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A Model Fungal Gene Regulatory Mechanism: the *GAL* Genes of *Saccharomyces Cerevisiae*

MARK JOHNSTON

Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

INTRODUCTION

Studies of gene regulation in fungi have contributed greatly to our understanding of mechanisms of eucaryotic gene expression. Because the yeast *Saccharomyces cerevisiae* has long been a favorite organism for genetic analysis, the regulatory circuits of a number of yeast genes have been well characterized. Among the most intensively studied and best understood genetic regulatory circuits in yeast cells is the one that acts on the *GAL* genes, which encode the enzymes of galactose utilization. I review here what is known about the mechanism of regulation of the *GAL* genes. I will first briefly present the current model of *GAL* gene regulation, followed by detailed descriptions of each component of the regulatory circuit. The *GAL* gene regulatory mechanism will then be compared with other fungal gene regulatory circuits, and I will suggest that knowledge of the details of the mechanism of *GAL* gene expression might help in understanding how genes are regulated in higher eucaryotes. This subject was last reviewed in 1982 (114).

GALACTOSE UTILIZATION PATHWAY AND ENZYMES

Galactose is utilized by *S. cerevisiae*, as by almost all other organisms, by its conversion to glucose-6-phosphate, catalyzed by the enzymes of the Leloir pathway (75, 82) (Fig. 1). These enzymes are encoded by *GAL1* (kinase), *GAL7* (transferase), *GAL10* (epimerase), and *GAL5* (mutase) (29–32, 107). Galactose in the form of melibiose is also

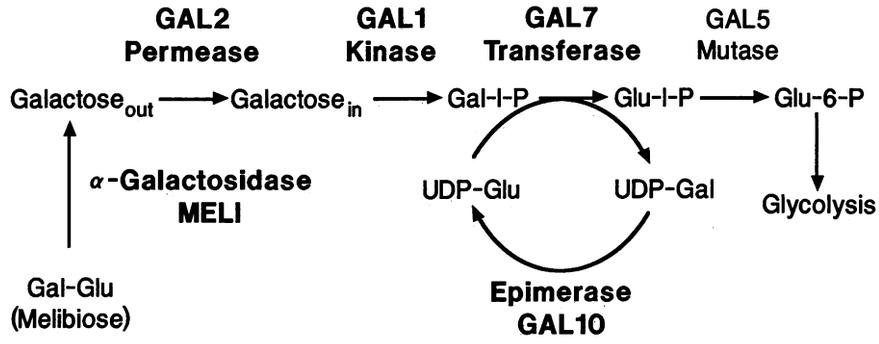


FIG. 1. Pathway of galactose utilization. The enzymes are galactokinase (EC 2.7.1.6, encoded by *GAL1*), galactose-1-phosphate uridylyltransferase (EC 2.7.7.12, encoded by *GAL7*), uridine diphosphoglucose 4-epimerase (EC 5.1.3.2, encoded by *GAL10*), phosphoglucomutase (EC 2.7.5.1, encoded by *GAL5*), and α-galactosidase (EC 3.2.1.22, encoded by *MEL1*). Shown in boldface are the genes whose expression is regulated by galactose.

available to yeast cells after cleavage of this disaccharide catalyzed by α-galactosidase, encoded by the *MEL1* gene (19, 79, 80, 157). Galactose enters yeast cells through a specific permease, encoded by *GAL2* (24, 31, 151).

Expression of the genes encoding these enzymes (except for *GAL5*) is closely regulated: their expression is induced by growth on galactose and repressed during growth on glucose, as described below. The *GAL5* gene (encoding phosphoglucomutase) is unregulated, being expressed under all conditions (10).

MODEL FOR *GAL* GENE REGULATION

The circuit responsible for the regulation of expression of the *GAL* genes is well defined and has served as a paradigm for gene regulatory circuits in fungi. The major features of the model were established by Douglas and Hawthorne in the 1960s (31–35), and further refined by subsequent work. Because of the nature of the organism and the inclinations of the investigators, much of what we know about the mechanism of *GAL* gene regulation is based on genetic evidence. Only recently have the regulatory models been confirmed by more direct biochemical experiments. Before describing in detail what is known about each component of the regulatory circuit, I will first briefly present the current model of *GAL* gene regulation. Shown in Fig. 2 are the essential elements of the *GAL* gene regulatory circuit. The *GAL1*, *GAL7*, and *GAL10* genes are clustered, but separately transcribed from individual promoters. *GAL2* and *MEL1* lie on different chromosomes. *GAL4* encodes a protein that activates transcription of these five genes by binding to sites located upstream of each gene. The *GAL80* gene encodes a protein that binds directly to GAL4 protein, preventing it from activating transcription. The inducer prevents GAL80 protein from inhibiting GAL4 protein, presumably by binding to the GAL80 protein. The inducer may dissociate GAL80 protein from GAL4 protein, or it may prevent GAL80 protein from inhibiting the function of GAL4 protein without

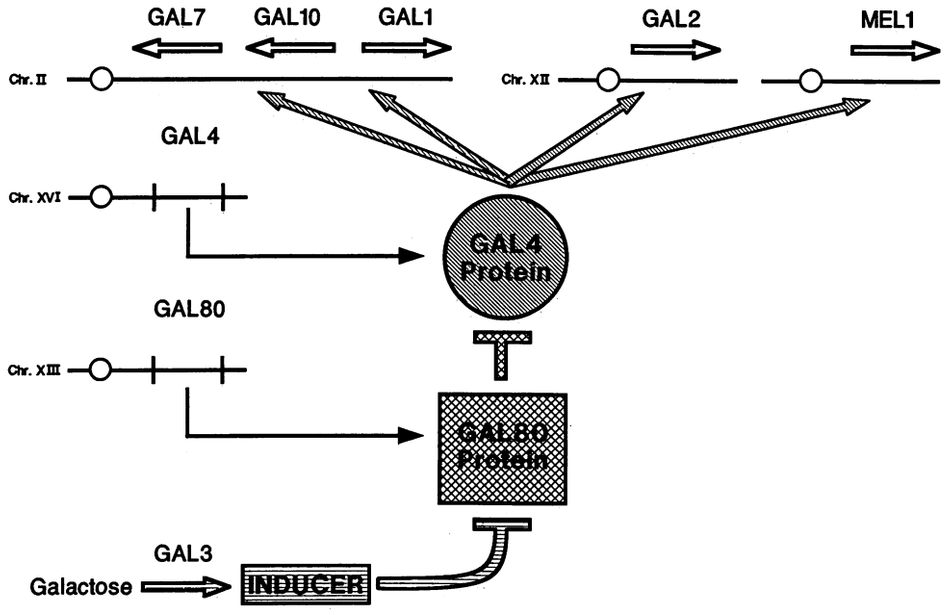


FIG. 2. Components of the *GAL* gene regulatory circuit. Bold lines with arrows denote stimulation of activity; those with bars denote inhibition of activity.

disrupting the protein complex. Thus, in the absence of galactose, GAL80 protein binds to GAL4 protein, preventing it from activating expression of *GAL1*, *-2*, *-7*, and *-10* and *MEL1*; during growth on galactose, the inducer prevents GAL80 protein from inhibiting GAL4 protein function, allowing expression of these five genes. The inducer is an unidentified small molecule whose synthesis is probably catalyzed by the product of the *GAL3* gene. Growth on glucose (even in the presence of galactose) prevents *GAL* gene expression through a regulatory circuit termed catabolite repression, about which little is known.

While this model for *GAL* gene regulation is widely accepted and generally well supported by the experimental evidence, the details of the regulatory mechanism remain to be revealed. In the following sections I will describe the evidence for each component of the model and attempt to point out those features of the mechanism that remain to be elucidated.

ORGANIZATION AND TRANSCRIPTION OF THE *GAL* GENES

The *GAL1*, *GAL7*, and *GAL10* genes, which encode the enzymes of the Leloir pathway, are clustered near the centromere of chromosome II (Fig. 2) (7, 32, 142). These genes have been isolated (26, 126, 141) and sequenced (25, 65, 147, 161), their transcripts have been identified and mapped (142, 143), and their sites of transcription initiation have been located (25, 65, 110). The *GAL2* gene, which encodes the galactose permease (24, 31, 151), and *MEL1*, which encodes α-galactosidase (19, 79, 80, 83), lie on separate chromosomes. The regulatory genes *GAL4*, *GAL80*, and *GAL3* also reside on separate chromosomes. Not shown in Fig. 2, or discussed further here, is *GAL11*, a putative *GAL* regulatory gene. Mutants of *gal11* are unable to achieve full levels of *GAL* gene expression (109). No function has been ascribed to this gene, and no further reports have appeared since the only existing *gal11* mutant was initially described.

The *GAL1*, *GAL7*, and *GAL10* genes are separately transcribed from individual promoters (61, 128, 142, 143). This fact was apparent even before its direct demonstration by analysis of the transcripts of these genes {by Northern [ribonucleic acid (RNA)] blot analysis} (142), because nonsense mutations in one gene were shown not to have a polar effect on the expression of the other *GAL* genes (16). In addition, the primary translation product of each gene appears to be a separate protein (16). Detection of the *GAL7* and *GAL10* messenger RNAs by an in vitro translation assay also showed that they are separate molecules (61). Their direction of transcription is shown in Fig. 2.

Transcription of the *GAL* genes is closely regulated (29, 142, 151). The *GAL1*, *GAL7*, *GAL10*, and *GAL2* genes are unexpressed in cells grown without galactose and are induced approximately 1,000-fold after growth on galactose; *MEL1* is expressed at a basal level that is induced about 100-fold after growth on galactose (72, 117). These genes are highly expressed: the *GAL1*-, *-7*-, and *-10*-encoded proteins each constitute 0.3 to 1.5% of total cell protein (40, 125, 127); their transcripts each comprise 0.25 to 1% of total polyadenylated RNA (142).

GAL4 PROTEIN BINDS TO DNA AND ACTIVATES TRANSCRIPTION

The GAL4 protein is the focus of much of the current interest in the mechanism of *GAL* gene regulation. This protein is required for the transcription of *GAL1*, *-2*, *-7*, and *-10*: *gal4* mutants fail to transcribe these genes (60, 61, 141, 142). The GAL4 protein is also responsible for increasing the basal level of *MEL1* transcription (117). As described below, GAL4 protein binds to deoxyribonucleic acid (DNA) upstream of these genes; the events that occur subsequent to DNA binding that lead to transcription activation are unknown.

