Intragenic Suppression of Gal3^C Interaction With Gal80 in the *Saccharomyces cerevisiae* GAL Gene Switch

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ABSTRACT

Gal4-mediated activation of *GAL* gene transcription in *Saccharomyces cerevisiae* requires the interaction of Gal3 with Gal80, the Gal4 inhibitor protein. While it is known that galactose and ATP activates Gal3 interaction with Gal80, neither the mechanism of activation nor the surface that binds to Gal80 is known. We addressed this through intragenic suppression of *GAL3^C* alleles that cause galactose-independent Gal3–Gal80 interaction. We created a new allele, *GAL3^{SOC}*, and showed that it suppressed a new *GAL3^C* allele. We tested the effect of *GAL3^{SOC}* on several newly isolated and existing *GAL3^C* alleles that map throughout the gene. All except one *GAL3^C* allele, D368V, were suppressible by *GAL3^{SOC}*. *GAL3^{SOC}* and all *GAL3^C* alleles were localized on a Gal3 homology model that is based on the structure of the highly related Gal1 protein. These results provide evidence for allostery in the galactose- and ATP-activation of Gal3 binding to Gal80. In addition, because D368V and residues corresponding to Gal80-nonbinder mutations colocalized to a domain that is absent in homologous proteins that do not bind to Gal80, we suggest that D368 is a part of the Gal80-binding surface.

REGULATION of gene expression is a predominant way for cells to react and adapt to environmental signals. This is typically carried out by genetic switches that either activate or repress the transcription of a specific subset of genes. One example is the well-studied *GAL* gene switch that is used by *Saccharomyces cerevisiae* (*Sc*) and *Kluyveromyces lactis* (*Kl*) and related yeasts to adapt to the presence of galactose, melibiose, and lactose by activating the expression of the *GAL* genes that encode enzymes for metabolizing these carbon sources (JOHNSTON 1987; LOHR *et al.* 1995; ZENKE *et al.* 1996; RUBIO-TEXEIRA 2005; SELICK and REECE 2005).

The *S. cerevisiae* *GAL* gene switch operates through the activities of three regulatory proteins: a transcriptional activator *ScGal4*, its inhibitor *ScGal80*, and the galactose sensor Gal3. *ScGal4* binds to a consensus DNA sequence (*UAS_{GAL}*) within the *GAL* gene promoters independently of galactose. In the presence of galactose the *ScGal4* transcription activation domain activates transcription, but in the absence of galactose the *ScGal4* activation domain is inhibited through its binding to *ScGal80* (TORCHIA *et al.* 1984; YOCUM and JOHNSTON 1984; BRAM and KORNBERG 1985; MA and PTASHNE 1987; SELLECK and MAJORS 1987; LEUTHER and JOHNSTON 1992). Relief

of this inhibition occurs when galactose- and ATP-activated Gal3 binds to *ScGal80* (BAJWA *et al.* 1988; BHAT and HOPPER 1992; SUZUKI-FUJIMOTO *et al.* 1996; BLANK *et al.* 1997; YANO and FUKASAWA 1997; PLATT and REECE 1998; PENG and HOPPER 2002; PILAURI *et al.* 2005). The *S. cerevisiae* *GAL* gene switch acts rapidly, with galactose-induced *GAL* gene mRNAs appearing within minutes of exposure to galactose (YARGER *et al.* 1984; TORCHIA and HOPPER 1986; BRYANT and PTASHNE 2003).

Gal3 is paralogous with the galactokinase enzyme *ScGal1* in *S. cerevisiae* (WOLFE and SHIELDS 1997) and shares 72% amino acid identity with it. *ScGal1* is a bi-functional protein that serves as a galactokinase and is activated by galactose and ATP to bind to *ScGal80* (BHAT and HOPPER 1991, 1992; PLATT and REECE 1998; TIMSON *et al.* 2002). However, *ScGal1* does not contribute to the normally rapid *GAL* gene switch in response to galactose since in contrast to *GAL3*, the *ScGAL1* gene is not expressed at detectable levels in the absence of galactose (ST. JOHN and DAVIS 1981). The capacity of galactose- and ATP-activated *ScGal1* to bind to *ScGal80* and relieve inhibition of *ScGal4* is independent of its galactokinase activity (BHAT and HOPPER 1992). Moreover, Gal3 does not possess galactokinase activity (BHAT *et al.* 1990), but can acquire such activity with the insertion of a serine and alanine at a highly conserved P-loop motif (PLATT *et al.* 2000). On the basis of all of the above, it is likely therefore that Gal3 lost its galactokinase activity in the process of evolving to become a more specialized component of the *GAL* gene switch in *S. cerevisiae*.

