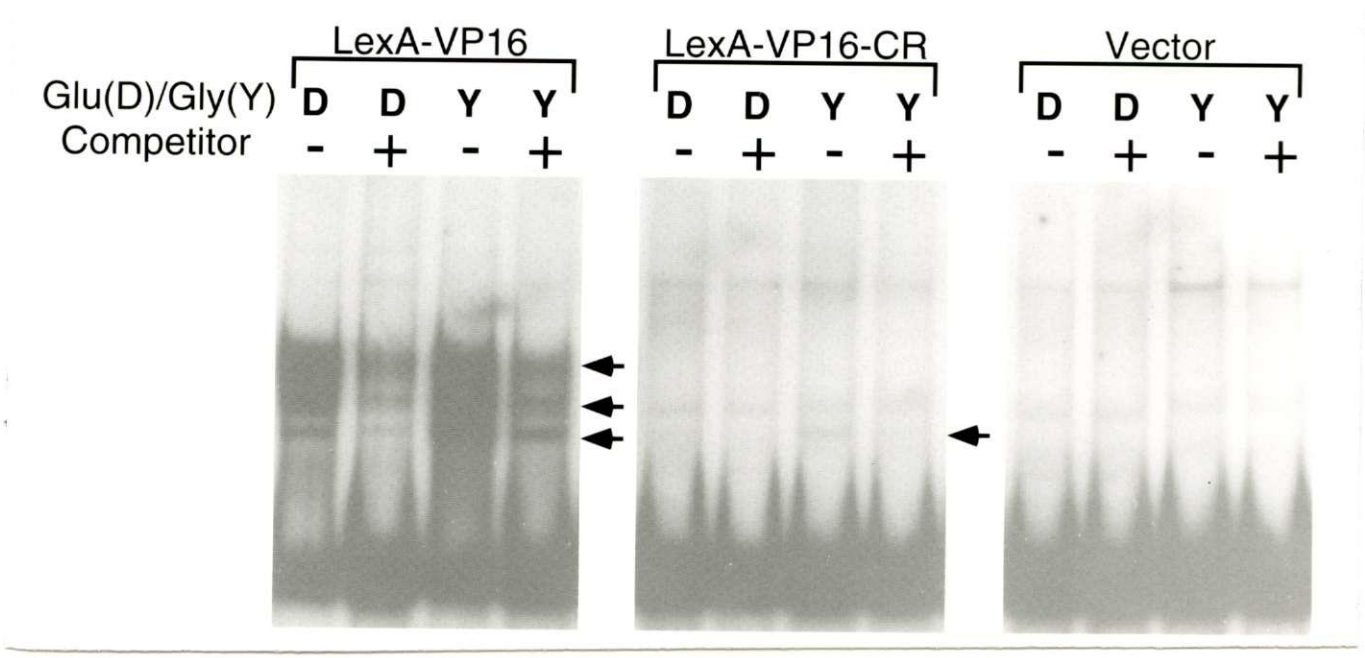
Glucose inhibition of the LexA-VP16 derivatives could be via one of two mechanisms. Either activation is impaired by masking or disrupting the VP16 activation domain, or DNA binding via LexA is disrupted. To test whether the central region regulates DNA binding, I performed a series of *in vitro* DNA binding experiments with LexA-VP16 derivatives. Figure 10a shows that LexA-VP16 expressed from yeast grown in either glucose (D) or glycerol (Y) is capable of binding a [^{32}P]-labeled LexA operator site. Three bands are clearly visible,

