Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6971–6975, November 1982
Genetics

Isolation of the yeast regulatory gene *GAL4* and analysis of its dosage effects on the galactose/melibiose regulon

(eukaryotic gene regulation/recombinant DNA)

STEPHEN A. JOHNSTON AND JAMES E. HOPPER

Department of Biological Chemistry, and the Specialized Cancer Research Center, The M. S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

Communicated by Oliver E. Nelson, Jr., August 31, 1982

ABSTRACT ***GAL4* is a classically defined positive regulatory gene controlling the five inducible structural genes of galactose/melibiose utilization in yeast. The positive regulatory function of the *GAL4* gene product in turn is controlled by the product of another gene, the negative regulator *GAL80*. We have cloned a 3.1-kilobase fragment containing *GAL4* by homologous complementation using the multicopy chimeric vector YEp24 and demonstrated that multiple copies of *GAL4* in yeast have pronounced dosage effects on the expression of the structural genes. Yeast transformed with *GAL4*-bearing plasmid become constitutive for expression of the galactose/melibiose genes, even in normally repressing (glucose) medium. Multiple copies of the *GAL4* plasmid also increase expression of the structural genes in inducing (galactose) medium and can partially overcome the effects of a dominant super-repressor mutant, *GAL80*^{S}. Using an internal deletion in *GAL4*, we have demonstrated that these dosage effects are due to overproduction of *GAL4* positive regulatory product rather than an effect of the flanking sequences titrating out a negative regulator. These results point to the importance of competitive interplay between the positive and negative regulatory proteins in the control of this system. We have also used the dosage effect of *GAL4* plasmid in combination with different *GAL4* and *GAL80* alleles to create new phenotypes. We interpret these phenotypes as indicating that (*i*) the repressing effects of glucose, at least in part, are mediated by the product of the negative regulatory gene, *GAL80*, and (*ii*) the *GAL80* protein may have specific interactions with the control regions of the structural genes.**

A well-defined system for delineating aspects of eukaryotic regulation is the galactose/melibiose utilization regulon in the yeast *Saccharomyces cerevisiae*. The *GAL4* gene in this system encodes a positive regulatory protein required to express transcriptionally the structural genes for galactose/melibiose metabolism (1–3): *GAL1* (galactokinase, EC 2.7.1.6), *GAL7* (α-D-galactose-1-phosphate uridyltransferase, EC 2.7.7.12), *GAL10* (uridine diphosphoglucose 4-epimerase, EC 5.1.3.2), and *MEL1* (α-galactosidase, EC 2.1.1.22) (4, 5). *GAL4* controls expression at transcription (3, 6–8). The system also involves a negative regulatory gene, *GAL80*, which codes for a protein that prevents expression of the structural genes in the absence of inducer (9). At least three allelic states exist for both *GAL4* and *GAL80* (Table 1).

Douglas and Hawthorne (10) proposed an operator/repressor control circuit for the galactose regulon, analogous to bacterial systems. In their model, *GAL80* protein ("i protein" by their nomenclature) represses *GAL4* transcription under noninducing conditions by binding at the operator for *GAL4*. Induction involves release of *GAL80* repressor, *de novo GAL4* transcription and translation, and *GAL4* protein-mediated transcription of the structural genes. However, later experiments have forced a revision of this model. First, different allelic combinations of *GAL80*^{S} and *GAL4*^{c} have quite different phenotypes, suggesting *GAL80-GAL4* protein–protein interactions (11). Second, two different experimental approaches (2, 6) have supported the conclusion that *GAL4* protein is produced constitutively, not derepressed as predicted by the Douglas–Hawthorne model. And third, it has been demonstrated that the *GAL4*^{c} mutations map within the protein coding region of *GAL4* (12), not at one end of the gene as might have been expected for operator mutations at *GAL4* (*GAL4*^{c}) and envisioned by the Douglas–Hawthorne model.

Taking into account this new evidence, two groups of workers proposed another model (2, 6) in which the system is kept off in noninducing conditions as a result of binding of constitutively produced *GAL4* protein by the *GAL80* protein to form an inactive complex. In the presence of inducer, the *GAL4* protein is freed (or the *GAL4–GAL80* protein complex is altered), allowing it to activate transcription of the structural genes. Recently, the results of Laughon and Gesteland (13) have lent more support to one aspect of this model by showing that the *GAL4* mRNA transcript is constitutively produced.