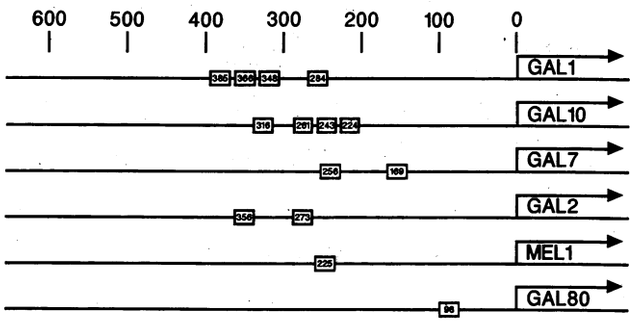
The sequences to which GAL4 protein binds are termed upstream activation sequences, or UAS (48). UAS is a generic term for sequences located upstream of yeast genes that provide for activation of transcription, presumably by binding transcriptional regulatory proteins. Several UAS elements, including UAS_{GAL}, have been shown to be binding sites for transcriptional regulatory proteins. Because UAS elements mediate transcription activation in either orientation and at variable distances, they have been compared with enhancer sequences of higher eucaryotes (47). A central question in the study of eucaryotic gene regulation is how proteins that activate transcription, which presumably act by binding to enhancer elements, can work over relatively great distances. The GAL4 protein provides an excellent opportunity to understand this phenomenon.

DNA Sequences Responsible for Activation of Transcription

The sequences that mediate activation of transcription by GAL4 protein were first identified by locating a piece of DNA between the *GAL1* and *GAL10* genes that is able to confer *GAL4*-dependent expression on a second gene (48, 144). The region upstream of the yeast *CYC1* or *HIS3* genes (neither of which are regulated by galactose) was replaced with a 365-base pair DNA fragment that lies upstream of *GAL1* and *GAL10*. This made expression of *CYC1* and *HIS3* dependent on GAL4 protein, suggesting that the recognition site for GAL4 protein (UAS_{GAL}) lies in that piece of DNA. The UAS_{GAL} was further localized to a region of 108 base pairs that is unusually guanine-plus-cytosine rich and lies approximately equidistant between *GAL1* and *GAL10* (65, 161). Detailed analysis of mutants deleted for this segment of DNA defined a small region that contains four short sequences of partial dyad symmetry, two of which are required for *GAL1* and *GAL10* expression (155). Two short sequences with partial dyad symmetry were also found to be required for expression of *GAL7* (148). These six sequences are homologous to each other, and also to sequences found upstream of the other two genes regulated by GAL4 protein, *GAL2*, and *MEL1* (13). These sequences have been shown to be binding sites for GAL4 protein (see below). Their locations (Fig. 3) vary from about 100 (for the site upstream of *GAL80*) to 385 (for the site most distal to *GAL1*) base pairs upstream of the transcription initiation site.

The sequences responsible for GAL4 protein-mediated gene activation are functional in the absence of surrounding *GAL* sequences. A 17- or 21-base pair synthetic oligonucleotide with a sequence similar to the consensus sequence of the GAL4 protein-binding sites confers high-level *GAL4*-dependent gene expression when placed upstream of any of several genes (42, 87; J. Flick, unpublished results). Therefore, only an intact GAL4 protein-binding site is specifically required for activation of transcription by GAL4 protein. Also required, of course, are general promoter elements, such as the "TATA box," which can be provided by any gene (144). The GAL4 protein-binding site, then, appears to be a sequence module whose only role is to bring GAL4 protein near a promoter.

While one GAL4 protein-binding site is sufficient for expression, genes with two sites are in some cases expressed



Consensus Binding Site

C G G A G/C G A C A
T C A G G/C A G G C
19 20 18 10 16 9 13 11 20

FIG. 3. Location of GAL4 protein-binding sites. The boxes each represent a 17-base pair sequence homologous to the consensus GAL4 protein-binding site (taken from reference 42) shown at the bottom. The numbers inside each box are the distance in nucleotides from the center of dyad symmetry of each sequence to the site of transcription initiation. The *GAL2* gene has multiple transcription initiation sites that have not been precisely mapped (to about 20 nucleotides); the estimated distance of the GAL4 protein-binding sites is to the most upstream initiation site (R. Bram, personal communication). The start sites of transcription for each gene are aligned, and the distance in nucleotides is marked at the top. The numbers at the bottom show the number of times each consensus nucleotide appears in the half-dyad of the 20 binding sites.

at higher levels (42, 87, 155), although more than two sites do not further increase levels of gene expression significantly (87). In other cases, however, one GAL4 protein-binding site provides for as much expression as do two sites (13, 117). The reason for these conflicting results may be that the GAL4 protein-binding sites (each of which deviates from the consensus sequence) are not equivalent. This is noteworthy because several of the *GAL* genes contain multiple GAL4 protein-binding sites: the region between *GAL1* and *GAL10* contains four sites, only two of which appear to be important for gene expression (the middle two sites in Fig. 3) (155); *GAL7* and *GAL2* also possess two GAL4 protein-binding sites.

The four genes with two or more GAL4 protein-binding sites (*GAL1*, *-2*, *-7*, *-10*) are not expressed in cells grown in the absence of galactose, and it has been suggested that at least two GAL4 protein-binding sites are required for repression of gene expression under these conditions (13). This idea comes from the observation that the *MEL1* and *GAL80* genes, both of which are activated by GAL4 protein and are expressed at basal levels in the absence of galactose (72, 117, 132), each have only one GAL4 protein-binding site (Fig. 3). Furthermore, hybrid genes with only one GAL4 protein-binding site are expressed in the absence of galactose at a low basal level that is abolished by the addition of a second binding site (13). The basis for this effect is unknown; it may reflect requirements for GAL80 protein function (see below).

The DNA between *GAL1* and *GAL10* that contains the GAL4 protein-binding sites is unusually sensitive to digestion by deoxyribonuclease I in nuclei (85, 118). This is true when the genes are expressed, as well as when they are unexpressed. Thus, this region of DNA seems to be free of nucleosomes and therefore accessible to GAL4 protein. The extreme sensitivity to deoxyribonuclease I digestion of the sequences that immediately flank the GAL4 protein-binding sites is thought to reflect binding of GAL4 protein to this region of DNA (86).

Three features of the GAL4 protein-binding sites have led to their comparison to enhancer sequences of higher eucaryotes (47, 144). First, they are modular sequence elements that can enhance transcription of presumably any gene and are active only under certain conditions (that is, when GAL4 protein is active). Second, they are functional in either orientation. Finally, UAS_{GAL} can work at variable distances from the site of transcription initiation. However, the flexibility of UAS_{GAL} function only extends over a few hundred base pairs (160a), while many mammalian enhancers can work over several thousand base pairs. Another significant difference between UAS_{GAL} and enhancer sequences is that the GAL4 protein-binding sites are incapable of activating transcription when placed downstream of the transcription initiation site (144, 160a). These differences between the properties of UAS_{GAL} and enhancer elements may reflect constraints on GAL4 protein function to which enhancer binding proteins are not subject.

GAL4 Protein Binds to DNA

The possibility that *GAL4* encodes a DNA-binding protein has long been entertained based on genetic evidence that it encodes the direct regulator of *GAL* gene expression (33, 73, 74). This has indeed been demonstrated recently by several methods. The most direct demonstration of the ability of GAL4 protein to bind to DNA comes from analysis of GAL4 protein produced in *S. cerevisiae* and in *Escherichia coli*. The GAL4 protein was partially purified from yeast strains that overexpress it (12, 13), or produced in *E. coli* (66, 71), and shown to bind in vitro specifically to DNA upstream of the *GAL* genes, using a nitrocellulose filter-binding assay. The exact sequences to which GAL4 protein binds were found, by deoxyribonuclease I "footprint" analysis, to coincide with the sequences required for *GAL* gene expression described in the previous section (12, 13, 71). The ability of GAL4 protein to bind to DNA in vivo was also revealed based on its ability to alter the reactivity of DNA sequences to which it is bound to various treatments, including dimethyl sulfate (42) and ultraviolet light (129, 130). Because GAL4 protein makes the same in vivo contacts with its binding site in *E. coli* as it does in yeasts (42, 129), no other proteins appear to be required for it to bind to DNA. Taken together, these results demonstrate that GAL4 protein binds to the several 17-base pair sequences whose consensus sequence and location upstream of the *GAL* genes are shown in Fig. 3. As noted above, this 17-base pair consensus sequence, when appropriately located in a promoter, is sufficient for GAL4 protein to activate transcription.

The consensus sequence of the 10 GAL4 protein-binding sites is shown in Fig. 3. It is dyad symmetric, suggesting that GAL4 protein binds to DNA as a multimer, probably a dimer or tetramer. It is somewhat surprising that a protein as large as GAL4 protein (99 kilodaltons) binds as a multimer to such a short sequence. The most highly conserved bases in the binding site are the outermost CGG residues. The central G residue, which is invariant in all 20 half sites, is consistently protected from methylation by dimethyl sulfate when GAL4 protein binds, suggesting that the protein makes an important contact to it (42). Indeed, a mutation that changes the invariant G residue to C in one of the binding sites upstream of *GAL7* drastically reduces expression of that gene (148).

Conditions under Which GAL4 Protein Binds to DNA

The conditions under which GAL4 protein binds to yeast DNA in vivo have been analyzed by assaying its ability to reduce or enhance the reactivity of its binding site and surrounding sequences to dimethyl sulfate (42), ultraviolet light (129, 130), or deoxyribonuclease I (86). The results of those experiments suggest that GAL4 protein binds to DNA in both the presence and the absence of galactose (86, 130; Giniger et al., unpublished data, cited in reference 42). Therefore, induction of *GAL* gene expression must involve modification of the transcription activation function, but not the DNA-binding function of GAL4 protein. Apparently, in the absence of inducer, the GAL80 protein interacts with GAL4 protein that is bound to DNA and prevents it from activating transcription, without drastically changing its DNA-binding properties. The GAL80 protein may, however, subtly alter the DNA-binding properties of GAL4 protein because it slightly alters the effect of GAL4 protein on the pattern of DNA modifications (42, 130). The GAL80 protein appears not to make specific DNA contacts (42, 130).