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The *GAL* gene switches of *S. cerevisiae* and *K. lactis* are overall similar but are not interchangeable due to several differences. *K. lactis* lacks a *GAL3* gene, and the *K. lactis* *GAL* gene switch activity corresponding to that of Gal3 is carried out by the bifunctional *KIGal1* protein, which serves as a galactokinase and a galactose- and ATP-dependent *KIGal80*-binding protein (MEYER *et al.* 1991; ZENKE *et al.* 1996; VOLLENBROICH *et al.* 1999; MENEZES *et al.* 2003). Unlike *ScGAL1*, *KIGAL1* is expressed at appreciable levels in the absence of galactose, providing ample levels of the *KIGal1* protein (DICKSON and RILEY 1989). Although *ScGal3* is 57% identical to *KIGal1*, it binds only poorly to *KIGal80* and supports only low levels of *KIGal4* activation (ZENKE *et al.* 1996). Another difference between the *S. cerevisiae* and *K. lactis* *GAL* gene switches is that *KIGAL4*, but not *ScGAL4*, is subjected to autoregulation due the presence of a *UAS_{GAL}* in its promoter (CZYZ *et al.* 1993).

Genetic studies of Gal3 and *KIGal1* have yielded mutants that affect their interactions with *ScGal80* and *KIGal80*, respectively. Constitutive mutants have been identified and characterized for both *GAL3* (*GAL3^c* alleles) and *KIGAL1*. They cause galactose-independent interaction with *ScGal80*/*KIGal80* and galactose-independent activation of the switch (BLANK *et al.* 1997; VOLLENBROICH *et al.* 1999; MENEZES *et al.* 2003). Mutants of *gal3* and *Klgal1* have been isolated and shown to cause defects in interactions with *ScGal80* and *KIGal80*, respectively (SUZUKI-FUJIMOTO *et al.* 1996; VOLLENBROICH *et al.* 1999; MENEZES *et al.* 2003). However, the amino acid changes in the *gal3* mutants are not known.

On the basis of sequence homologies, the Gal3, *ScGal1*, and *KIGal1* proteins belong to the GHMP superfamily of small molecule kinases that includes galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (BORK *et al.* 1993). The structures of several members of the superfamily have been determined, including homoserine kinase, mevalonate kinase, phosphomevalonate kinase, and four galactokinases. The galactokinases are those from *Lactococcus lactis* (THODEN and HOLDEN 2003), *Pyrococcus furiosus* (HARTLEY *et al.* 2004), human (THODEN *et al.* 2005b), and *Saccharomyces cerevisiae* (THODEN *et al.* 2005a).

KIGAL1 constitutive and *Klgal1* interaction-defective mutants have been mapped to a *KIGal1* homology model based on the structure of the mevalonate kinase (MVK) from *Methanococcus jannaschii*, a protein that shares only 13% amino acid identity with *KIGal1*. The *KIGAL1* constitutive mutants were shown to localize at a putative "hinge region" of the model, whereas the *Klgal1* interaction-defective mutants localized predominantly within the so-called "upper-" and "lower-" lip domains of the model. On the basis of the localization of the various mutations on the *KIGal1* homology model, it has been proposed that galactose and ATP induce a conformational change within *KIGal1* that brings the two separated upper- and lower-lip domains together to form

the common binding surface for *KIGal80* (MENEZES *et al.* 2003). However, the predictive value of the *KIGal1* homology model is limited, as it does not account for a third of the amino acid sequence of Gal3 and *KIGal1*, precluding reliable mapping of a subset of mutants in the model.