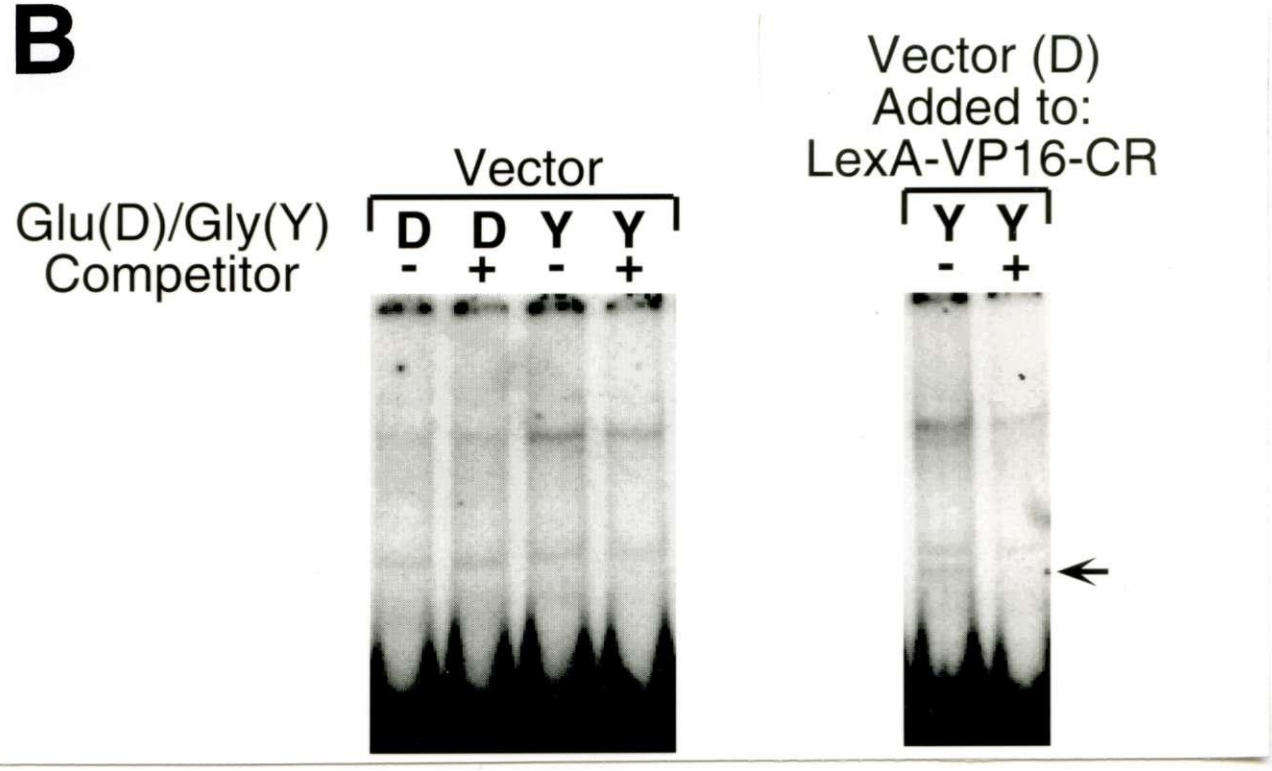
Figure 10. LexA Derivatives Inhibited by the Central Region do not Bind DNA

Yeast strain YT6::6LexOp bearing plasmids expressing derivative 6-12 (LexA-VP16), derivative 9-1 (LexA-VP16-CR), or plasmid YCplac22 (vector) were grown to mid-log phase in either glucose (D) or glycerol (Y).

A) DNA-binding of LexA derivative proteins to a labeled LexA operator was measured in crude extracts. The binding reactions were performed in the absence (- Competitor) and presence (+ Competitor) of excess unlabeled LexA operator. Migration of the bands indicated with arrows is retarded when antibodies against VP16 are included in the binding reaction (not shown).

B) Reactions were performed as in (A), except crude extracts of 9-1 (cells grown in glycerol) and YCplac22 (cells grown in glucose) were mixed and incubated 15 minutes at room temperature, followed by addition of the binding reaction mix.


A
LexA-VP16
LexA-VP16-CR
Vector
Glu(D)/Gly(Y)
Competitor
D D Y Y
- + - +


B
Vector
Glu(D)/Gly(Y)
Competitor
D D Y Y
- + - +
Vector (D)
Added to:
LexA-VP16-CR
Y Y
- +

corresponding to a number of protein-DNA complexes, all of which can be partially competed off with excess cold operator DNA (+ competitor), or shifted with LexA specific antibodies (data not shown). Other bands visible on the gel are also seen when a negative control (YCPLac22) is used, and are likely the result of non-specific binding. When 9-1 is assayed for mobility shifting, however, I only see one band specific to the protein, corresponding to a single DNA-Protein complex. This band is only seen when the derivative is grown in glycerol, despite the fact that 9-1 is produced at similar levels in both carbon sources (as seen by western blotting). The specific complex in glycerol can also be competed with cold oligo (+ competitor) and shifted with LexA antibodies (not shown). This suggests that growth in glucose is affecting DNA binding of 9-1.

If glucose inhibition is occurring, and is inhibiting DNA binding, it may be the result of a factor which reversibly binds or otherwise modifies the central region. If this is the case, incubation of a glucose grown yeast extract with extract from glycerol grown yeast expressing 9-1 may result in a loss of DNA binding. To test this, I incubated the 9-1 glycerol extract with the YCPLac22 glucose extract for 15 minutes prior to incubation with radiolabeled LexA operator. As Figure 10b shows, DNA binding is still occurring after this incubation, suggesting that modification for glucose inhibition either occurs during GAL4 translation (a hypothesis inconsistent with my nuclear run-on result that repression occurs within 30 minutes) or more likely requires a metabolite that has been diluted from the yeast extract.

Gel mobility shift assays were also attempted with GAL4 deletion mutants. Unfortunately, no bands unique to these derivatives were seen on gels when compared to control lanes. While *In vitro* mobility shifting of GAL4 has been reported, control mobility shifts without GAL4 protein were never attempted (Corton and Johnston, 1989). Comparing their putative mobility shifts with my own results, I surmise that the shifts they observe are the result of non-specific binding.