As a part of our efforts to discover how *GAL4* and *GAL80* genes regulate gene expression, we have cloned *GAL4* on a multicopy plasmid by complementation in yeast. We have determined that multiple copies of *GAL4* produce constitutive expression of the structural genes. A model based on noncatalytic *GAL4–GAL80* protein interactions predicts that multiple copies of constitutively expressed *GAL4* gene might produce enough *GAL4* protein to titrate out the negative *GAL80* protein, allowing expression of the structural genes under noninducing conditions. This prediction is also of interest because the presumed lack of dosage effect of a regulatory gene has been used to distinguish regulatory from structural genes (14). One report used this criterion to support the conclusion that *GAL4* was a regulatory gene (15).

Having established that multiple copies of *GAL4* can produce constitutive galactose/melibiose regulon expression, we then determined whether new phenotypes produced by combining multiple copies of *GAL4* with various *GAL4* and *GAL80* alleles could provide further insight into the circuitry and mechanism of regulation.

MATERIALS AND METHODS

Yeast and *E. coli* Strains and Media. Yeast strain 21 was *a gal4*-2 *ura*3-52 *leu*2-3 *leu*2-112 *ade*1 *MEL*1; and strain 21R was a *GAL4* revertant of 21 having normal induced galactokinase and uridyltransferase levels (compared to a standard laboratory wild-type strain, Sc128). For the N-series strains (see Tables 4 and 5), spontaneous *gal80*-100 and *GAL4*^{c}-100 mutations were isolated from strain 7d (α *GAL80*^{S}-100 *ura*3-52 *leu*2-3 *leu*2-112

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: kb, kilobase(s).

Table 1. Regulatory alleles of the galactose system and their interactions

Genotype	Phenotype
GAL4 GAL80	Wild-type; repressed by glucose; induced by galactose
gal4 GAL80*	Uninducible; recessive (4)
GAL4^{c} GAL80*	Constitutive; dominant (10)
*GAL4 gal80**	Constitutive; recessive (10)
*GAL4 GAL80^{S}**	Uninducible; dominant (9)
gal4 gal80	Uninducible
GAL4^{c} GAL80^{S}	Constitutive; uninducible or inducible depending on alleles involved (11)

Recessive or dominant refers to the allele marked *.

*MEL*1). These isolates and the parent were crossed to 21, and segregants with the desired *GAL4* and *GAL80* allelic combinations were obtained. All isolates were *ura*3-52 *leu*2-3 *leu*2-112 *MEL*1 and had a coefficient of kinship of 0.35. In each strain the *GAL4* and *GAL80* wild-type alleles were identical. Unless otherwise noted, yeast strains were grown on complete medium (3) lacking uracil, to which one of the following carbon sources was added (wt/vol); 2% glucose, 2% galactose, or 3% glycerol plus 2% lactic acid.

Plasmid was propagated in an *Escherichia coli* K-12 derivative, RR1 (F^- pro^- leu^- thi^- rK^- mK^- $endoI^-$), provided by K. Tatchell. Growth was in Luria broth.

Transformation. Yeast transformation was by the method of Hinnen *et al.* (16) with minor modifications and gave a transformation frequency of 10^{-2} per viable spheroplast. *E. coli* transformation was by the protocol of Villa-Komaroff *et al.* (17).

Cloning and Subcloning *GAL4*. Yeast strain 21 was transformed to Ura^+ by using a recombinant DNA pool of yeast genomic DNA in the vector YEp24 provided by S. Falco, M. Carlson, and D. Botstein. YEp24 has the yeast *URA3* gene inserted into the *Hin*dIII site of pBR322 and the small *Eco*RI fragment of yeast 2-μm circle (B-form) into the *Eco*RI site of pBR322 (18). Plates containing Ura^+ transformed colonies of strain 21 were poked with a florist's frog and rinsed with sterile water. Aliquots of the rinse were spread on complete/galactose plates at a density of 10^4 cells per plate in order to select GAL^+ transformants. GAL^+ colonies appeared in 4–6 days. These were restreaked on medium containing galactose and lacking uracil to isolate single colonies. Plasmid was extracted from yeast (19) and used to transform *E. coli* strain RR1 to ampicillin resistance. Plasmid isolated from single *E. coli* colonies (20) was used to retransform yeast and for restriction analysis. All plasmids capable of retransforming strain 21 to Gal^+ had inserts with overlapping restriction patterns.

A purified plasmid preparation was made (21) of one plasmid, SJ3. SJ3 was totally digested by *Bam*HI and then partially digested by *Sau*3A. The restriction fragments were religated into the single *Bam*HI site of YEp24. A plasmid pool was made from these ligations and used to transform yeast strain 21 to Gal^+. Plasmid was reisolated from individual Gal^+ transformants and used to transform *E. coli* RR1 to ampicillin resistance, and the insert size of each plasmid was determined as above. The plasmid with the smallest insert [3.1 kilobases (kb)] was SJ4.