In this work, we address the mechanism by which the binding of galactose and ATP activates Gal3 binding to *ScGal80*. We identified a suppressor (*GAL3^{soc}*) of a new *GAL3^c* mutation and determined its *in vivo* and *in vitro* suppression activities for several new and existing *GAL3^c* alleles. *GAL3^{soc}* suppressed all but one of the *GAL3^c* alleles. We localized the *GAL3* and *KIGAL1* mutations on a Gal3 homology model based on a crystal structure of the *ScGal1* protein that provided a full template for Gal3, including a 100 amino acid domain that was not accounted for in the *KIGal1* homology model. The *GAL3^{soc}* allele and the suppressible *GAL3^c* alleles colocalized to one region of the model whereas the single nonsuppressible allele mapped to another region along with *Klgal1* interaction-defective mutations. We suggest that the first region is involved in allostereism, while the second region is involved in binding to *ScGal80*.

MATERIALS AND METHODS

Media and growth conditions: Yeast were cultured at 30° on either standard nonselective YEP medium or selective SC medium (SHERMAN 1991). For culture under uninduced conditions the carbon source used was 0.05% (final concentration) glucose, 3% glycerol, and 2% lactic acid (pH adjusted to 5.7 with potassium hydroxide). For culture under inducing conditions the above media was supplemented with 2% (final concentration) galactose. Bacteria were cultured at 37° in LB broth or agar plates supplemented with 50 mg/liter of ampicillin.

Yeast and bacteria strains: The yeast strain AH109 (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 GAL2_{UAS}-GAL2_{TATA}-ADE2 URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*) was used for the yeast two-hybrid selection (CLONTECH). The yeast strains Sc781 (*MATa ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 MEL1 LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 gal3Δ gal1Δ*) and Sc787 (*MATa ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 MEL1 LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 gal3Δ gal80Δ gal1Δ*) were derived from strain SJ21R (JOHNSTON and HOPPER 1982), as previously described (BLANK *et al.* 1997). Both Sc781 and Sc787 strains carry the chromosomal *MEL1* gene that has a single Gal4-binding site within its promoter and an integrated *HIS3* gene (expressed from a *GAL* promoter bearing four Gal4-binding sites (*P_{GAL}HIS3*) (FLICK and JOHNSTON 1990). The bacteria *Escherichia coli* MG7-α was used to propagate plasmids (GRIFFITH and GIETZ 2003).

PCR mutagenesis of *GAL3*, gap-repair, and yeast two-hybrid selection: Mutagenesis of the *GAL3* gene was performed using Taq DNA polymerase (Sigma, St. Louis) and the manganese (Mn)-dITP error-prone PCR method (XU *et al.* 1999; FENTON *et al.* 2002). The plasmid pAKS46 carrying the *GAL3* gene fused to the DNA-binding domain of *ScGAL4* was used as the template for the PCR reactions. The *GAL3* gene was divided into four regions by using four sets of primers: region-I, DIEP42 (5'-GCGACATCATCATCGGAAGAGAGTAG-3') and DIEP43 (5'-GCCAGATGAGAGTCCACCACCAGTAGG-3'); region-II, DIEP44 (5'-GACGAAAAAATCCATCCATTACCTTAAC-3')

and DIEP45 (5'-CAGACGTTGCTTGATCCATACCACC-3'); region-III, DIEP46 (5'-GCTCCGGAAGATTTAATAATACAC CC-3') and DIEP47 (5'-GCATCTTGAGTAAACGTTCAATAC CAG-3'); and region-IV, DIEP48 (5'-GAGTAATAGAGGTAA CAGTTGCTG-3') and DIEP49 (5'-CTTTCGCGCCACTTCCT TGCCGT-3'). A PCR product pool was generated for each region separately and was used with the cognate-gapped *GAL3* gene in pAKS46 to reconstitute the complete plasmid, using a homologous recombination-based gap-repair method (MUHLRAD *et al.* 1992) as described previously (BLANK *et al.* 1997). The four cognate-gapped plasmids were created by the following restriction digestions: gap-I, *EcoRI*/*Bgl*II; gap-II, *Bln*I; gap-III, *Bgl*II/*Xcm*I; gap-IV, *Xcm*I/*Nco*I. Each PCR product pool and cognate-gapped plasmid were combined and used to transform the yeast strain AH109 carrying pVP16GAL80. We used $\sim 1 \times 10^5$ cells for each gap-repair transformation, resulting in ~ 800 primary Trp⁺ Leu⁺ prototrophs for each gap. The transformants were then replica-plated to select for Trp⁺ Leu⁺ Ade⁺ prototrophs in the absence of galactose. For each gap, ~ 80 Trp⁺ Leu⁺ Ade⁺ prototrophs arose. Plasmid was isolated from the prototrophs and retransformed into AH109 carrying pVP16GAL80 to retest for the two-hybrid interaction. Candidates that repeated the two-hybrid interaction in the absence of galactose were sequenced and subjected to further analysis.