9. Titration of a GAL4 Inhibiting Factor

If a factor is required for the glucose inhibition of GAL4, it should be titratable. To test this, a multicopy plasmid transcribing the construct GG19 (Gill and Ptashne, 1988; Figure 11a) was co-transformed into YT6::171 with a series of CR containing constructs expressed from the ARS-CEN single copy plasmid. Plasmid pGG19 (expressing GAL4 residues 74-881) produces a construct unable to bind to DNA or activate transcription, but is able to bind any factors that might be interacting with the CR containing constructs. GG19 was titrated against glucose inhibited derivatives containing the full central region, ID1, or the central region to residue 412. The results indicate that full length CR is capable of titrating out a factor that prevents GAL4 activity in the presence of glucose, but deletion of amino acids 479-767 results in no titration. Only the construct containing the full central region shows greater activity in the presence of GG19 than under control conditions.

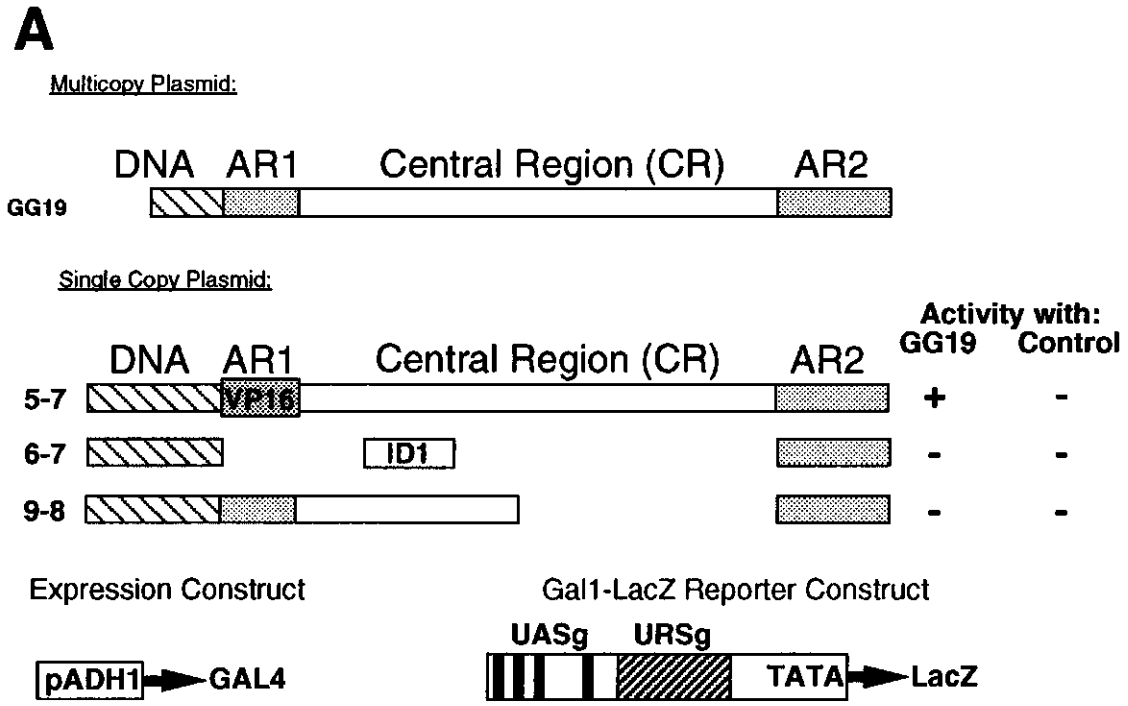
Another series of deletions implicates residues 693 to 767 in titration of a factor. Data summarized in figure 11b shows further titrations of construct 9-1 (expressed from a single copy plasmid) with multicopy expression of a nested set of C-terminal GAL4 deletions (Ma and Ptashne, 1987b). While full length GAL4 and 5-2 are able to titrate a factor in glucose, Δ692 and Δ677 are unable to titrate, suggesting residues between 693 and 767 are required for interaction of GAL4 with a glucose responsive factor.