In contrast of these results, GAL4 protein does not bind to DNA in cells grown on glucose (42, 86, 130), a condition that causes "catabolite repression" of *GAL* gene expression. Therefore, one mechanism of glucose repression appears to be the prevention of DNA binding by GAL4 protein. Whether this is due to the action of GAL80 protein or to other unidentified gene products is not known (see section, "Catabolite Repression of *GAL* Gene Expression").

Functions of GAL4 Protein

Much of the current interest in *GAL* gene regulation centers on the mechanism by which GAL4 protein activates transcription. The *GAL4* gene has been sequenced and encodes a relatively large protein of 881 amino acids (99,350 daltons) (77) that is present in very low levels in *S. cerevisiae* (possibly only one or two molecules per cell [12]). Substantial progress has been made recently in defining some of the regions of the protein responsible for its several functions, which include (i) DNA binding, (ii) transcription activation, (iii) the ability to specifically enter the nucleus, (iv) interaction with the GAL80 protein, (v) possible direct involvement in catabolite repression, and, presumably, (vi) multimer formation. The locations of functional domains in GAL4 protein are diagramed in Fig. 4 and described in detail below.

(i) DNA binding. The DNA-binding domain resides in the N-terminal 74 amino acids of GAL4 protein. This region of the protein functions independently: a *GAL4-lacZ* hybrid protein consisting of the amino-terminal 74 residues of GAL4 protein fused to most of β-galactosidase appears to have DNA-binding properties identical to GAL4 protein, both in vivo and in vitro (71). The DNA-binding domain is shown in Fig. 4 to include amino acids 10 to 51, because mutations that affect the DNA-binding activity of GAL4 protein are limited to this sequence (see below).

This region of the protein is homologous to regions in several other eucaryotic DNA-binding and transcriptional regulatory proteins (examples cited in reference 66) and is thought to form a structure called the "cysteine-zinc DNA binding finger." The proposed structure of this region in GAL4 protein is diagramed in Fig. 5A. The two pairs of cysteine residues are proposed to chelate a zinc ion, with the intervening amino acids looping out to form a "finger" of protein that is thought to contact DNA. This hypothesis is based on the observation that another transcriptional regulatory protein that binds to DNA (TFIIIA from *Xenopus laevis*) could form nine cysteine-zinc fingers and has been shown to bind nine zinc ions (102) that are required for its DNA-binding activity (49). The cysteine-zinc finger of GAL4 protein is especially homologous to similar regions in several other transcriptional regulatory proteins from *S. cerevisiae* and one from the filamentous fungus *Neurospora crassa* (Fig. 5B). The consensus sequence shown at the bottom of Fig. 5 shows that these proteins have identical amino acids at 8 of 23 positions; 6 other positions have amino acids with very similar properties in at least five of the seven proteins. That in all of these proteins the regions between the pairs of cysteines are rich in amino acids known to interact with DNA is consistent with the proposed direct role of this region in binding to DNA.

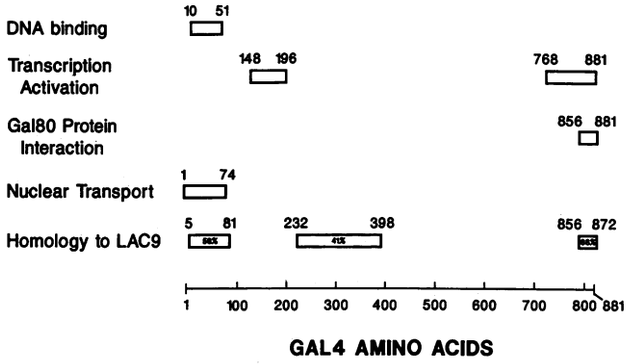


FIG. 4. Functional domains of GAL4 protein. The length of the GAL4 protein is marked off in amino acid residues at the bottom. The boxes delimit regions (the boundary amino acids are listed above the boxes) identified to be involved in each function of the protein. The references providing the evidence for these domain assignments are as follows: GAL80 protein interaction (68, 91); DNA binding (64a, 66, 71); nuclear transport (134); transcription activation (90); *LAC9* homology (124, 158).

Direct evidence that the cysteine-zinc finger is involved in DNA binding comes from the identification of *gal4* mutations that alter this structure and abolish the DNA-binding activity of GAL4 protein (66) (Fig. 6). The 20 amino acids immediately adjacent (C terminal) to the cysteine-zinc finger in GAL4 protein also appear to be involved in DNA binding because mutations that alter amino acids in this region of the protein abolish or severely reduce its DNA-binding activity (Fig. 6) (66). Because the cysteine-zinc fingers of the seven fungal proteins listed in Fig. 5B are highly homologous, despite the fact that they bind to different DNA sequences, this structure may be a general DNA-binding domain that does not provide sequence specificity (124). The high proportion of lysine and arginine residues in the finger, which could bind to the phosphate backbone of DNA, is consistent with this idea. The sequence specificity of GAL4 protein DNA binding might reside in the region adjacent to the cysteine-zinc finger (124), which shows little apparent homology among the five proteins listed in Fig. 5B. The DNA binding domain of GAL4 protein, then, may consist of two regions (Fig. 6): one (the cysteine-zinc finger) that binds nonspecifically to DNA, and another (the specificity domain) that provides for the sequence specificity of binding.

There is good evidence that the cysteine-zinc finger of GAL4 protein indeed contains a zinc ion that is required for its DNA-binding activity (64a). One of the mutations that alters this structure (Pro26>Leu) results in a GAL4 protein that is unable to bind to DNA due to its reduced affinity for

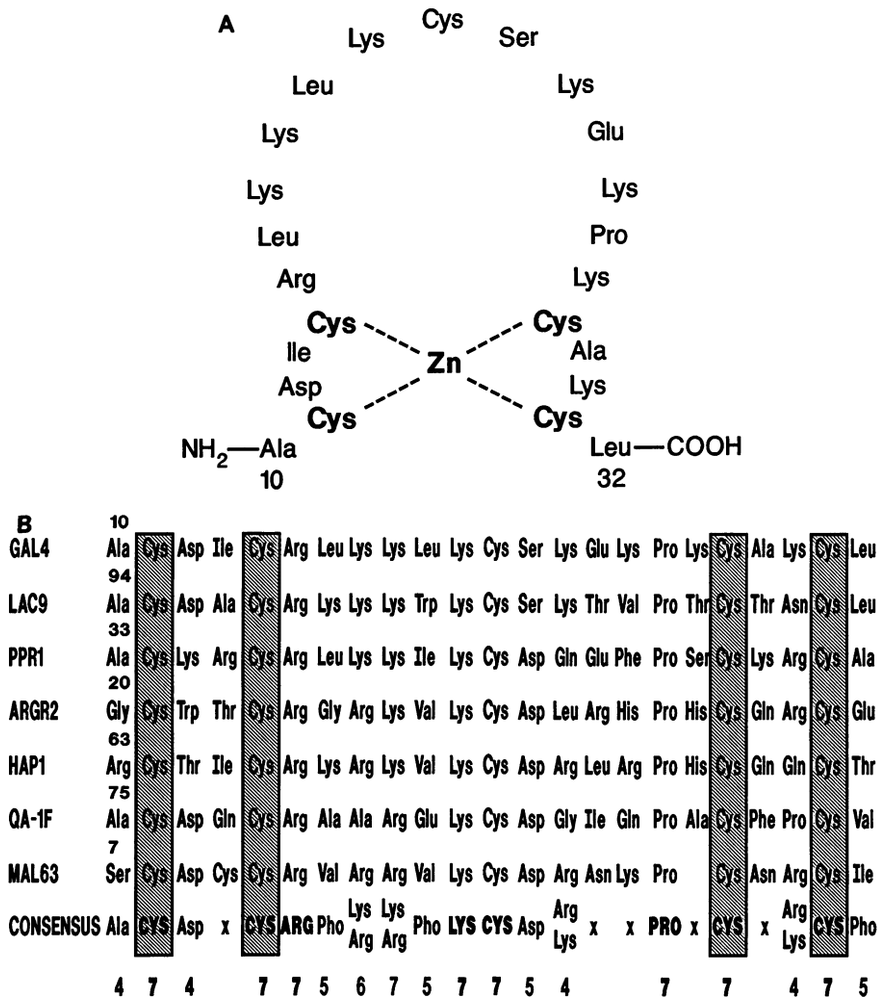


FIG. 5. Proposed structure of the cysteine-zinc DNA binding finger of GAL4 protein (A) and homologies among the cysteine-zinc DNA binding fingers of several fungal transcriptional regulatory proteins (B). (A) The sequence of GAL4 protein from amino acids 10 to 32 is shown. The two pairs of cysteine residues shown in boldface are thought to chelate a zinc ion. (B) The number above the first amino acid of each protein is its residue number in that protein. Shown at the bottom is the consensus sequence (Pho = hydrophobic amino acid; X = any amino acid). Shown below the consensus sequence is the number of proteins that each consensus amino acid appears in. The amino acids in bold-faced capital letters are present in all seven proteins. The sequences of these proteins are reported in references 77 (GAL4), 124 and 158 (LAC9), 70 (PPR1), 99 (ARGRII), and 8 (QA-1F); the sequences of HAP1 (Pfeiffer and Guarente, personal communication) and MAL63 (Kim and Michels, personal communication) are unpublished.

zinc ions. This conclusion is inferred from the observation that the Gal$^-$ phenotype of mutants carrying this *gal4* mutation is corrected by high concentrations of zinc in the medium. Furthermore, the ability of the altered GAL4 protein to bind to DNA in vitro is restored in the presence of zinc. It seems likely that the proline-26 residue (located just in front of the second pair of cysteines; Fig. 5A) causes a bend in the protein that brings the two pairs of cysteines close enough together to allow them to tightly chelate a zinc ion. It appears, then, that zinc plays an essential structural role in the DNA-binding domain of GAL4 protein, as well as of several other transcriptional regulatory proteins.