Assays. For enzyme assays, cells were grown in media lacking uracil and supplemented with a carbon source. Cells were harvested in midlogarithmic phase and a crude extract made as described (22). The α-galactosidase (5), galactokinase (23), and uridyltransferase levels were assayed in the crude extract. Uridyltransferase was assayed by mixing 37.5 μl of diluted enzyme extract with 12.5 μl of premix (0.5 M glycylglycine, pH 7.5/17.2 mM UDP-glucose/4 mM [^{14}C]galactose-1-*P* at 1,000 dpm/nmol or 4,000 dpm/nmol). This was incubated at 30°C for 30 min and then boiled for 1 min. *E. coli* alkaline phosphatase (5 μl; 0.6 unit) was added and the mixture was incubated at 37°C for 90 min. Water (50 μl) was added, the solution was spun at 12,000 × *g* in a Microfuge, and 50 μl of the supernatant spotted on a Whatman DE81 filter. The filter was washed in water, dried, and assayed in Liquifluor (New England Nuclear). Protein determinations were done by the method of Bradford (24).

Plasmid copy number was determined essentially by the method of Zakian and Scott (25) except that cells were grown to midlogarithmic phase in medium lacking uracil and the DNA was labeled with [8-^{14}C]adenine (1.3×10^5 dpm/nmol).

Materials. *p*-Nitrophenyl α-D-galactopyranoside for the α-galactoside assay and *E. coli* alkaline phosphatase were from Sigma. The D-[^{14}C]galactose, [^{14}C]galactose-1-*P*, and [8-^{14}C]-adenine were from New England Nuclear. Restriction endonucleases were from Bethesda Research Laboratories and New England BioLabs; T4 DNA ligase was also from New England BioLabs.

RESULTS

Cloning the *GAL4* Gene. The plasmid isolated, SJ3, that complemented the chromosomal *gal4* lesion in yeast strain 21 had an insert of 7.7 kb. Its restriction map as well as that of subclone SJ4 are shown in Fig. 1. SJ4 comprises 3.1 kb of the left end of the insert of SJ3 inserted into the *Bam*HI site of YEp24 in the opposite orientation.

When strain 21 transformed with SJ3 (21[SJ3]) was grown with uracil in the medium, the plasmid was lost from approximately 50% of the cells after 10 generations. There was strict concomitant loss of the Ura^+ and Gal^+ phenotypes, supporting the conclusion that the gene responsible for the Gal^+ phenotype was plasmid-borne. The following strategy was used to show that this gene was *GAL4*. First, an isolate with SJ3 integrated into the chromosome was found. This was made easy by the fact that multiple copies of SJ3 caused constitutive expression of α-galactosidase (*MEL1*). Colonies with free plasmid appeared yellow in a plate assay for α-galactosidase (26) on medium containing glucose and lacking uracil; integrants were white. In this way a mitotic segregant of strain 21[SJ3] that stably expressed the Ura^+, Gal^+ phenotype was picked. This isolate, 21-I, was crossed to a *gal4 ura3* sib, the diploid was induced to sporulate, and tetrads were dissected.

All 11 tetrads dissected were parental ditype (Ura^+, Gal^+ or Ura^-, Gal^-) indicating that the genes responsible for the Ura^+ and Gal^+ phenotypes were closely linked. Integration at the chromosomal *GAL4* locus by homologous recombination would strongly indicate that SJ3 contained *GAL4* because *URA3* and *GAL4* are on different chromosomes (27). This was demonstrated by backcrossing 21-I with its *GAL4 ura3* parent. All 24 tetrads dissected from this cross showed 2:2 segregation for Ura phenotype and no Gal^- segregants. Therefore, the plasmid had integrated at or near the *GAL4* locus. Because the *GAL4* region has been extensively mapped genetically (12) and no other gene that relieves the requirement for functional *GAL4* gene is

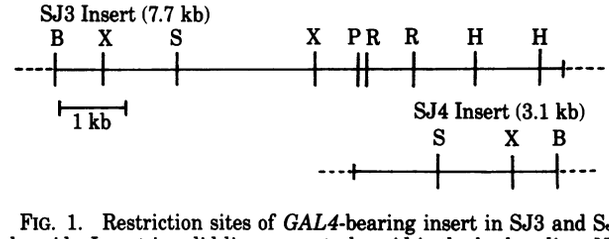

FIG. 1. Restriction sites of *GAL4*-bearing insert in SJ3 and SJ4 plasmids. Insert is solid line; parent plasmid is the broken line. Not all of the plasmid is shown. B, *Bam*HI; X, *Xho* I; S, *Sal* I; P, *Pst* I; R, *Eco*RI; H, *Hin*dIII.

known, we conclude that SJ3 contains *GAL4*.