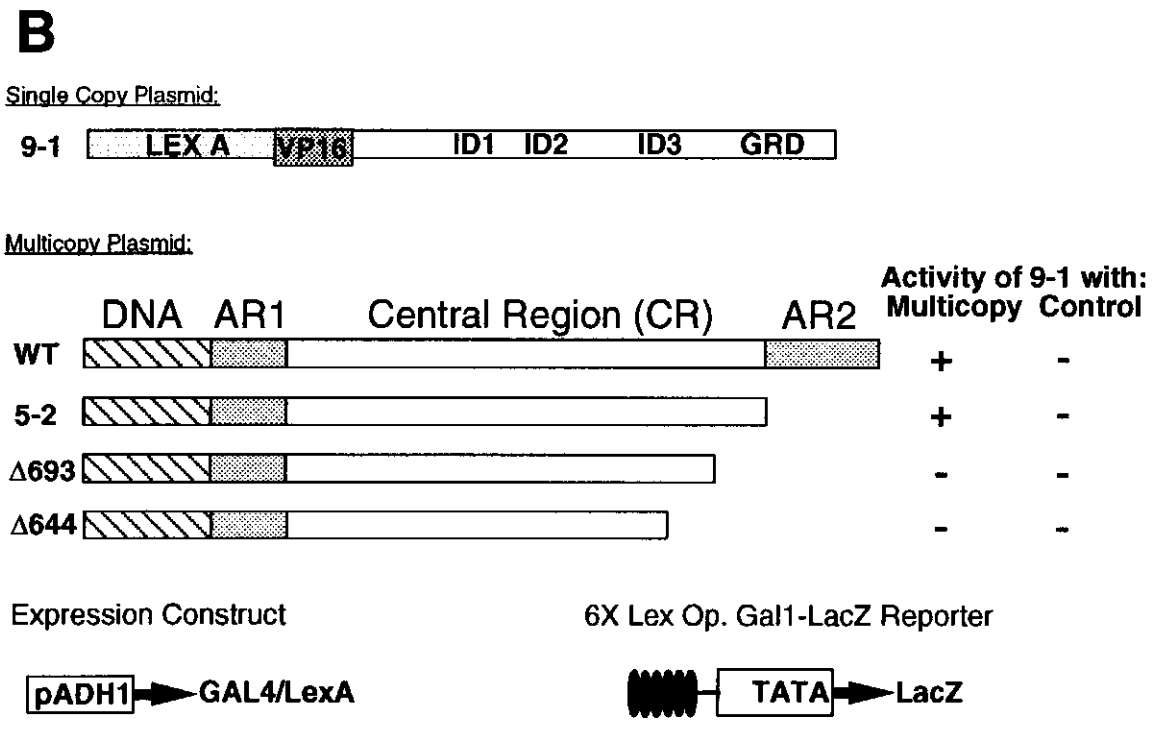
Figure 11. The Glucose Response Domain (GRD) Binds a Titratable Factor which Renders a Heterologous Glucose Inhibited Protein Active in Glucose.

Activity of heterologous proteins was determined by noting the colour change of yeast colonies on X-gal laced selective media plates containing 2% glucose. Yeast were co-transformed with a multicopy and a single copy plasmid expressing derivatives as indicated. Control assays were yeast co-transformed with the construct indicated and a control vector not expressing protein.

A) A GAL4 mutant unable to bind DNA contained on a multicopy 2μ plasmid was co-transformed with one of three GAL4 derivatives contained on ARS-CEN single copy plasmids. Derivatives are expressed from the *ADH1* promoter (bottom, left) in the yeast strain YT6::171, bearing a *GAL1-LacZ* reporter gene containing both UASg and URSg (bottom, right).

B) A LexA heterologous protein on a single copy ARS-CEN plasmid was co-transformed with one of four GAL4 deletion mutants contained on multicopy 2μ plasmids. Derivatives were expressed from the *ADH1* promoter (bottom left) in the yeast strain YT6::6LexOp, bearing a *GAL1-LacZ* fusion reporter gene in which both the UASg and URSg were deleted and replaced with 6 LexA operators (bottom, right).


A
Multicopy Plasmid:
DNA AR1 Central Region (CR) AR2
GG19
Single Copy Plasmid:
Activity with:
GG19 Control
DNA AR1 Central Region (CR) AR2
5-7 VP16 + -
6-7 ID1 - -
9-8 - -
Expression Construct
pADH1 GAL4
Gal1-LacZ Reporter Construct
UASg URSg
TATA LacZ


B
Single Copy Plasmid:
9-1 LEX A VP16 ID1 ID2 ID3 GRD
Multicopy Plasmid:
Activity of 9-1 with:
Multicopy Control
DNA AR1 Central Region (CR) AR2
WT + -
5-2 + -
Δ693 - -
Δ644 - -
Expression Construct
pADH1 GAL4/LexA
6X Lex Op. Gal1-LacZ Reporter
TATA LacZ

Discussion

1. Inhibition by the Central Region

My data summarized in Figure 5 shows that the central region is capable of inhibiting GAL4 activity. Deletion of either activating region results in a large decrease in activity for constructs containing either a GAL4 activation region or the VP16 activation region. Subsequent deletion of the central region results in the return to moderate levels of activity. This data indicates that the central region may be playing a role in the regulation of GAL4 activity, a role which is revealed by the deletion of one activation region.

2. Immunoprecipitations and Phosphorylations

Immunoprecipitations of the proteins produced by various deletions derivatives indicate that all proteins are being produced at comparable levels, whether or not they are capable of activating transcription. The stability of these proteins, indicated by the absence of degradation products, suggests that they are not denatured within the cell to such an extent that it would affect activation by AR1, AR2, or VP16.