Plasmid constructions: The plasmid pAKS46 (DBD-GAL3) was constructed as follows. The *GAL3* gene was amplified by PCR using the primers G1 (5'-CGGATCCGCATGAATACAAA CGTTCCAATA-3') and BLNK06 (5'-AGCTGGCGAAAGGGG GATGTG-3') from the template pTEB16 (BLANK *et al.* 1997). The ~ 2.1 -kb product was digested with *Bam*HI and then ligated to pGBT9 (CLONTECH) digested with *Bam*HI. The plasmids pVP16GAL80 (SL *et al.* 1999), pTEB16 (*GAL3* under its native promoter), pMPW60 (GSTGAL3 under the *ADH2* promoter), pMPW61 (GST under the *ADH2* promoter), and pMPW82 (*ScGAL80* under the *ADH2* promoter) were constructed as previously described (BLANK *et al.* 1997). Plasmids pGP160 and pGP161 were derived from pTEB16 (BLANK *et al.* 1997) by site-directed mutagenesis. All other plasmids were generated by standard restriction digestion and ligation.

Spot assay for cell growth: Sc781 transformants were grown to an OD₆₀₀ of ~ 1.0 in selective media. Each culture was adjusted to the same number of cells, and serial 10-fold dilutions were made with dH₂O. A total of 6 μ l of a 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} dilution of each cell culture was spotted onto tryptophan and histidine dropout agar plates supplemented with 10 mM 3-AT (3-amino-1,2,4-triazole). The growth of cells was evaluated on plates incubated for 3–6 days.

MEL1 α -galactosidase enzyme activity assay: To determine the expression level of the *MEL1* α -galactosidase gene, Sc781 cells harboring the indicated plasmids were grown to an OD₆₀₀ of ~ 0.5 in 5 ml of selective media (–galactose, uninduced cultures). To induce the cultures, galactose was added to the uninduced cultures to a final concentration of 2%. Both the uninduced and the induced cultures were incubated for an additional 3 hr. A total of 1 ml of each culture was centrifuged and the supernatant was assayed for α -galactosidase activity as follows. A total of 120 μ l of the supernatant was mixed with 360 μ l of assay buffer [2 volumes of 0.5 M sodium acetate, pH 4.5, and 1 volume of 100 mM *p*-nitrophenyl α -D-galactopyranoside (PNP- α -Gal, Sigma)]. The reaction was incubated at 30° for 5 hr and terminated by adding 520 μ l of stop buffer (1 M sodium carbonate), and the OD₄₁₀ was recorded (HAO *et al.* 2004). The selective media served as the blank. Three independent transformants for each plasmid were assayed and the average and standard deviation were reported. The amount of PNP- α -Gal hydrolyzed can be determined as previously described (POST-BEITENMILLER *et al.* 1984) by calculating the concen-

tration of *p*-nitrophenol using its molar extinction coefficient (18.3×10^3).