Dosage Effects of SJ3. To study the effects of multiple copies of *GAL4* on the structural genes it controls, enzyme levels in various media were determined in the following strains: (*i*) 21[SJ3] (*gal4 ura3* with SJ3 plasmid), (*ii*) 21R[SJ3] (a *GAL4* revertant of 21, also transformed with SJ3), (*iii*) 21-I (21 with SJ3 stably integrated), and (*iv*) 21R[YEp24] (21R transformed with the parent plasmid). Although 21R was a revertant at *GAL4*, it had a wild-type phenotype (qualitatively and quantitatively) when tested against a standard wild-type Gal$^+$ strain (data not shown) and, therefore, is referred to as "wild-type" below. All strains were grown in media lacking uracil to maintain selection for cells containing the plasmid. Both α-galactosidase (*MEL1*) and uridyltransferase (*GAL7*) levels were measured in each strain to determine if their response to excess *GAL4* was parallel. It should be pointed out that 21, the *gal4* strain, had no detectable α-galactosidase or transferase activity when grown in any of the media.

From Table 2 it is clear that plasmid-borne *GAL4* produced constitutive expression of both *MEL1* and *GAL7* in glucose medium, conditions under which a single chromosomal copy of *GAL4* produced no detectable expression (21R[YEp24] and 21-I). The effect of multiple copies of plasmid-borne *GAL4* was also evident in inducing medium (galactose). In galactose medium, both 21[SJ3] and 21R[SJ3] had substantially higher levels of both enzymes compared to wild-type induced (21R[YEp24]), suggesting that *GAL4* protein may be a limiting factor in transcription of *MEL1* and *GAL7*. In strain 21[SJ3] (or 21R[SJ3]) the levels of both enzymes relative to wild-type induced were comparable in glucose and galactose media. However, in glycerol/lactic acid medium (noninducing, nonrepressing) there was a large difference in response between the two enzyme levels (92% wild-type induced α-galactosidase versus 11% for uridyltransferase). It appears, then, that there were quantitatively different responses of *MEL1* and *GAL7* to extra copies of *GAL4* in glycerol/lactic acid medium. Galactokinase (GAL1) had the same pattern of response as uridyltransferase (data not shown).

It was possible that the effect of plasmid-borne *GAL4* was due to the particular *GAL4* allele cloned rather than to a dosage effect. However, 21-I with the plasmid integrated had a qualitatively wild-type response in all three media (Table 2). The difference in levels between 21-I and 21R[YEp24] may be due in part to allelic differences in *GAL4* or to the fact that only 85–90% of the cells had free plasmid (were Ura$^+$) and therefore could grow, in the culture of 21R[YEp24].

The cultures of both 21[SJ3] and 21R[SJ3] grown in glucose (Table 2) were estimated to have 5–7 plasmids per cell by direct physical determination (25). Struhl *et al.* (28) and B. Hyman (personal communication) have estimated similar plasmids to have 5–10 copies per cell. Thus, the constitutive expression of *MEL1* and *GAL7* in glucose media was caused by multiple copies of a normal *GAL4* allele.

A direct physical estimate of plasmid copy number was not made for the other cultures in Table 2. Because the composition of the growth medium may influence plasmid copy number, the estimate of 5–7 copies may not apply to the other cultures in Table 2 or cultures reported in tables below. However, because none of the comparisons, except one discussed below, are between different transformed strains or different media, the actual plasmid copy number per cell is not essential to the arguments presented.

GAL4 DNA or GAL4 Protein Effects. There is strong indirect evidence that *GAL4* gene mediates its effect on transcription (3, 6–8) of the structural genes through its encoded protein (1, 3, 9, 12). Nevertheless, the dosage effect of SJ3 may have resulted from a response to more *GAL4* DNA sequences rather than from a product of *GAL4* gene. For example, more *GAL4* promoters or flanking regions could titrate out a repressing element. To test this possibility, a plasmid bearing an internal deletion in *GAL4* was constructed [based on information provided by A. Laughon (personal communication)] by restriction enzyme deletion of the *Xho* I–*Sal* I fragment of the insert in SJ4 (Fig. 1). This leaves intact the flanking 5′ and 3′ ends of *GAL4*. The *GAL4 ura3* strain, 21R, was transformed with this plasmid and the α-galactosidase enzyme levels were measured after growth in glucose and glycerol/lactic acid media. There was no increased activity over 21R[YEp24] (Table 3), indicating that the dosage effect of SJ3 was due to *GAL4* gene product rather than noncoding flanking sequences. We conclude that the effects on the enzyme levels observed were produced by increased levels of *GAL4* protein due to multiple copies of a normal *GAL4* allele.