My results are consistent with the model that GAL4 must be bound to the UASg and in the process of transcriptional activation before phosphorylation occurs. This result is especially striking when immunoprecipitations of 6-3, 6-4, and 6-5 are compared. All three constructs contain at least two phosphorylation sites, as shown by mobility shifts of the constructs 6-4 and 6-5. Yet construct 6-3, which is inactive, shows no mobility shift despite the fact that the protein appears to be stable, contains all the sequences of 6-4 and 6-5, and is produced at similar levels to 6-4 and 6-5 in the cell. Previous work has shown that one phosphorylation site is at Serine 837 (Sadowski et al., 1991) and I have determined by immunoprecipitation that another site lies between residues 683 and 768 (data not shown). As the one characterized

phosphorylation site, Serine 837, has structural similarity to the RNA polymerase "tail" (a series of repeated amino acid motifs that are phosphorylated when RNA polymerase becomes activated) it is possible that the same kinase acts on both proteins. In the case of RNA polymerase II, a long stretch of Pro-Thr-Ser-Pro-Ser-Tyr-Ser is thought to extend from the C-terminal of the protein, acting as a substrate for a specific kinase which phosphorylates RNA polymerase when it is in the process of initiating transcription.

3. Glucose Repression Via the Central Region

Inhibition of GAL4 by its central region appears to be involved in GAL4 glucose regulation. In all GAL4 derivatives, the presence of the central region contributes from 5 to 20 fold more glucose repression. This data supports the working hypothesis that the central region is involved in glucose mediated inhibition of GAL4 activity, whereas the inhibitory domains act only in the presence of glucose. Construct 5-10 indicates that in addition, the central region is not capable in and of itself of activating transcription, showing that, in terms of glucose regulation, CR is not acting as a glucose repressed activation domain but rather must act on other domains of GAL4 for glucose repression to occur. The absence of glucose repression in cells expressing wildtype GAL4 from *ADH1* is, as has been mentioned previously, a factor of its abnormally high concentration when expressed from the *ADH1* promoter.

When expressed at physiologically relevant concentrations, wildtype GAL4 activity is also inhibited after long term growth in glucose, independent of the URSg and GAL80 mechanisms. This inhibition is dependent on the presence of the central region, consistent with the model that the central region mediates this glucose response. While the effect is small, it does not preclude the idea that more glucose inhibition occurs in the short term response to glucose. Why are deletion derivatives of GAL4 glucose inhibited, even when expressed at high levels? For construct 5-1,

the inhibitory domains have been positioned closer to the DNA binding domain, possibly resulting in less DNA binding. For construct 5-2 and 9-1, the activation domains are internal, and thus may be less accessible to general initiation factors.

4. Nuclear Run On Assay of Glucose Repression

My nuclear run-on results demonstrate that the central region of GAL4 is capable of repressing GAL4 activity at significant levels in the short term. This repression, which occurs thirty minutes after glucose addition, can be eliminated by the deletion of the central region. Because these results are independent of GAL80 and URSg repression of both *GAL4* and *GAL1* transcription, they provide conclusive evidence that the GAL4 protein, in and of itself, is involved in glucose repression of activation. GAL4ΔCR results prove that this repression requires the presence of CR.

The presence of high levels of glucose repression within 30 minutes is also consistent with direct repression of *GAL* genes by CR. URSg and GAL80 mediated glucose repression requires the down-regulation of *GAL4*, *GAL2* and *GAL3* mRNA. Since the half-life of *GAL4* mRNA and protein is known to be more than thirty minutes, also likely to be the case for *GAL2* and *GAL3* mRNA, the affect of URSg and GAL80 mediated repression would not be seen within the thirty minute time span. URSg and GAL80 are more likely to act after four hours of glucose induction, so that if the endogenous GAL4 promoter and GAL80 were used in my nuclear run on experiment, GAL4 and GAL4ΔCR would both have the expected 100 fold repression.

After four hours, glucose inhibition mediated by the central region decreases markedly, especially for *GAL10* transcription. The reason is for this decrease is unclear, but may relate to cooperative binding and to changes in the cell environment after long term growth in glucose. For long term glucose repression, the cell may rely on the URSg and GAL80 pathways to maintain *GAL* gene repression.

5. Characterization of the Inhibitory and Glucose Response Domains

GAL4's ability to respond to glucose has been shown to require the inhibitory domains and the glucose response domain. The inhibitory domains consist of at least three segments between residues 238 and 600 (ID1, ID2, and ID3). These have been shown to constitutively inhibit activity of both GAL4 and heterologous transcriptional activators. The best characterized of the inhibitory domains are ID1 and ID2. Unfortunately, only weak (around 12%) identity can be found when comparing the sequences of the inhibitory domains. Perhaps, as is the case for the Glucocorticoid Receptor HSP90 binding domains (Binart et al., 1989), a particular spacing of charged residues acts as a target within the inhibitory domains, while the rest of the HSP90 binding sequence contains little identity. Alternatively, the ID regions may independently repress GAL4 activity, perhaps as a function of their tertiary structure, a structure which is masked and unmasked by the GRD.

Constitutive repression by the inhibitory domains is overcome by the addition of the glucose response domain. The glucose response domain does not itself activate or severely inhibit transcription. Analyzing these results, I have concluded that the GRD is the regulator of the GAL4 inhibitory domains. The mechanism of this regulation still must be determined, perhaps through characterization of GRD and inhibitory domain mutants. As well, constructs 9-6, 9-7 and 9-8 illustrate that the GRD response also takes place on the GAL4 protein in the presence of endogenous DNA binding, dimerization and activation domains.