Pull-down assay for GSTGal3 and ScGal80 interaction: Sc787 cells cotransformed with plasmids carrying GSTGAL3 and *ScGAL80* were grown to mid-log phase for preparation of yeast whole-cell extracts. The pull-down binding assay utilizing GSTGal3 fusion was carried out as previously described with the following modifications. Whole-cell extracts containing GSTGal3 and *ScGal80* were prepared as described (BLANK *et al.* 1997) using a modified lysis buffer (20 mM HEPES, pH 7.4, 0.5% Triton X-100, 200 mM NaCl, 0.5 mM EDTA, 2 mM DTT, and 5 mM MgCl₂). Protease inhibitor cocktails (PIC-D: 88 mg/ml PMSF and 1 mg/ml pepstatin A in DMSO; PIC-W: 157 mg/ml benzamidine, 0.5 mg leupeptin, and 0.5 mg bestatin in water) were added to all lysis buffer and all subsequent solutions at 1:1000 dilutions. The whole-cell extract (1–8 mg) was brought up to a volume of 400 μ l with lysis buffer containing 2 mM ATP (–galactose) or lysis buffer containing 2 mM ATP and 25 mM galactose (+galactose). Glutathione sepharose beads (Amersham Biosciences, Piscataway, NJ) were equilibrated with the lysis buffer and resuspended in the lysis buffer as a 50% slurry. The whole-cell extracts were then incubated with 50 μ l of 50% glutathione sepharose beads on a rotator at 4° for 4 hr. The beads were pelleted and washed three times with 500 μ l of either lysis buffer containing 2 mM ATP or lysis buffer containing 2 mM ATP and 25 mM galactose. The beads were then boiled for 5 min in 40 μ l of 1 $\times$ SDS–PAGE sample-loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT, and 0.003% Pyronin Y) before analysis by standard SDS–PAGE and Western blot. Antibodies for GST and *ScGal80* were used together at a 1:200 dilution.

Multiple sequence alignment: The alignment was generated by the T-Coffee server (3DCoffee, regular mode) (POIROT *et al.* 2004). The results were saved as ALN and MSF files and edited further using the GeneDoc program (<http://www.psc.edu/biomed/genedoc/>), CLUSTAL_X (THOMPSON *et al.* 1997), and Microsoft Word.

Homology modeling: The Gal3 homology model was based on the structure of *ScGal1* (THODEN *et al.* 2005a), which shares 72% protein-sequence identity with Gal3. The homology model was obtained using the threading path as implemented in Prime v1.2. Energy minimization of main chain and side chain interactions was performed as standard in this protocol. The backbone angles of the resulting structure fell within the allowed regions of a Ramachandran map, indicating that the energy minimization had not perturbed the geometry of the structure. Residues 290–297 were not modeled because this region was disordered in the *ScGal1* structure. This precluded the mapping of the Gal3-equivalent positions of the *KIGAL1* constitutive mutants E285K and G286F in Figure 5. The model was rendered using the programs GRASP (NICHOLLS *et al.* 1991) and MOLSCRIPT (KRAULIS 1991).

RESULTS

Isolation of new *GAL3^c* alleles: A previously conducted extensive selection for *GAL3^c* mutations that cause galactose-independent expression of a P_{GAL}HIS3 reporter yielded five mutations that occurred at only four discrete positions throughout the *GAL3* gene (BLANK *et al.* 1997). We sought to isolate additional *GAL3^c* mutations representing, if possible, other locations within the gene. To isolate new *GAL3^c* alleles, we carried out a yeast two-hybrid selection for *GAL3* mutations that cause Gal3 to bind to *ScGal80* independently

TABLE 1
***GAL3* and *KIGAL1* mutations**

Mutation	Reference	
<i>GAL3</i> ^{soc} allele D68S	This study	
<i>GAL3</i> ^c alleles		
L50P	This study	
V69E	This study	
V69E/D70V	BLANK <i>et al.</i> (1997)	
V203I	This study	
F237Y	BLANK <i>et al.</i> (1997)	
D368V	BLANK <i>et al.</i> (1997)	
V396A	This study	
F414L	This study	
S509D	This study	
S509P/L	BLANK <i>et al.</i> (1997)	
K510E	This study	
<i>KIGAL1</i> constitutives	Equivalent Gal3 position	Reference
S40F	S44	MENEZES <i>et al.</i> (2003)
E285K	H291	VOLLENBROICH <i>et al.</i> (1999)
G286F	S295	MENEZES <i>et al.</i> (2003)
L385F	L394	MENEZES <i>et al.</i> (2003)
<i>KIGAL1</i> reg ⁻ kin ⁺		
R94C	R97	VOLLENBROICH <i>et al.</i> (1999)
D108A	D111	MENEZES <i>et al.</i> (2003)
W114R	W117	MENEZES <i>et al.</i> (2003)
E254C	E260	VOLLENBROICH <i>et al.</i> (1999)
F368S/M409S	F377/M422	MENEZES <i>et al.</i> (2003)
<i>KIGAL1</i> reg ⁻ kin ⁻		
T74P/F91L	L78/F94	MENEZES <i>et al.</i> (2003)
V146F	C152	VOLLENBROICH <i>et al.</i> (1999)

of galactose. The wild-type Gal3 protein interacts with ScGal80 in the two-hybrid assay only in the presence of galactose. We identified six novel *GAL3* missense variants that exhibited a two-hybrid interaction with ScGal80 in the absence of galactose (data not shown). Their locations are given in Table 1.