GAL4 Dosage Effects in Strains Bearing Other GAL4 and GAL80 Alleles. To investigate further the dosage effects of *GAL4*, a series of related strains (N series) with different *GAL4* and *GAL80* alleles (see Table 1) were constructed and transformed with YEp24 and SJ3 plasmids. The levels of α-galactosidase and uridyltransferase activities were determined when the strains were grown in glucose medium. The results are presented in Table 4, and several comparisons are noteworthy.

First, all the strains responded dramatically to SJ3 dosage, with the exception of N-*GAL4^c GAL80*. Even the N-*GAL4 GAL80^S* strain, which normally is uninducible, was made constitutive with extra *GAL4* copies on SJ3. Although N-*GAL4^c GAL80* did not respond to SJ3 dosage in glucose or other media (data not shown), the other normally constitutive strain, N-*GAL4, gal80*, had a more than 10-fold increase in enzyme activities. The anomolous behavior of the *GAL4^c* strain might be explained if, as has been suggested from genetic studies, functional *GAL4* protein has a subunit structure (12) and heteromultimers composed of *GAL4^c* and *GAL4* subunits are not as effective as *GAL4^c* homomultimers in promoting transcription in glucose medium.

Table 2. Effect of SJ3 plasmid-bearing *GAL4* on α-galactosidase and uridyltransferase enzyme levels

	Enzyme activity in various media, %					
	α-Galactosidase			Uridyltransferase		
Strains	A	B	C	A	B	C
21[SJ3]	0.9	92	180	1.8	11	180
21-I	<0.1	9	140	<0.1	<0.1	120
21R[YEp24]	<0.1	3	100	<0.1	0.2	100
21R[SJ3]	1.2	180	250	2.7	15	210

21 is a *gal4 ura3* yeast strain and 21R is a spontaneous *GAL4* revertant. 21-I is 21 with SJ3 integrated. YEp24 is the vector without the *GAL4*-bearing insert. Enzyme levels are expressed as a percentage of 21R[YEp24] levels in galactose medium. The 100% values were: α-galactosidase, 0.24 μmol of *p*-nitrophenol formed per μg of protein per min; uridyltransferase, 0.15 nmol of product formed per μg of protein per min. Media: A, without uracil, with 2% glucose; B, with 3% glycerol and 2% lactic acid; C, with 2% galactose.

Table 3. Effect of a *GAL4* deletion-bearing plasmid on α-galactosidase levels in glucose and glycerol media

	Activity, %	
Strain	Glucose	Glycerol
21R[ΔSJ4]	<0.1	2.4
21R[YEp24]	<0.1	2.3

21R is a *GAL4 ura3* strain transformed with either a *GAL4* deletion plasmid ([ΔSJ4]) or the plasmid without the *GAL4* sequences ([YEp24]). Enzyme levels are expressed as a percentage of 21R[YEp24]-induced levels.

Table 4. Enzyme activity levels of α-galactosidase and uridyltransferase in yeast strains with different *GAL4* or *GAL80* alleles and with ([SJ3]) or without ([YEp24]) *GAL4*-bearing plasmid in glucose medium

Genotype	α-Galactosidase, %		Uridyltransferase, %	
	[SJ3]	[YEp24]	[SJ3]	[YEp24]
N-*GAL4 GAL80*	1.6	<0.1	2.1	<0.1
N-*GAL4 GAL80*^{S}	2.5	0.1	4.6	<0.1
N-*GAL4 gal80*	29	1.4	140	11
N-*gal4 GAL80*	0.6	<0.1	1.4	<0.1
N-*GAL4*^{c} *GAL80*	3.5	0.8	2.3	4.9

Enzyme levels are expressed as a percent of N-*GAL4 GAL80* [YEp24]-induced levels. The 100% values were: α-galactosidase, 0.35 μmol of *p*-nitrophenol formed per μg of protein per min; uridyltransferase, 0.17 nmol of product formed per μg of protein per min.

Second, the response of the *GAL4* strain (N-*GAL4*, *GAL80*) was similar to that of the *GAL4* revertant strain, 21R, reported in Table 2. Because N-*GAL4*, *GAL80* involves a wild-type *GAL4* allele, it implies that inferences drawn from data concerning 21R are applicable to wild-type *GAL4* alleles.

Third, there was a differential response of *MEL1* (α-galactosidase) and *GAL7* (transferase) in N-*GAL4 gal80*. Recall from Table 2 that, in glycerol/lactic acid medium, 21R[SJ3] had a much higher α-galactosidase than transferase level. In contrast, in N-*GAL4 gal80*[SJ3] the transferase level (as a percent of wild-type induced) was higher than the α-galactosidase level.