6. Glucose Regulation of DNA Binding

Gel-retardation assays of LexA constructs provide evidence that the glucose regulated inhibition of GAL4 affects DNA binding. The multiple bands for LexA-VP16 gel retardation are likely a consequence of complexes of LexA-VP16, either with itself, or with other proteins. For instance, because VP16 is such a strong activator, the yeast general initiation factors may form stable complexes with it

during the gel retardation. Only a single band is seen for LexA-VP16-CR, perhaps due to slight masking of VP16 activity by the CR, evident in the more than two fold decrease in activity between non glucose repressed LexA-VP16-CR and LexA-VP16. This band is much weaker than the band seen for LexA-VP16, and there are a number of explanations for this discrepancy. Firstly, assays of activity were performed on a set of six LexA binding sites. It is known that GAL4 binds cooperatively to DNA, and that the segment of GAL4 responsible for cooperative binding is outside region 1-147 and 767-847. Therefore a cooperativity site may exist on CR, and may play a key role in the glucose regulation mechanism along with the DNA and dimerization domains. If this is the case, LexA-VP16-CR would bind cooperatively to the six LexA operator sites, accentuating it's activity in glycerol. Even if LexA-VP16-CR binds very weakly to a single LexA site (possibly due to residual ID inhibition even in glycerol) cooperative binding to six sites would alleviate this affect. When assayed with only a single LexA site in the gel retardation assay, however, binding would be much weaker, especially as the LexA site used is not a consensus binding site. Therefore, weak binding would be observed for the gel retardation despite the ability of 9-1 to activate strongly from six LexA binding sites.

7. Titrations

Titration experiments have implicated GAL4 residues 693 to 767 in the binding of a glucose responsive factor. Titration of a construct whose activity is glucose repressed with another GAL4 construct containing residues 693 to 767 resulted in a loss of glucose repression, while titration with a GAL4 construct containing residues to 693 did not titrate out a glucose repression factor. Therefore 693 to 767 may bind a glucose responsive factor, which then results in inhibition of GAL4 activation. Such a factor may be a small molecule or a protein. If the inhibitory domains do not require a factor to inhibit GAL4, but rather act independently, the role of the GRD may be to sterically hinder the ID region in the

absence of glucose. In the presence of glucose, a factor may bind region 693 to 767, unmasking the ID region and resulting in inhibition of transcriptional activation.

8. Model of Glucose Regulation

Having considered the experimental evidence, I propose a model for the direct glucose regulation of GAL4. This model involves the interaction of the glucose response and inhibitory domains in repressing the UASg binding of the GAL4 protein (Figure 12). In the presence of glucose, the inhibitory domains are free from down-regulation by GRD and repress the GAL4 DNA binding or dimerization domains, resulting in loss of GAL4 activity. In the absence of glucose, the glucose response domain is able to mask the inhibitory domains, resulting in the binding of GAL4 to the UASg and subsequent transcriptional activation. In the presence of glucose, it is likely some factor inhibits the interaction of GRD and ID. It has been shown that GAL4 inhibitory domains function in mammalian cells (Bell, Stone and Sadowski, unpublished), where expression of 5-1 and 5-8 are repressed in the glucose media used to culture these cells. This suggests that a factor mediating inhibition would be highly conserved amongst eukaryotes. A factor may also be involved in interaction of the ID region and its target. When 6-7 is expressed in *E. coli*, it is capable of binding a GAL4 binding site *in vitro* (Kang and Sadowski, unpublished). This suggests that ID is unable to repress DNA binding when a construct is not expressed in a eukaryotic cell, possibly because a particular factor is not produced in prokaryotes. As well, it is possible that phosphorylations or other protein modifications in ID could be required for ID region activity.

There are a number of further observations that are consistent with my model of glucose regulation. The results of Gineger et al. (1985) which show that the *GAL1* UASg is devoid of GAL4 in the presence of glucose, though complicated by the parallel down-regulation of *GAL4* transcription, does suggest DNA binding as one target of the glucose response mechanism. Also, several point mutations have been found within ID1 which severely repress the activity of full length GAL4 (Johnston