(ii) Transcription activation. The portion of GAL4 protein responsible for activation of transcription lies in the C-terminal 90% of the protein, and this domain can function independently of the DNA-binding domain. This was clev-

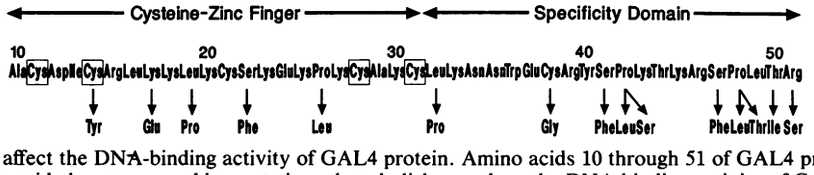


FIG. 6. Mutations that affect the DNA-binding activity of GAL4 protein. Amino acids 10 through 51 of GAL4 protein are shown. Below the sequence are the amino acid changes caused by mutations that abolish or reduce the DNA-binding activity of GAL4 protein (66). Shown above the line are the proposed subdomains in the DNA-binding domain of GAL4 protein.

erly demonstrated by constructing a hybrid gene encoding a GAL4 protein whose DNA-binding domain is replaced by the DNA-binding domain of a bacterial repressor (encoded by *lexA* of *E. coli*) (15). This hybrid protein is able to activate transcription of yeast genes, provided their promoters contain the binding site for the bacterial repressor (*lexO*). This suggests that the only role of the DNA-binding domain is to appropriately position the transcription activation domain near promoters; any functional DNA-binding domain will serve this purpose. This conclusion is supported by the observation that proteins that possess only the DNA-binding domain of GAL4 protein do not activate transcription in yeast cells, even though they are capable of binding to DNA (71). Conversely, GAL4 proteins that lack a DNA-binding domain are unable to activate transcription (66, 69).

It seems likely that GAL4 protein activates gene expression through contacts between its transcription activation domain and other proteins more directly responsible for transcription (e.g., RNA polymerase). A major goal of research on GAL4 protein is to locate the regions of the protein that interact with other proteins and to identify the interacting proteins. Attempts to isolate *gal4* mutations that inactivate its transcription activation function yielded few missense mutations: nearly all mutations that lie in the transcription activation domain are of the nonsense or frameshift type (66). This suggests that there may be multiple regions of the protein that activate transcription; single amino acid changes in any one region may not drastically reduce function of the protein. This indeed appears to be the case: two regions of GAL4 protein with transcription activation function have been identified by analysis of *gal4* mutants deleted for various portions of the protein (90). One of these domains is adjacent to the DNA-binding domain; the other encompasses the C terminus (Fig. 4).

Potential transcription activation domains have also been identified by comparing the sequence of GAL4 protein from *S. cerevisiae* with its counterpart (encoded by *LAC9*) from the distantly related yeast *Kluyveromyces lactis* (124, 158). These two proteins can substitute for each other in both organisms (121, 124, 158), but are surprisingly nonhomologous except in three regions: the DNA-binding domain (58% homology), a region of 163 amino acids near the center of the sequence (41% homology), and 18 amino acids near the C terminus (88% homology) (Fig. 4). The latter region coincides with one of the transcription activation domains defined by deletion analysis of *GAL4* (90). The central region of homology contains three of the only four *gal4* missense mutations that lie outside the DNA-binding domain (66; M. Johnston and J. Dover, unpublished data). However, this region of GAL4 protein appears not to have a role in transcription activation, because it can be deleted without loss of function of the protein (90).

The two transcription activation domains of GAL4 protein have a large net negative charge (90), and it appears that the acidic character of this region of GAL4 protein (and not any specific sequence of amino acids) is all that is required for its function (41a). This conclusion comes from the observation that many different sequences are able to provide the transcription activation function when attached to the carboxyl terminus of GAL4 protein, provided they are predominantly composed of acidic amino acids (91a). In this respect, the transcription activation domain of GAL4 protein is similar to the analogous domain of another yeast protein, encoded by *GCN4*, that activates transcription of several genes involved in amino acid biosynthesis (58). This may signify that a common mechanism is involved in the activation of transcription by yeast regulatory proteins. That no specific sequence appears to be required for the transcription activation function also explains the dearth of missense mutations affecting the transcription activation domain of GAL4 protein (66). In summary, the available evidence suggests that GAL4 protein activates transcription, after it is positioned near the appropriate promoter elements by the DNA-binding domain, by interactions between two distinct acidic transcription activation domains and other proteins of the transcriptional apparatus. A major future advance will be the identification of the proteins with which GAL4 protein interacts, which should lend insight into the mechanism of transcription activation.

How the GAL4 protein is able to activate transcription over variable distances is a question central to the mechanism of GAL4 protein function and is relevant to the mechanism of action of enhancer elements of higher eucaryotes. It has been suggested that this flexibility is a property of DNA, which bends to bring the GAL4 protein-binding site (or enhancer) close to the sites upon which the protein acts (119). Another possibility (not necessarily exclusive of the first model) is that the flexibility resides in the GAL4 (or enhancer-binding) protein, which might consist of transcription activation domains linked to the DNA-binding domain by a flexible arm. In this model, the only function of much of the protein is to allow the transcription activation domains to extend to their site of action on DNA (presumably the TATA box). It is interesting that most of the GAL4 protein (excepting the small DNA-binding domain) is remarkably insensitive to mutation (66). Even more remarkable is the observation that nearly 80% of the GAL4 protein between the transcription activation domains can be deleted with little loss of function (90). Perhaps this region insensitive to mutation encodes the "flexible arm" that allows GAL4 protein to function over variable distances. This hypothesis predicts that smaller GAL4 proteins that retain function will be unable to work over distances as great as the full-length protein.

(iii) GAL80 protein interaction. Based on evidence described more fully in the next section, GAL80 protein probably inhibits function of GAL4 protein by directly binding to it. Because GAL80 protein appears to specifically inhibit the transcription activation function of GAL4 protein, localizing its interaction domain within GAL4 protein is likely to shed light on the mechanism of transcription activation. This domain of GAL4 protein must lie in the region distal to the DNA-binding domain because GAL4 proteins consisting only of the C-terminal 90% (missing the first 74 to 78 residues) appear to retain their ability to interact with GAL80 protein (15, 69). This conclusion is based on the observation that, when such proteins are present at high levels in yeast cells, they cause *GAL* gene expression to be constitutive in strains that possess a normal *GAL4* gene. This result is interpreted to mean that the high levels of the altered GAL4 protein bind all of the GAL80 protein, freeing the normal GAL4 protein to activate transcription in the absence of inducer.

The domain of GAL4 protein responsible for its interaction with GAL80 protein is likely to be encoded by the portion of the *GAL4* gene wherein *GAL81* mutations lie. These are a class of *GAL4* mutations thought to prevent GAL80 protein from inhibiting GAL4 protein activity (93). The sequence change caused by one of these mutations has been determined; it lacks the C-terminal 30 amino acids of GAL4 protein (68). Furthermore, high levels in *S. cerevisiae* of a peptide with the sequence of this C-terminal region of

GAL4 protein causes titration of GAL80 protein (91); overexpression in *S. cerevisiae* of a GAL4 protein that lacks this sequence does not cause GAL80 protein to be titrated (68). This suggests that the transcription activation domain that resides in the C terminus of GAL4 protein is recognized by GAL80 protein and suggests a simple model for the mechanism of GAL80 protein action: GAL80 protein prevents transcription activation by covering the transcription activation domain in GAL4 protein, preventing it from interacting with a component(s) of the transcription apparatus. Perhaps GAL80 protein has a domain similar to a region of the protein with which GAL4 protein interacts to activate transcription.

(iv) Nuclear transport. Since the GAL4 protein is synthesized in the cytoplasm but acts in the nucleus, it must be able to pass through the nuclear envelope. It seems likely that a specific transport system accomplishes this feat, because the large size of GAL4 protein (99 kilodaltons) compared with the nuclear pore (~9 nm) makes it difficult to imagine that it is due simply to diffusion. Indeed, GAL4 protein sequences responsible for its nuclear localization lie in the N-terminal region that also contains the DNA-binding domain (Fig. 4): hybrid proteins that possess this GAL4 protein domain fused to β-galactosidase become concentrated in the yeast nucleus (134). (However, GAL4 protein must be able to diffuse into the nucleus at some level, because hybrid GAL4 proteins that lack the nuclear localization domain, and are therefore not specifically concentrated in the nucleus, are still able to activate gene expression [15].) The ability of a protein to bind to DNA in *S. cerevisiae* is not sufficient for it to be localized to the nucleus, so the domains responsible for DNA binding and nuclear localization appear to be functionally separate (135). Mutations that abolish the ability of GAL4 protein sequences to provide nuclear localization have been identified (133); it is not yet clear whether these mutations directly affect the mechanism of nuclear transport.

GAL80 PROTEIN INHIBITS GAL4 PROTEIN

The product of the *GAL80* gene has genetic properties of a repressor, since *gal80* mutants express the *GAL* genes constitutively (35, 149). The conclusion that the GAL80 protein indirectly represses *GAL* gene expression through its inhibition of GAL4 protein is based on the fact that *gal4* mutations are epistatic to *gal80* mutations (32, 149). That is, the *gal4 gal80* double mutant has the same phenotype (Gal^-) as *gal4* single mutants, indicating that the *GAL4* function acts after the *GAL80* function. At the time these experiments were first done the prevailing model of gene regulation was based on the *lac* operon of *E. coli*, so it was originally proposed that GAL80 protein is a repressor of *GAL4* transcription. It is now clear that *GAL4* is expressed constitutively and that its activity is inhibited by GAL80 protein posttranslationally.

GAL4 Is Expressed Constitutively

That *GAL4* is expressed in cells grown in the absence of galactose was first suggested by results of experiments that measured the kinetics of induction of *GAL* gene expression (94). A *gal4* mutant that makes a thermolabile GAL4 protein was grown at the nonpermissive temperature, thereby inactivating GAL4 protein. The cells were then shifted to the permissive temperature, and galactose was added to the culture. The lag time observed under these conditions for induction of *GAL* gene expression in this mutant (35 min) presumably represents the time required to resynthesize GAL4 protein. If GAL80 protein prevents *GAL4* gene expression in the absence of galactose, then wild-type cells should show a similar lag time for induction of *GAL* gene expression following addition of galactose to the culture. However, a much shorter lag time was observed (15 min), implying that GAL4 protein preexists in cells prior to galactose addition.

Direct support for constitutive expression of *GAL4* is the observation that induction of *GAL* gene transcription can occur in the absence of protein synthesis (115). In addition, it has been shown by direct analysis of the *GAL4* messenger RNA (by Northern blots) that transcription of *GAL4* is constitutive (78). These observations, along with the result of a clever experiment that shows that GAL80 protein can block the function of preexisting GAL4 protein (115), lead to the conclusion that GAL80 protein regulates *GAL4* posttranslationally.