Fourth, and unexpectedly, N-*GAL4 GAL80*^{S}[SJ3] had higher levels of both α-galactosidase and transferase activities than did N-*GAL4 GAL80*[SJ3]. One might have expected that, even if multiple copies of *GAL4* could overcome the repressing effects of *GAL80*^{S}, the *GAL80*^{S} strain would still have lower levels of activity than the wild-type, *GAL80* strain, not higher levels as observed. This difference between *GAL80* and *GAL80*^{S} transformed strains was repeatable (see Table 5 for glucose) and was evident even in comparisons of whole-cell α-galactosidase assays involving the untransformed *GAL80* and *GAL80*^{S} strains, but differences were less pronounced (data not shown).

In order to assess whether or not catabolite repression (glucose) contributed to the difference between the *GAL80*^{S}[SJ3] and *GAL80*[SJ3] strains, the enzyme levels in these strains grown in neutral (glycerol/lactic acid) and inducing (galactose) media were determined. As expected, in galactose medium N-*GAL4 GAL80*[SJ3] had higher enzyme levels than N-*GAL4 GAL80*^{S}[SJ3], the reverse of the situation in glucose medium (Table 5).

In glycerol/lactic acid medium the interactions were complex. As with 21R[SJ3] in Table 2, for both N-*GAL4 GAL80*-[SJ3] and N-*GAL4 GAL80*^{S}[SJ3] the α-galactosidase activity was higher than the transferase relative to induced levels. However, there was a differential response of the two activities to *GAL80* and *GAL80*^{S} alleles—i.e., for α-galactosidase, N-*GAL4 GAL80* had higher levels; and for transferase N-*GAL4*, *GAL80*^{S} had higher levels. In summary, *MEL1* (α-galactosidase) and *GAL7* (transferase) responded differently to carbon source and allelic state of *GAL80*. Again, *GAL1* followed the same pattern as *GAL7* (data not shown).

DISCUSSION

Only two other classically defined eukaryotic regulatory genes have been cloned besides *GAL4* (29, 30). Little is known about the mode of action of the regulatory proteins encoded by these genes or their interactions with other regulatory elements. We have shown that overproduction of *GAL4* protein dramatically changes the regulation of the galactose/melibiose system in yeast. This suggests that, in this eukaryotic regulon at least, control rests in a competitive interplay between the positive and negative regulatory elements and that the phenotype of the system can be substantially altered by changing the relative abundance of the two. Metzenberg and Nelson (31) had proposed that such interactions might be important in eukaryotic regulation and presented data on the regulation of the phosphorus-utilization system in *Neurospora* to support the hypothesis.

We have used the cloned *GAL4* regulatory gene to test a central feature of the *GAL4* protein–*GAL80* protein interaction model proposed by Matsumoto *et al.* (2) and Perlman and Hopper (6). The model predicts that excess *GAL4* protein should titrate out the inhibiting effects of *GAL80* protein. We have found this to be the case not only in neutral medium but even in repressing (glucose) medium (Table 2). Constitutive expression of the *MEL1* and *GAL7* genes appears to be produced by *GAL4* protein rather than by *GAL4* flanking region DNA because a plasmid bearing a *GAL4* gene with an internal deletion did not elicit constitutive expression (Table 3). If noncoding sequences of *GAL4* were able to titrate out a repressing element [for example, *GAL80* protein in the Douglas–Hawthorne model (10)] one would have expected the *GAL4* deletion-bearing plasmid to produce greater than wild-type expression of *GAL7* and *MEL1* in noninducing media.

Multiple copies of *GAL4* also increased the level of expression of *MEL1* and *GAL7* above wild-type even in galactose medium (Tables 2 and 5), implying that even in inducing conditions the level of active *GAL4* is a limiting factor in the expression of the structural genes. We conclude that in cells with a single copy of *GAL4* grown in galactose medium there is a residual inhibition by *GAL80* protein (or some as yet undefined other regulatory element) or rate-limiting levels of the *GAL4* protein.

The *GAL4* protein–*GAL80* protein interaction model (2, 6) envisioned induction of the regulon as involving galactose, or a derivative, interacting with either *GAL4* or *GAL80* protein to produce either free *GAL4* protein or an activated *GAL4* protein–*GAL80* protein complex. We report that multiple copies of *GAL4* can produce wild-type induced levels of α-galactosidase in a wild-type strain grown in neutral medium (Tables 2 and 5) and wild-type induced levels of transferase in a *gal80* strain grown in glucose medium (Table 4). Both these results strongly support the conclusion that galactose, or a metabolite of it, is not directly required for the promotion of transcription by wild-type *GAL4* protein and that, with respect to the galactose/melibiose regulon, the results of addition of galactose or of excess wild-type *GAL4* are operationally the same.