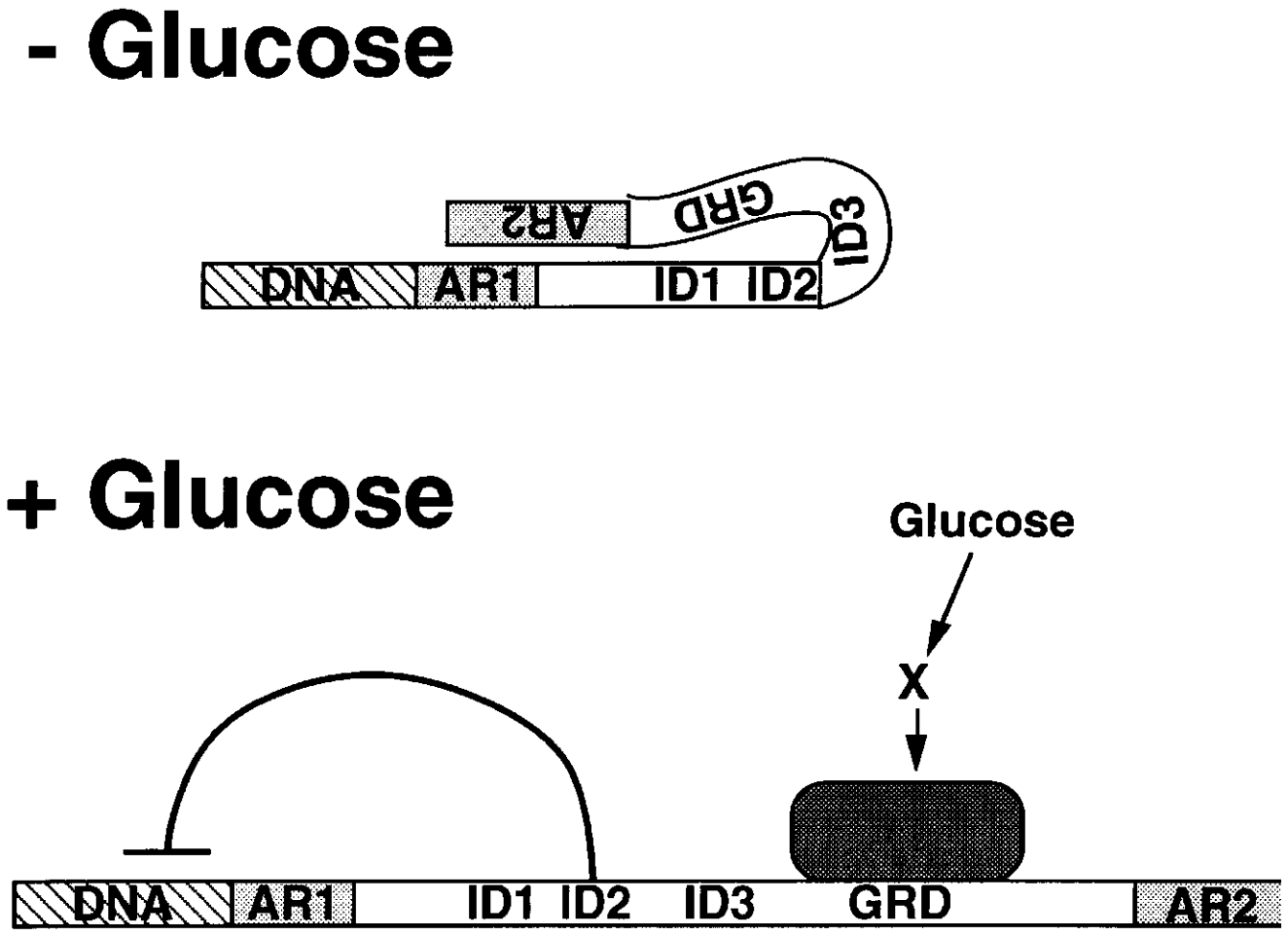


Figure 12. A Model for Glucose Repression by the GAL4 Central Region

In the absence of glucose (- Glucose), the glucose response domain (GRD) antagonizes the function of the inhibitory domains (ID1, ID2, and ID3). Upon addition of glucose (+ Glucose), a metabolite or a signal (X) is generated which causes inhibition of the glucose response domain by a factor (shaded); this allows free access of the inhibitory domains with the DNA binding domain. Interaction with the ID region decreases the affinity of DNA binding domain for its binding sites on DNA, perhaps through steric hindrance or by preventing the formation of dimers.

and Dover, 1988). These mutations may be the result of ID1 becoming a potentiated repressor, able to repress DNA binding of GAL4 even in the presence of GRD. These point mutations of ID1 could therefore be used to characterize the position of GRD potentiating mutants.

The observation that GAL4 binds cooperatively to the UASg provides a number of explanations for my experimental results. That glucose repression can be overcome by increases in *GAL4* transcription is possibly due to passing the saturation point for cooperativity, so that glucose regulation by CR would no longer have a strong effect. GAL4 would be able to bind to all four sites on the UASg without the aid of a cooperativity domain.

How might the presence of glucose induce the GAL4 inhibitory domain response? While it is possible that glucose could interact directly with GAL4, genetic studies (Flick and Johnston, 1990) suggest the possibility that direct repression of GAL4 is mediated by a series of gene products. Flick and Johnston note that some glucose response mutants of yeast are involved in the pathway of URSg regulation, while other mutants must act through a second mechanism. If this second mechanism is the one I have characterized, a diagram of glucose regulation would be as in Figure 13. In this model, the genes SNF1, GAL83, REG1, GRR1, and SSN6 act together to form a signal "X" from glucose. This signal then affects the MIG1 and URR gene products, mediating the URSg response, or follows an alternate pathway with the gene products GAL82 and HXK2 to mediate direct GAL4 repression. Genetic studies are already underway to analyze the relationship of direct GAL4 repression and these glucose response genes. Another mechanism of glucose response is phosphorylation. Mylin et al. (1989) showed that when grown in glucose, GAL4 is de-phosphorylated. However, Sadowski et al. (1991) have shown that some GAL4 phosphorylation occurs as a consequence of activity, making it difficult to distinguish loss of phosphorylation due to glucose repression, and that caused by the inability of GAL4 to activate transcription. In general, my results