GAL4 and GAL80 Proteins Interact

The first evidence for a direct interaction between GAL4 and GAL80 proteins came from analysis of mutations that are now thought to alter the interaction between these two proteins. These mutations are of two types: (i) $GAL80^s$ mutations are dominant and cause a Gal^- phenotype, presumably because they make the GAL80 protein unable to bind inducer (34, 111); (ii) *GAL81* mutations are a class of semidominant alleles of *GAL4* that cause constitutive *GAL* gene expression, presumably because GAL80 protein is unable to bind to GAL4 protein in these mutants (33). A significant observation made with these mutants was that certain $GAL80^s$ and *GAL81* alleles appear to partially suppress each other's defect in an allele-specific manner (112), which suggests that these two proteins interact. The subsequent finding that *GAL81* mutations lie within the *GAL4* coding sequence (93) rules out the possibility that these mutations alter a binding site in the DNA (e.g., an operator) through which GAL80 protein represses *GAL4* gene expression and further supports the idea that these two proteins directly interact.

The interaction between GAL4 and GAL80 proteins has recently been directly observed in vitro in three experiments (88). First, antibody to GAL4 protein precipitates GAL80 protein if GAL4 protein is present in the reaction. Second, GAL80 protein alters the electrophoretic mobility of the GAL4 protein-UAS_{GAL} complex. Since GAL80 protein was shown not to bind to UAS_{GAL}, this altered mobility must be due to GAL80 protein binding to GAL4 protein in the GAL4 protein-DNA complex. Finally, GAL80 protein is retained on a GAL4 protein affinity column (generated by binding GAL4 protein to UAS_{GAL} coupled to Sepharose). No other yeast proteins appear to be necessary for the interaction because it occurs with GAL4 and GAL80 proteins made by transcription and translation in vitro. The binding of GAL80 protein to GAL4 protein appears to be stoichiometric and very tight, with an estimated binding constant of 5×10^{-9} M.

While it has been suggested that GAL80 protein binds directly to DNA, based on circumstantial evidence (67, 113), the GAL80 protein was found not to bind to DNA and is able to bind to GAL4 protein in the absence of DNA (88). The lack of any *GAL80*-dependent effects on in vivo footprints of the *GAL* regulatory sequences also rules against a direct role for GAL80 protein in DNA binding (42, 130). However, the

GAL4 protein-binding sites may play an indirect role in GAL80 protein function, because of the observation (discussed above) that two GAL4 protein-binding sites are required for complete repression by GAL80 protein (13). Perhaps the affinity of GAL80 protein for GAL4 protein is increased when it interacts as an oligomer with two GAL4 protein molecules.

Because the GAL4 and GAL80 proteins directly interact, a precise balance in the levels of the two proteins should be essential for proper *GAL* gene regulation: with too much GAL4 protein, there should not be enough GAL80 protein to maintain repression of the *GAL* genes. This has in fact been observed. A *GAL4* gene present in *S. cerevisiae* on a high-copy plasmid results in expression of the *GAL* genes in the absence of inducer (52, 67); addition to this strain of *GAL80* on a high-copy plasmid restores repression of *GAL* gene expression, presumably by restoring the proper balance in the levels of the two proteins (52). Furthermore, yeast strains with multiple copies of *GAL80* grown on galactose have less *GAL* gene expression than strains with only a single copy (113). This last result suggests that there is not enough inducer to saturate all GAL80 protein when these strains grow on galactose.

The GAL80 protein may remain bound to GAL4 protein in the presence of inducer, although the observation that GAL80 protein binds to the C-terminal transcription activation domain suggests that the inducer must cause GAL80 protein to be released from GAL4 protein (68, 91). There is suggestive evidence that inducer causes GAL80 protein to dissociate from GAL4 protein. When a *GAL4 GAL80* strain, grown on galactose, was mated to a *gal4 GAL80*^{s} strain, the resultant zygote was unable to express the *GAL* genes (115). This was interpreted to mean that the GAL80^s protein (which is insensitive to inducer) from one parent was able to inactivate the GAL4 protein of the other parent. If in the presence of inducer GAL80 protein is stably bound to GAL4 protein, the GAL4 protein in the *GAL80* parent should have been complexed with GAL80 protein and therefore unavailable for binding by the GAL80^s protein in the zygote. This appears not to be the case.

In summary, GAL4 protein is expressed constitutively; its transcription activation activity is inhibited by GAL80 protein posttranslationally by a direct interaction between these two proteins. The *GAL81* and *GAL80*^{s} mutations probably alter the sites through which these proteins interact.

GAL80 Is Regulated by GAL4 Protein

Transcription of the *GAL80* gene is regulated by the GAL4 protein: its basal level of expression is increased approximately fivefold by growth on galactose and is altered by the various *gal4* and *gal80* mutations in the expected way (132). This is presumably due to the presence of a binding site for GAL4 protein located in the *GAL80* promoter (13). The effect of this regulation is to dampen the induction of *GAL* gene expression, because it results in a requirement for continuously higher galactose concentrations to maintain high levels of *GAL* gene expression. It also explains the observation that inducer levels in cells growing on galactose appear to be insufficient to saturate all of the GAL80 protein in these cells (113, 149, 162). The mutually antagonistic effects of inducer, that is, to increase GAL80 protein levels while reducing GAL80 protein activity, are perhaps a way by which yeasts maintain homeostasis of *GAL* gene expression during its induction.

Model for Action of the GAL4 and GAL80 Proteins

As a summary of the results discussed above, a model of how the GAL4 and GAL80 proteins work is shown in Fig. 7. Many of the features of this model have been proposed by others (42, 68, 71, 86, 90, 130, 146). In the absence of inducer (Fig. 7A), the GAL4 protein is bound to UAS_{GAL} but is prevented from activating transcription by GAL80 protein, which covers the C-terminal transcription activation domain. In the presence of galactose (Fig. 7B), the inducer prevents GAL80 protein from binding to the transcription activation domain of GAL4 protein, allowing it to interact with a protein such as a TATA box binding factor (129), whose presence is essential for transcription. Under conditions of catabolite repression (growth on glucose; Fig. 7C), GAL4 protein function is presumably inhibited at two levels: GAL80 protein binds to the transcription activation domain, and another protein (a catabolite-repressing protein) prevents it from binding to DNA.

It should be pointed out that the effects of the GAL4 and GAL80 proteins on the several genes regulated by these proteins vary (Table 1). Expression of the *GAL1*, *-2*, *-7*, and *-10* genes absolutely requires GAL4 protein (column 2) and is completely inhibited by GAL80 protein (column 3) (142, 151). Expression of *MEL1* also requires GAL4 protein, but is not completely inhibited by GAL80 protein (117). Finally, the basal level of expression of *GAL80* does not depend on GAL4 protein, but is increased moderately by GAL4 protein (in the absence of GAL80 protein [column 4]) (132). An understanding of the basis of these diverse effects of GAL4 and GAL80 proteins promises to reveal important facts about the details of how these proteins work.

SYNTHESIS AND ACTION OF INDUCER

Inducer Inhibits GAL80 Protein Function

There are two pieces of genetic evidence that indicate that the inducer acts upon GAL80 protein. First, *gal80* mutants are constitutive for *GAL* gene expression, demonstrating that inducer is not needed for expression of the *GAL* genes in the absence of GAL80 protein (32, 35, 113, 149, 162). Second, some mutations that lie within *GAL80* coding se-

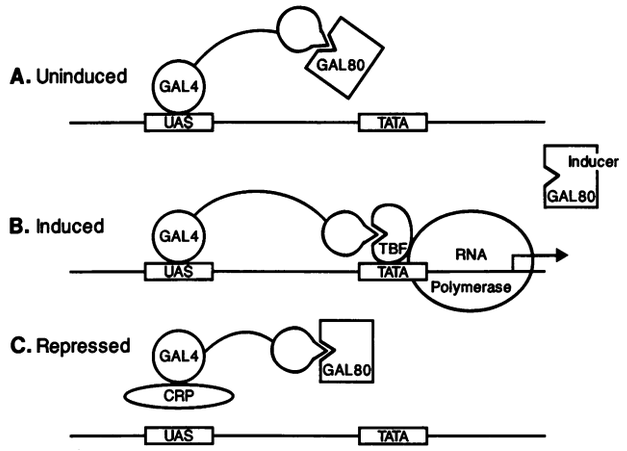


FIG. 7. Model for mechanism of action of GAL4 and GAL80 proteins. See text for explanation. TBF, TATA box binding factor; CRP, catabolite-repressing protein; UAS, upstream activation sequence.

TABLE 1. Expression state of GAL4-regulated genes under various conditions

Gene	Expression conditions[a]			
	gal4⁻	Uninduced	Induced	Repressed
GAL1, -2, -7, -10	Inactive	Inactive	Active (high)	Inactive
MEL1	Inactive	Active (basal)	Active (high)	Inactive
GAL80	Active (basal)	Active (basal)	Active (moderate)	Active (basal)

[a] Uninduced means growth in the absence of galactose (e.g., on glycerol), induced means growth on galactose or *GAL* expression in a *gal80* strain, and repressed denotes growth in the presence of glucose. See text for further explanation and references.

quences (*GAL80*^{s}) appear to make GAL80 protein unable to be recognized by inducer (34, 111). It seems likely that the inducer molecule binds directly to GAL80 protein, but no direct evidence for this has been obtained yet.

Synthesis of Inducer from Galactose Is Catalyzed by GAL3 Protein

Insight into the nature of the small molecule that is ultimately responsible for induction of *GAL* gene expression comes from studies of *gal3* mutants. It appears that *GAL3* encodes an enzyme that catalyzes synthesis of the inducer from galactose. Despite intensive analysis of these mutants that dates back to the 1940s, we still do not know the precise role of *GAL3* in *GAL* gene regulation (103, 122, 136–140, 156). The phenotype of *gal3* mutants is slow induction of *GAL* gene expression: *gal3* mutants reach full rates of *GAL* gene transcription 3 to 5 days after addition of galactose to the culture (2, 140, 150, 156); wild type does so in 20 min (1, 142). Once induced, *gal3* mutants maintain full levels of *GAL* gene expression. It is clear that this phenomenon is due to adaptation of the entire culture of *gal3* mutants to growth on galactose and not to selective growth of a few variants (103, 140, 152). It appears that *GAL3* is involved in the process of induction, because it is dispensable in mutants that do not require inducer for GAL gene expression (i.e., *gal80* mutations suppress the defect of *gal3* mutants [35]). The *GAL3* gene has been isolated and found to encode two transcripts (150).