The results discussed above support the idea that a form of intimate interplay and balance between the *GAL4* and *GAL80* proteins comprises the central feature of regulation in this system. Our data comparing the *MEL1* gene responses versus the *GAL7* gene responses to excess *GAL4* protein suggest that both *GAL4* and *GAL80* proteins bind DNA at the structural genes. The rationale for this is as follows. In comparisons of α-galactosidase and transferase activity levels within extracts from 21[SJ3] and 21R[SJ3] (Table 2), it is clear that *MEL1* is more responsive to excess *GAL4* than is *GAL7* in neutral medium. This difference may be due to differential affinity of *GAL4* protein for the control regions of *GAL7* and *MEL1*. However, two other within-extract comparisons suggest another possible explanation. First, the relative levels of transferase and α-galactosidase activities varied in opposite directions in the *GAL80*[SJ3] and *GAL80*^{S}[SJ3] strains (Table 5). Second, it was the transferase rather than the α-galactosidase level that was much higher in the *gal80*[SJ3] strain (Table 4). The fact that different allelic states of *GAL80* can produce qualitatively different responses in *MEL1* and *GAL7* suggests a direct interaction between *GAL80* protein and the regulatory sequences of the structural genes.

Table 5. α-Galactosidase (α-Gal) and uridyltransferase (Transf) of *GAL80* and *GAL80^S* strains transformed with SJ3 (a *GAL4*-bearing plasmid) and grown in various media

Genotype	Glucose			Galactose			Glycerol/lactic acid		
	α-Gal	Transf	With plasmid, %	α-Gal	Transf	With plasmid, %	α-Gal	Transf	With plasmid, %
N-*GAL4 GAL80*[SJ3]	1.7	1.5	82	170	120	57	110	16	76
N-*GAL4 GAL80^S*[SJ3]	2.4	5.2	79	93	70	95	74	25	76

The percentage of cells with plasmid was the number of viable cells that were Ura$^+$ at the time cells were collected for enzyme extraction. Enzyme levels are expressed as a percent of N-*GAL4 GAL80*[YEp24] induced levels. The 100% values were α-galactosidase, 0.35 μmol of *p*-nitrophenol formed per μg of protein per min; uridyltransferase, 0.17 nmol of product formed per μg of protein per min.

An additional ancillary indication from the data we reported is that *GAL80* protein may transmit the glucose-repressive effect. Matsumoto *et al.* (32) published data suggesting that either *GAL4* or *GAL80* protein normally transmits the glucose-repressive effect. We found that not only could multiple copies of *GAL4* gene partially overcome the effects of a *GAL80^S* allele, but also the levels of expression of both enzymes in the *GAL80^S*[SJ3] strain grown in glucose medium was observed to be higher than in the *GAL80*[SJ3] strain (Tables 4 and 5). Although plasmid copy number in the *GAL80*[SJ3] and *GAL80^S*[SJ3] strains may not have been the same, the differences in enzyme levels are probably real because they are also evident in the untransformed *GAL80* and *GAL80^S* strains. In extracts from the *gal80*[SJ3] strain grown in glucose medium there was wild-type induced level of transferase activity (Table 4). The phenotypes of both the *GAL80^S* and *gal80* strains could be explained if the lesions in the *GAL80* gene that produced the *GAL80^S* or *gal80* alleles altered the response of *GAL80* protein not only to galactose but also to glucose, so that the *GAL80^S* and *gal80* proteins were not as effective in transmitting the catabolite repression signal as the *GAL80* protein.

Any model for the regulation of the galactose/melibiose system should accommodate several lines of experimental data which now exist: (*i*) the system is inducible; (*ii*) both *GAL4* and *GAL80* proteins exist in the cell prior to induction and exhibit allele-dependent functional interactions; (*iii*) the *GAL4* protein is absolutely required for induction regardless of the allelic state of *GAL80*; (*iv*) addition of multiple copies of an intact *GAL4* gene (but not one carrying an internal deletion) produces constitutive expression of the structural genes; and (*v*) inducer is not required for *GAL4* positive regulatory function. In addition, we interpret our data to suggest that the *GAL80* protein mediates a glucose-repression effect and that there is a *GAL80*-mediated differential response of *GAL7* and *MEL1* genes to excess *GAL4*.