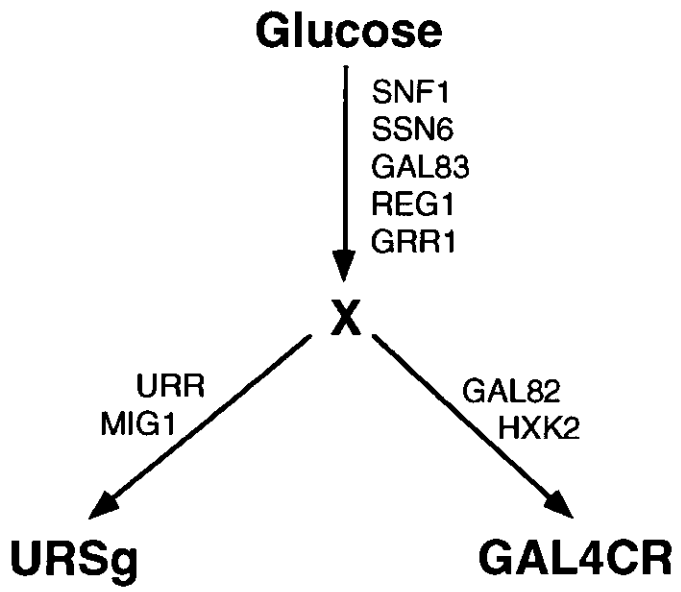


Figure 13. Yeast Genes Involved in the Glucose Repression of GAL4 Follow One of Two Pathways.

Glucose is assumed to create a derivative "X", involved in both glucose repression through URSg and that mediated by the GAL4 central region. A variety of yeast mutants have been implicated in either the creation of "X", the repression via URSg, or repression of a separate mechanism which may mediate central region inhibition.

suggest that only active derivatives are phosphorylated as observed by mobility shift on SDS-PAGE, and any glucose regulated phosphorylations are not observable. GAL4 homology to a variety of yeast transcriptional activators suggests that the model I am proposing may have general application to the transcriptional regulation of proteins required for various metabolic pathways. LEU3 (Zhou et al., 1987), PPR1 (Kammerer et al., 1984), and PUT3 (Brandiss and Marczak, 1991) of *S. cerevisiae*, and LAC9 (Salmeron and Johnston, 1986) of *Kluyveromyces lactis*, all contain within a large central portion of the protein a region of homology to ID1, and in addition are all involved in the regulation of a metabolic pathway as described in Figure 7. They are all stimulated by a specific metabolic precursor, and both GAL4 and LAC9 are also known to be repressed by the presence of glucose. My results suggest that ID1 is involved in negative regulation of DNA binding, and this may be the case for the other proteins as well. I constructed a GAL4 heterologous protein containing the GAL4 or LexA DNA binding domain and the activator VP16 fused to the LAC9 region of homology to ID1. In both instances, LAC9 was able to repress activity, consistent with the results of GAL4 ID1. Thus ID1 may be a common regulatory motif in transcriptional activators, regulating in response to a variety of signals. A region homologous to GRD is not seen in the other activator proteins, making it possible that they contain regions mediating regulation from more suitable signals, such as the over abundance of a metabolite they regulate.

9. Conclusion

The regulation of the *GAL* genes in *S. cerevisiae* is tightly controlled by the carbon sources available in the media. My research has shown that glucose regulation of the *GAL* genes involves direct repression of the *GAL* gene transcriptional activator, GAL4. This repression occurs rapidly, within 30 minutes, and involves the large central region of the protein. The N-terminal half of the central region contains a series of domains that mediate constitutive repression of GAL4 activity, possibly by affecting DNA binding. The C-terminal half of the

central region regulates this inhibitory response such that it only occurs when glucose is used as carbon source. This repression may be the earliest response in switching from galactose to glucose utilization. An early response to glucose would then provide a selective advantage to yeast living a "feast or famine" existence in the wild. Yeast able to metabolize glucose earlier could begin exponential growth while glucose is still available in the environment. While glucose metabolism genes are expressed constitutively, the prescence of *GAL* gene products may inhibit full utilization of glucose *in vivo*.

My research suggests a novel mechanism for regulation of transcriptional activation, providing a better understanding of eukaryotic gene regulation in the yeast system. These results can now be applied to higher eukaryotes, suggesting models for gene regulation in as yet uncharacterized systems. Presently, the glucocorticoid receptor remains the best characterized transcriptional activator which contains an inhibitory domain region. My results suggest that such a region may in fact be a common regulatory motif that was present before the evolutionary development of mammals.

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