A significant advance toward understanding the role of *GAL3* was the observation that the inducer is probably an intermediate of galactose metabolism. While *gal3* mutants become induced for *GAL* gene expression slowly, *gal3 gal1*, *gal3 gal7*, and *gal3 gal10* double mutants never become induced (16). This result is interpreted to mean that the inducer, an intermediate of galactose breakdown, is initially produced from galactose in *gal3* mutants by the action of very low basal levels of the GAL enzymes. As inducer accumulates, *GAL* gene expression increases, resulting in higher levels of inducer that ultimately lead to full levels of induction. This process apparently takes 3 to 5 days. In wild-type cells, inducer synthesis is presumably rapidly catalyzed by the *GAL3*-encoded protein. Once cells are induced, the action of either the *GAL3*-encoded protein or the GAL pathway enzymes is required continuously to maintain full levels of *GAL* gene expression (108, 150).

The identity of the inducer is unknown. It must be a small molecule, because addition of extracts of wild-type cells to cultures of *gal3* mutants causes induction of GAL gene expression in these cells, and the molecule responsible appears not to be DNA, RNA, or protein (122). It has been proposed that uridine 5′-diphosphate–glucose is the inducer, but it is difficult to see how it could provide specificity since it is known to be synthesized in the absence of galactose (108, 153). Because of this, it has been suggested that *GAL3* might be responsible for synthesis of a coinducer (perhaps uridine 5′-diphosphate-glucose) that acts together with galactose as the inducer (153). It is significant that the inducer appears to be produced by the action of the GAL enzymes. In this respect *GAL* gene regulation is similar to that of the *lac* operon of *E. coli*, whose small-molecule effector (allolactose) is also produced by the action of one of the enzymes of the lactose utilization pathway. Perhaps this is a mechanism by which yeasts ensure that they are able to utilize galactose before they become committed to synthesizing the enzymes for its breakdown. The identification of the inducer is essential for achieving the important future goal of reproducing *GAL* gene regulation in vitro.

The pathway of inducer synthesis may be complex, because two other functions besides *GAL3* appear to be involved. The *GAL3*-mediated pathway of inducer synthesis requires either mitochondrial function or the product of the nuclear-encoded *IMP* gene. This is based on the observation that *GAL3* strains become rapidly induced for *GAL* gene expression if they are either *IMP* or [*rho*$^{+}$] but not if they are *imp* [*rho*$^{-}$] (3). The pathway of inducer synthesis catalyzed by the GAL enzymes (i.e., in a *gal3* strain) requires mitochondrial function, because *gal3* [*rho*$^{-}$] strains never become induced for *GAL* gene expression (35, 152). Not enough is known about the *IMP*-encoded and mitochondrial functions to be able to propose their roles in inducer synthesis.

CATABOLITE REPRESSION OF GAL GENE EXPRESSION

Since glucose enters the glycolytic pathway directly, *S. cerevisiae* cells prefer to utilize it instead of other sugars, such as galactose, that require conversion to glycolytic intermediates before they can be utilized. Accordingly, expression of the *GAL* genes is repressed during growth on glucose (1, 32, 65, 95, 142, 161, 162). The regulatory circuit responsible for this phenomenon, termed catabolite repression, is superimposed upon the circuit responsible for induction of *GAL* gene expression; very little is known about its mechanism. While genes responsible for catabolite repression have been identified, their roles in this process have not yet been elucidated. Our knowledge of the *GAL4-GAL80* regulatory circuit allows us to envision possible mechanisms of catabolite repression. These are described below, along with the available data pertaining to them.

Properties of Catabolite Repression

Growth of *S. cerevisiae* on glucose, even in the presence of galactose, completely represses *GAL* gene expression at the level of transcription (142); addition of glucose to yeast cultures already growing on galactose causes a reduced rate of *GAL* gene expression that is preceded by a severe transient repression of expression (1). The differences in the

severity of repression under these two conditions may reflect multiple levels of action of catabolite repression on *GAL* gene expression, one acting to inhibit galactose uptake or inducer synthesis or both, for example, and another possibly acting to inhibit GAL4 protein.

Growth on glucose also affects the kinetics of induction of *GAL* gene expression. Yeast cells grown on glucose become induced for *GAL* gene expression 3 to 5 h after the addition of galactose to the culture (washed free of glucose) (1); cells grown on a non-catabolite-repressing carbon source, such as glycerol, require only 10 to 20 min to become induced for *GAL* gene expression (1, 142). Possible reasons for this long lag time in induction of glucose-grown cells follow: (i) glucose may prevent production of proteins, such as permease or GAL3 protein, that are required for inducer synthesis, and the induction lag may be the time required to resynthesize these proteins; (ii) growth on glucose may remove GAL4 protein, which might require 3 to 5 h to be resynthesized; (iii) growth on glucose may cause the production of a long-lived repressor that must be diluted out before catabolite repression is relieved. The second possibility can probably be ruled out, because the time required to resynthesize enough GAL4 protein to resume *GAL* gene expression is only 30 to 40 min, not 3 to 5 h (94).

Cyclic adenosine 5'-monophosphate is not involved in catabolite repression in *S. cerevisiae*, unlike the case in *E. coli*. The addition of high levels of cyclic adenosine 5'-monophosphate to cultures of mutants able to transport this compound does not affect catabolite repression of the *GAL* genes (97). Furthermore, catabolite repression of *GAL* gene expression is normal in mutants unable to synthesize cyclic adenosine 5'-monophosphate (96).

Several genes responsible for catabolite repression of *GAL* gene expression have been identified (Table 2). Most of these were identified by the characterization of mutants that express the *GAL* genes during growth on glucose. The *snf* mutants were isolated based on their inability to express *SUC2* (another gene subject to catabolite repression) and were found also to be defective in *GAL* gene expression (20, 105). Most of these genes appear to be involved in repression of several genes; only *GAL82* acts specifically upon the *GAL* genes. The functions encoded by only two of these genes have been identified (see below).

Mechanisms of Catabolite Repression

The expression of the *GAL* genes could be regulated by catabolite repression at several points, as diagramed in Fig. 8. The first suggestion that there is more than one pathway of

TABLE 2. Genes involved in catabolite repression of *GAL* gene expression[a]

Gene	*GAL* specific?	*gal80* dependent?	Reference
REG1	No	Yes	98
GRR1	No	No	4
GRR2	No	No	Flick (unpublished)
HXK2	No	No	39, 89
GAL82	Yes	No	95, 98
GAL83	No	Yes	95, 98
SNF1 (CCR1)	No	NA	20
SNF2, -4, -5	No	NA	105
SSN20	No	NA	106

[a] See text for further explanation. NA, Not applicable.

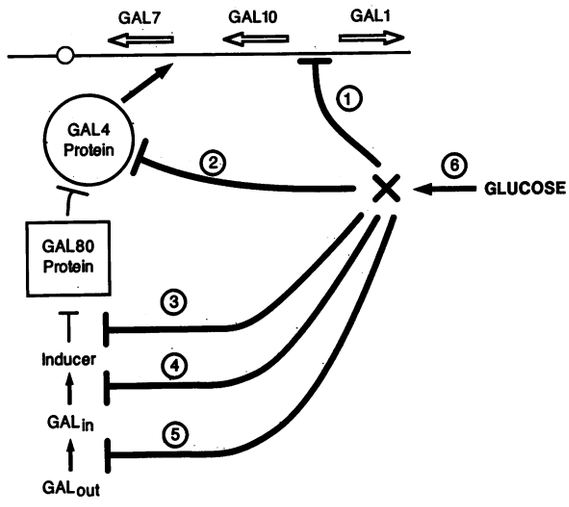


FIG. 8. Possible mechanisms of catabolite repression of *GAL* gene expression. See text for explanation.

catabolite repression came from cases in which two genes must be inactivated to abolish repression of *GAL* gene expression: mutations in some of the genes listed in Table 2 (*GAL83*, for example) cause full resistance to catabolite repression only when coupled with a *gal80* mutation (95, 98). One interpretation of this result is that *GAL83* is involved in one circuit of catabolite repression (for example, those labeled 1 and 2 in Fig. 8) and *GAL80* is part of another independent regulatory pathway (those labeled 3, 4, and 5 in Fig. 8). Genes in which mutations cause full resistance to catabolite repression even in a *GAL80* background (e.g., *GRR1*) might be involved in a step common to both pathways, for example, synthesis of a co-repressor ("X" in Fig. 8) that signals catabolite repression (step 6 in Fig. 8). An important but as yet unaccomplished goal of studies on catabolite repression of the *GAL* genes is to assign the genes listed in Table 2 to the potential pathways of catabolite repression described below.

(i) Direct repression of the *GAL* promoters. One component of glucose repression appears to act directly on the *GAL* promoters (pathway 1 in Fig. 8). This conclusion is based on our observation that *GAL* gene regulatory sequences located between UAS_{GAL} and the transcription initiation site are sufficient to provide for catabolite repression. The hybrid promoter diagrammed in Fig. 9 (line 2), which consists of the UAS from the non-glucose-regulated gene *LEU2* in place of UAS_{GAL}, is repressed during growth on glucose (Flick, unpublished data). Therefore, we conclude that the *GAL* sequences present in this promoter are targets for catabolite repression. This is consistent with the observation that deletion of these sequences from a normal *GAL* promoter partially relieves catabolite repression (155). However, there is no direct evidence that a protein responsible for catabolite repression binds to this region of the promoter because no in vivo footprints specific to the catabolite-repressed state are apparent in this region of DNA (130).