In light of these considerations, one way of envisioning the regulation of the system is the following. Under noninducing conditions, a *GAL4* protein–*GAL80* protein complex binds DNA at the structural genes, repressing *GAL4* protein-promoted transcription. *GAL80* protein mediates a catabolite repression effect. In the absence of the repressive effects of glucose (neutral medium or modification of *GAL80* protein) the *GAL4* protein–*GAL80* protein complex binds the regulatory sequences of *MEL1* less than those of *GAL1,7,10*, allowing limited expression of *MEL1* in response to any free *GAL4* protein. Induction would involve galactose-mediated relaxation of *GAL80*-protein binding to *GAL4*-protein and DNA, permitting *GAL4* protein to promote transcription. The essential modifications of the protein–protein interaction model (2, 6) suggested from the data we report here is that the *GAL4* protein–*GAL80* protein complex would be poised at the structural genes in the uninduced state and that both *GAL4* and *GAL80* proteins recognize regulatory sites at these genes. Both of these predictions should be directly testable.

We thank David Botstein for providing the YEp24 yeast pool and Allen Laughon and Ray Gesteland for communicating unpublished results. We appreciate the comments of Anita Hopper, Chuck Hill, Eugene Davidson, and Ross Shiman. S.A.J. was supported by a Rockefeller Foundation Postdoctoral Fellowship. The work was supported by National Institutes of Health Research Grant GM 27925.

1. Klar, A. J. & Halvorson, H. O. (1974) *Mol. Gen. Genet.* **135**, 203–212.
2. Matsumoto, K., Toh-e, A. & Oshima, Y. (1978) *J. Bacteriol.* **134**, 446–457.
3. Hopper, J. E., Broach, J. R. & Rowe, L. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2878–2882.
4. Douglas, H. C. & Hawthorne, D. C. (1964) *Genetics* **49**, 837–844.
5. Kew, O. M. & Douglas, H. C. (1976) *J. Bacteriol.* **125**, 33–41.
6. Perlman, D. & Hopper, J. E. (1979) *Cell* **16**, 89–95.
7. Hopper, J. E. & Rowe, L. B. (1978) *J. Biol. Chem.* **253**, 7566–7569.
8. St. John, T. P. & Davis, R. W. (1979) *Cell* **16**, 443–452.
9. Douglas, H. C. & Hawthorne, D. C. (1972) *J. Bacteriol.* **109**, 1139–1143.
10. Douglas, H. C. & Hawthorne, D. C. (1966) *Genetics* **54**, 911–916.
11. Nogi, Y., Matsumoto, K., Toh-e, A. & Oshima, Y. (1977) *Mol. Gen. Genet.* **152**, 137–144.
12. Matsumoto, K., Adachi, Y., Toh-e, A. & Oshima, Y. (1980) *J. Bacteriol.* **141**, 508–527.
13. Laughon, A. & Gesteland, R. F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6827–6831.
14. Carlson, P. S. (1972) *Mol. Gen. Genet.* **114**, 273–280.
15. Klar, A. J. & Halvorson, H. O. (1976) *J. Bacteriol.* **125**, 379–381.
16. Hinnen, A., Hicks, J. B. & Fink, G. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1929–1933.
17. Villa-Komaroff, L., Efstratiadis, A., Broome, S., Lomedico, P., Tizard, S. P. N., Chick, W. L. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3727–3731.
18. Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K. & Davis, R. W. (1979) *Gene* **8**, 17–24.
19. Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369.
20. Holmes, D. S. & Quigley, M. (1981) *Anal. Biochem.* **114**, 193–197.
21. Wensink, P. C., Finnegan, D. J., Donelson, J. E. & Hogness, D. S. (1974) *Cell* **3**, 315–325.
22. Bostian, K., Lemire, J. M., Cannon, L. E. & Halvorson, H. O. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4505–4508.
23. Blume, K. & Beutler, E. (1975) *Methods Enzymol.* **42**, 47–53.
24. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–252.
25. Zakian, V. A. & Scott, J. F. (1982) *Mol. Cell. Biol.* **2**, 221–232.
26. Buckholz, R. G. & Adams, B. G. (1981) *Mol. Gen. Genet.* **182**, 77–81.
27. Mortimer, R. K. & Schild, D. (1980) *Microb. Rev.* **44**, 519–571.
28. Struhl, K., Stinchcomb, D. T., Scherer, S. & Davis, R. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1035–1039.
29. Patel, V. B., Schweizer, M., Dykstra, C. C., Kushner, S. R. & Giles, N. H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5783–5787.
30. Losson, R. & Lacroute, F. (1981) *Mol. Gen. Genet.* **184**, 394–399.
31. Metzenberg, R. L. & Nelson, R. E. (1977) in *Molecular Approaches to Eukaryotic Genetic Systems*, eds. Wilcox, G. & Abelson, J. (Academic, New York), pp. 253–268.
32. Matsumoto, K., Toh-e, A. & Oshima, Y. (1981) *Mol. Cell. Biol.* **1**, 83–93.