(ii) Inhibition of GAL4 protein DNA binding. One mechanism of glucose repression is to prevent binding of GAL4 protein to DNA (pathway 2 in Fig. 8). This is apparent because the footprint of GAL4 protein on its binding site in vivo (42, 130), as well as the pattern of sites hypersensitive to DNase I cleavage that it causes (86), is absent in cells grown on glucose. This could be due to action of GAL80

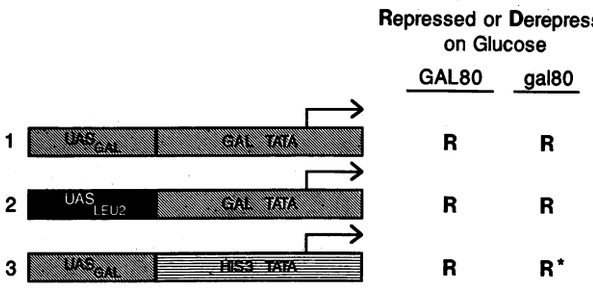


FIG. 9. Susceptibility of various hybrid *GAL* promoters to catabolite repression, summarizing our results from quantifying expression of these promoters (by Northern blots) in strains grown on galactose plus glucose. All of these promoters are completely repressed by glucose, except for the promoter shown on line 3 (R^*), which is only slightly resistant to glucose repression (~1 to 2%) in a *gal80* strain (Flick, unpublished data).

protein or other unidentified gene products. We favor a role for an unidentified gene product based on results of an experiment summarized in Fig. 9. The hybrid promoter diagramed on line 3 requires GAL4 protein for expression because of the presence of UAS_{GAL}; all other sequences come from the non-glucose-repressed *HIS3* promoter. Expression from this promoter, which reflects GAL4 protein activity, is repressed by glucose in a *GAL80* strain; in a *gal80* strain this promoter is still mostly repressed during growth on glucose (Flick, unpublished data). This suggests that a protein(s) other than GAL80 protein is responsible for preventing GAL4 protein from binding to DNA. A direct role for GAL4 protein in catabolite repression is also suggested by the observation that *GAL4* can provide glucose repression of galactose-regulated genes in the yeast *K. lactis*, which is not normally sensitive to this regulatory mechanism (121).

(iii) Inhibition of inducer-GAL80 protein interaction. Glucose could cause repression of *GAL* gene expression by preventing the inducer from inactivating GAL80 protein activity (pathway 3 in Fig. 8). Effects of glucose on inducer levels appear to be responsible for some amount of glucose repression (48), but it cannot be the sole mechanism of catabolite repression because mutants with no GAL80 protein (due to deletion of the gene) are almost fully repressed for *GAL* gene expression during growth on glucose (line 1, Fig. 9) (113, 149, 162).

(iv) Inhibition of inducer synthesis. The *GAL3* gene is regulated by the GAL4 and GAL80 proteins (cited in reference 150). Therefore, glucose also apparently inhibits synthesis of the inducer (pathway 4 in Fig. 8) by repressing transcription of *GAL3*.

(v) Inhibition of galactose transport. The transport of galactose into yeast cells is inhibited by glucose at two levels (pathway 5 in Fig. 8). First, synthesis of the permease is repressed during growth on glucose, because *GAL2* expression is subject to catabolite repression (151). In addition, glucose inactivates preexisting permease molecules, a process termed "catabolite inactivation" (57, 92). This is thought to occur by activation of one or several proteases during growth on glucose. This interesting regulatory mechanism has attracted little attention because most of the interest in *GAL* gene regulation has focused on transcriptional control. These two controls of permease levels ensure close regulation of galactose transport: catabolite inactivation of the permease acts immediately to reduce transport rates; catabolite repression of *GAL2* causes a more severe but delayed reduction of galactose transport.

In summary, catabolite repression acts on at least three separate steps in the *GAL* gene regulatory circuit: directly on the promoters (between UAS_{GAL} and the transcription initiation site), on GAL4 protein (to prevent it from binding to DNA), and on inducer levels (by repression of *GAL3* and by inhibition of galactose transport). It is not clear why redundant mechanisms of catabolite repression evolved, but it seems that yeast cells able to carefully control which carbon source they utilize have a significant advantage over cells that are less careful about this matter. Perhaps these multiple regulatory mechanisms evolved in a manner analogous to evolution of the repressor of the *trp* operon of *E. coli*. Since the *trp* operon is controlled by attenuation, repression mediated by the *trp* repressor would seem to be redundant. However, the *trp* repressor also acts upon the *aro* operon (45), and it has been proposed that control of *aro* expression is the role for which the *trp* repressor initially evolved; only later was it recruited for repression of *trp* expression (160). Perhaps some of the mechanisms of catabolite repression of *GAL* gene expression initially evolved as the sole means of controlling other genes and later became adapted for repression of the *GAL* genes.

Common Functions for Catabolite Repression of Several Genes

Some functions of the mechanism of catabolite repression must be shared among several genes whose expression is regulated by glucose, because mutations in most of the genes listed in Table 2 affect repression of several genes. These genes may be involved in the production of a common signal for catabolite repression (step 6 in Fig. 8), or they may be responsible for functions more directly involved in repression of gene expression. Possible roles for two of these genes are suggested by the identification of the functions they encode.

The *HXK2* gene encodes hexokinase PII, one of the three enzymes in yeasts that phosphorylates glucose (84). Mutations in *HXK2* cause several genes to be resistant to catabolite repression (37, 89). Because some *hxk2* mutations relieve catabolite repression without affecting hexokinase activity, it has been proposed that the enzyme has separable catalytic and regulatory functions (38, 39). However, these two domains are apparently not localized in distinct regions of the protein (H. Ma and D. Botstein, unpublished data cited in reference 89). Because hexokinase catalyzes an early step in the metabolism of glucose, it is likely to be involved in one of the earliest steps of catabolite repression.

Several genes possibly involved in regulation of *GAL* gene expression (*SNF1*, *-2*, *-4*, and *-5* and *SSN20*) were identified by isolating mutants unable to express *SUC2* (encoding invertase) as well as other genes subject to catabolite repression, including the *GAL* genes (20, 105, 106). Only the product of the *SNF1* gene, which is a protein kinase, has been identified (22). This finding suggests that protein phosphorylation plays a role in catabolite repression in yeasts. Of great interest is the identity of the proteins phosphorylated by SNF1 protein. The SNF1 protein probably has separate targets in the *GAL* and *SUC* regulatory circuits, because suppressors of *snf1* mutations, selected for their effects on *SUC* expression (*ssn6*, for example [21]), do not suppress the defect in *GAL* gene expression caused by *snf1* mutations. A potential target of SNF1 protein in the *GAL* gene regulatory circuit is GAL4 protein. Consistent with this possibility

is our observation that *gal80* mutations do not suppress the defect in *GAL* gene expression caused by *snf1* mutations (Flick, unpublished data). Mutants (*crp*) of *E. coli* with pleiotrophic defects similar to those of yeast *snf* mutants were instrumental in elucidating mechanisms of catabolite repression in that organism, so an understanding of the roles of the various *SNF* genes is likely to contribute greatly to our knowledge of mechanisms of catabolite repression in yeasts.

GAL PROMOTER VECTORS FOR REGULATED HIGH-LEVEL GENE EXPRESSION IN *S. CEREVISIAE*

Because of the high level of gene expression that they direct, the *GAL* gene promoters are attractive for use in vectors designed for high-level regulated gene expression in *S. cerevisiae*. There are two advantages to using the *GAL* promoters to direct gene expression. First, the ability to grow cells under conditions that almost completely repress these promoters allows one to express genes whose products may be toxic to yeast cells (6). Second, these promoters are among the strongest in *S. cerevisiae*, directing transcription of 0.25 to 1% of the total polyadenylated RNA (142). Even higher levels of gene expression can be obtained if these promoters are present on high-copy-number yeast plasmids (5, 17).

Three types of vectors for directing gene expression from the *GAL* promoters are available. Probably the most popular are those that carry all *GAL* control sequences, including the transcription initiation site, but no ATG translation initiation codon (pBM150 in reference 65 and YEp51 and -52 in reference 18). Genes inserted in these vectors (at unique restriction sites located between the *GAL* transcription initiation sites and the *GAL* ATG codon) are expressed by using the control elements and transcription initiation sites of the *GAL* promoters; the ATG codon is provided by the inserted gene (for some examples, see references 6, 62, 76, and 154). There are also vectors that allow expression of a fused protein by use of the *GAL* ATG codons (YEp61 and -62 in reference 18, G1 in reference 46, and pBM756, -757, and -758 [M. Johnston, unpublished data]). Finally, vectors are available in which the gene placed under control of UAS_{GAL} provides both the transcription and the translation initiation functions (G2 in reference 46 and pBM126 [Johnston, unpublished data]). An alternative approach for making expression of a gene inducible by galactose is to replace its UAS with UAS_{GAL} (48) (for examples, see references 11, 63, and 104). All of these vectors have been successfully used to express genes in yeast cells.

COMPARISONS WITH OTHER REGULATORY CIRCUITS

The mechanism of regulation of *GAL* gene expression is probably the best understood regulatory circuit in yeasts. Because many features of the mechanism are common to several other yeast regulatory circuits, detailed knowledge of how this mechanism operates is sure to contribute to a more general understanding of mechanisms of yeast gene expression. The general properties of regulatory mechanisms shared by many yeast genes include (i) positive control of gene expression mediated by a protein that activates transcription (e.g., GAL4 protein) and (ii) negative control operating at the posttranscriptional or posttranslational level by a protein (e.g., GAL80 protein) that indirectly represses transcription by inactivating the activator protein. As discussed below, proteins whose mechanism of action appear very similar to those of GAL4 and GAL80 proteins mediate the regulation of many (and possibly most) yeast genes. In addition, certain aspects of *GAL* gene regulation are similar to genetic regulatory mechanisms in higher eucaryotes.

Similarities to Other Fungal Regulatory Circuits

(i) Direct positive controls. Because the first genetic regulatory mechanisms to be elucidated in bacteria were those that act to repress gene expression, it was initially thought that most genes are negatively controlled in *E. coli*. Although several examples of positive control of gene expression in *E. coli* are now known (120), negative control is still recognized as a common theme in the regulation of bacterial gene expression. In contrast, most fungal genes that have been studied are primarily under positive control. That is, the regulatory proteins act on DNA to activate gene expression; without their intervention the genes are unexpressed. Metzenberg has suggested that negative regulation is unacceptable as the predominant control mechanism for eucaryotic cells, because of their large size (100). The concentration of specific genes is much lower in eucaryotes than in the smaller bacterial cells, possibly leading to a requirement for much higher concentrations of repressor proteins to maintain suitably low levels of expression of negatively controlled genes. With positive control, lower levels of regulatory proteins would be required, although the cost of this efficiency might be lower maximal levels of gene expression. Metzenberg has proposed that the major advantage to the cell of keeping the protein concentration low is the attendant reduction in osmotic pressure inside the cell.

Listed in Table 3 are many of the fungal genes whose regulation is best understood (column 1). All of these genes, except the last three listed, are positively controlled: they require another protein to be expressed (or induced above basal levels of expression). I have termed this protein the "primary activator" (column 2), because it presumably acts directly on DNA to activate gene expression. Several of these proteins are known to be DNA-binding proteins (GAL4, GCN4 [59], QA1F [8], HAP1 [116], MATα1 [9]); most of the others probably also bind to DNA to activate transcription.

The mechanisms of action of these proteins are likely to be very similar. First, many of them probably bind to DNA by use of the cysteine-zinc finger motif. This has clearly been shown for GAL4 protein (64a, 66) and is almost certainly true for the other proteins that also contain this sequence motif (Fig. 5B). However, the GCN4 protein does not contain the cysteine-zinc finger and must therefore use some other sequence for its DNA-binding function (55).

There are suggestions that some of the proteins listed in Table 3 use a similar mechanism to activate transcription. It is generally assumed that proteins such as GAL4 protein activate transcription through interactions with proteins of the transcriptional apparatus (36, 119, 146). The regions of the GAL4 and GCN4 proteins responsible for transcription activation are unusually rich in acidic amino acids (58, 90). Portions of the GCN4 protein activation domain are functional provided they contain enough acidic amino acids (146). Perhaps all that is required for stimulation of transcription is interaction of these acidic protein domains, in a sequence-nonspecific manner, with some complementary (presumably basic) domain of another protein that acts close to the site of transcription initiation. In this regard, it is interesting to note that several of the proteins listed in Table

TABLE 3. Regulatory proteins for several fungal genes[a]

Target gene(s)	Primary activator	Constitutive mutation	Primary repressor	Secondary activator	Effector	Reference(s)
GAL1, -2, -7, -10	GAL4	*GAL81*	GAL80		Galactose	
PHO5	PHO4 + PHO2	*PHO82*	PHO80 + PHO85	PHO81	Inorganic PO_4	114
PHO8	PHO4	*PHO82*	PHO80 + PHO85	PHO81	Inorganic PO_4	114
HIS4 et al.	GCN4		GCD1 et al.	GCN1 et al.	Amino acids	44, 54
QA2, -3, -4 (N. crassa)	QA1F	*QA1F*[c]	QA1S		Quinic acid	41
PHO2, -3 (N. crassa)	NUC1	*NUC1*[c]	PREG + PGOV	NUC2	Inorganic PO_4	100
PRT (N. crassa)	CYS3		SCON		Sulfate	100
DAL1-4, DUR1-4	DAL81		DAL80		Allophanate	27
ADH2	ADR1	*ADR1*[c]	ADR4		Glucose	28
CHO1, INO1	INO2 + INO4	*OPI5*	OPI1			53
MAL41, -42	MAL43	*MAL43*[c]			Maltose	23
CYC1, -7	HAP1, -2, -3				Heme	116
ENO1, -2, TDH1-3	GCR1					55a
URA1, -3	PPR1					70
STE3 et al.	MATα1					9
LEU1, -2	LEU3				Leucine	163
CAR1, -2			CAR80	ARG80, -81, -82	Arginine	27
ARG3			ARG80, -81, -82		Arginine	27
STE6 et al.			MATα2			64

[a] See text for further explanation.

3 have regions that are unusually acidic. The most extreme case is LEU3 protein, in which 19 of 20 amino acids near the carboxyl terminus are acidic in character (163). Another striking example is PHO4 protein, which has a region of 77 amino acids near its amino terminus in which over 30% of the residues are acidic (81). Many of the other proteins listed in Table 3 (PHO2 [131], PPR1 [70], ARGRII [99], ADR1 [51], QA1F [8], MAL63 [equivalent to MAL43] [J. Kim and C. Michels, personal communication], and HAP1 [K. Pfeiffer and L. Guarente, personal communication]) also have stretches of amino acids that are highly acidic. In addition, some regulatory proteins in higher eucaryotes may also activate transcription in a similar manner: the virion-associated protein (VP16) of herpes simplex virus that activates transcription also has an unusually acidic domain responsible for this function (S. Triezenberg and S. McKnight, personal communication). It seems possible that all of these proteins activate transcription by a common mechanism, so a detailed understanding of how GAL4 protein works is likely to be of general significance to mechanisms of eucaryotic gene regulation.

There are, however, a few examples of direct negative control of yeast gene expression. Listed in the last three lines of Table 3 are genes known or thought to be directly regulated by repressor proteins. The best characterized of these repressors, encoded by the *MATα2* gene, represses transcription of genes involved in determining the cell type of yeasts (e.g., *STE6*) by binding to DNA upstream of these genes (64). Less well understood is how expression of *CAR1*, *CAR2*, and *ARG3* are repressed (27). The *ARG80*, *-81*, and *-82* genes encode proteins thought to constitute a molecule that represses transcription of *ARG3* (encoding an enzyme for arginine biosynthesis). This same repressor also indirectly activates expression of *CAR1* and *CAR2* (encoding enzymes for arginine catabolism) by inactivating the product of the *CAR80* gene, which is thought to be a direct repressor of *CAR1* and *CAR2* expression. It is somewhat surprising that one molecule (ARG80, -81, -82) can directly repress one gene (*ARG3*), presumably by binding to DNA, and indirectly activate another set of genes (*CAR1* and *-2*), presumably by binding to the CAR80 protein. By analogy to the *GAL* regulatory circuit, it seems possible that the CAR80 and ARG80, -81, and -82 proteins act on other as yet unidentified proteins that are direct primary activators of gene expression.

Similar negative regulatory mechanisms may also be operative on the *GAL* genes. Catabolite repression of *GAL* gene expression may be mediated by a protein that binds to the region of DNA between UAS_{GAL} and the TATA box. This may be similar to an artificial case of negative regulation in which a protein bound to DNA between UAS_{GAL} and the TATA box reduces *GAL* gene expression (14). Another component of catabolite repression that works on UAS_{GAL} seems to work at a distance and may be due to direct repressors that bind to that sequence (145). The details of how these potential negative regulatory mechanisms operate on the *GAL* genes remain to be elucidated.

(ii) Indirect negative control. Negative regulation of most of the genes listed in Table 3 is indirect: the repressor works by inhibiting the primary activator posttranscriptionally. The gene products responsible for this are designated "primary repressors" and are listed in Table 3 (column 4). Sites in the primary activator responsible for interaction with the repressors are presumably defined by the constitutive mutations in some of the activators (listed in column 3). Evidence for a posttranscriptional mode of action of the repressor is most extensive for the GAL80, GCD1 (54, 55), and PHO80+PHO85 (114) proteins; the other repressors listed probably also regulate the function of their respective activator proteins after transcription. Unlike the case for GAL80 protein, however, the GCD1 protein regulates the translation of *GCN4* (54); other *GCD* genes do seem to regulate *GCN4* transcription as well as translation (44, 50). The functions of the proteins encoded by *DAL80*, *ADR4*, and *OPI1* are less well defined, but they are good candidates for repressors that inactivate primary activator proteins. A repressor protein has not yet been identified for six of the genes listed in Table 3.

The specificity of regulation is due to the ability of the primary repressor (e.g., GAL80 protein) to specifically recognize the effector molecule (e.g., a metabolite of galactose). Hence, the effector indirectly influences gene expression by binding to proteins whose mode of action is at least one step removed from the final event. This is in contrast to the usual

situation in bacteria, in which the effector (e.g., allolactose) binds to the direct regulator of gene expression (e.g., the *lac* repressor). This generalization predicts that a repressor will be identified for *MAL61* and *MAL62*, genes whose transcription is induced by maltose. In the best-understood cases, the effector inactivates the repressor. This is even true for genes whose expression is repressed by the effector, such as the *PHO* genes of yeasts and filamentous fungi. These genes achieve the proper response to the effector through a third regulatory protein, termed the "secondary activator" (listed in column 5 of Table 3). The effector of *PHO* gene expression (inorganic phosphate) inhibits the secondary activator (encoded by *PHO81*), causing the primary repressor (PHO80+PHO85) to inactivate the primary activator (PHO4+PHO2), thereby repressing transcription; removal of the effector causes the secondary activator (PHO81) to inhibit the primary repressor (PHO80+PHO85), thereby freeing the primary activator to activate transcription. Such a cascade of regulatory events seems to be a general feature of fungal gene regulatory circuits.

Similarities to Regulatory Mechanisms in Higher Eucaryotes

The mechanism of *GAL* gene regulation is also relevant to understanding how genes are expressed in higher eucaryotes. The similarities between UAS elements of yeasts and enhancer elements of mammals have already been noted. In addition, many features of the gene regulatory circuit are remarkably similar to the mechanism by which expression of certain genes in mammalian cells is induced in response to steroid hormones. These hormones induce gene expression by binding to receptor proteins in the cytoplasm, which cause these proteins to enter the nucleus, bind to DNA, and activate transcription of certain genes (reviewed in reference 159). This family of proteins shares many properties with GAL4 protein.

First, the glucocorticoid hormone receptor binds to short sequence elements (termed glucocorticoid response elements or GRE) that lie upstream of genes that it regulates. Like UAS_{GAL}, GRE work over variable distances as modules that allow any gene that carries this sequence (in the proper location) to be induced by the hormone. Like GAL4 protein, the receptor protein probably uses a cysteine-zinc finger to bind to the GRE sequence. This sequence motif is also present in several other eucaryotic DNA-binding and transcriptional activator proteins (for a partial tabulation, see reference 66), implying that they all bind to DNA in a similar manner. The glucocorticoid receptor may also use sequences immediately adjacent to the cysteine-zinc finger to specifically recognize the GRE (123). This is similar to GAL4 protein, which may possess two subdomains responsible for general and sequence-specific DNA binding (Fig. 6). The glucocorticoid receptor also contains a domain responsible for activation of transcription that presumably interacts with a protein(s) of the transcriptional apparatus. However, unlike the case for GAL4 protein, this domain does not appear to be separable from the DNA-binding domain (56, 101).

The most striking similarity of the mechanism of action of steroid hormone receptors with the *GAL* gene regulatory circuit is the manner by which the inducer activates gene expression. The hormone binds to a domain in the receptor protein whose function is remarkably analogous to that of GAL80 protein. In the absence of hormone, this domain of the protein inhibits transcription activation, possibly by binding to the sequences of the receptor that interact with proteins of the transcriptional apparatus. Like GAL80 protein, this region of the receptor is presumably prevented from inhibiting transcription activation when inducer binds to it. Furthermore, deletion of the hormone-binding domain has the same effect on receptor function as deletion of *GAL80* has on GAL4 protein: it causes constitutive activation of transcription (43, 56). Perhaps higher eucaryotes regulate gene expression by using mechanisms similar to those responsible for regulation of the yeast GAL genes.

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