Each *GAL3* mutation was recreated as a GSTGal3 fusion and evaluated for its effect for binding to ScGal80 in an *in vitro* pull-down assay. Wild-type GSTGal3 interacts with ScGal80 only in the presence of galactose. In contrast, all six mutants had a galactose-independent interaction with ScGal80 (Figure 1A). The GST-fusions of all of the variants except K510E were present in cells at levels equivalent to that of the wild-type GSTGal3. The K510E variant was present at very low levels. The remaining mutants, except L50P, retained full galactose-

responsiveness, showing ScGal80-binding levels similar to wild-type GSTGal3 in the presence of galactose.

Since the new *GAL3* mutants exhibited galactose-independent interaction with ScGal80, we expected that they would cause constitutive activation of ScGal4 *in vivo*. To confirm this, we first established each mutation within an otherwise wild-type *GAL3* gene under its native promoter in the yeast strain Sc781. Each *GAL3* mutation was then evaluated for its effect on the *GAL* gene switch by carrying out a P_{GAL}*HIS3* expression-dependent colony-growth assay. Wild-type *GAL3* activated the *GAL* gene expression only in the presence of galactose. Each of the six *GAL3* mutations caused constitutive, galactose-independent activation of the *GAL* switch, as determined by colony growth in the absence of histidine and galactose (Figure 1B), indicating that they are new *GAL3*^c alleles. These new *GAL3*^c alleles, with the exception of L50P mutant, retained galactose-responsiveness and fully activated the *GAL* switch in the presence of galactose. The somewhat weaker galactose-responsiveness of the L50P that was observed might be due to its somewhat weaker interaction with ScGal80 in the presence of galactose (Figure 1A).

To quantify the constitutivity of these new *GAL3*^c alleles, expression levels of an endogenous, galactose-regulated gene, *MEL1* (POST-BEITENMILLER *et al.* 1984) was determined using an enzymatic-activity assay for its encoded α -galactosidase. Wild-type *GAL3* supported appreciable α -galactosidase activity only in the presence of galactose, whereas the L50P, V69E, and V396A alleles supported appreciable α -galactosidase activity independently of galactose. The remaining three *GAL3*^c alleles supported much lower levels of activity (Figure 1C). The L50P variant exhibited a weak response to galactose compared to wild-type *GAL3* and the other variants. The results in Figure 1 confirm that the mutations identified from the two-hybrid selection are indeed *GAL3*^c alleles.

Five of the six *GAL3*^c alleles isolated here were found to contain new mutations that are different from those of pre-existing *GAL3*^c alleles (BLANK *et al.* 1997). The V69E mutation was the only one previously identified as a double mutation V69E/D70V, but until now was not known to be the mutation conferring constitutivity. Clearly, the V69E alone is sufficient to cause the constitutive phenotype. The new *GAL3*^c alleles did not reveal any strong clustering within the *GAL3* sequence either as a group or when considered with the previously isolated *GAL3*^c alleles. The widespread distribution of *GAL3*^c throughout the gene might explain why previous attempts to isolate a single peptide of Gal3 or KGal1 able to interact with ScGal80 or KGal80, respectively, were unsuccessful (DIEP and HOPPER, unpublished data; VOLLENBROICH *et al.* 1999).

***GAL3*-D68S suppressed the constitutive phenotype conferred by *GAL3*-S509D:** In comparing the structures of homoserine kinase (HSK, PDB code 1H72) and the *L. lactis* galactokinase (PDB code 1PIE), we noticed a conserved interaction. In HSK, E31 formed a hydrogen

FIGURE 1.—Newly isolated *GAL3* mutations constitutively activate the *GAL* gene switch. (A) Galactose-independent interaction between GSTGal3 and Gal80 *in vitro*, as determined by a pull-down assay. Yeast whole-cell extracts containing GSTGal3 and Gal80 were incubated at 4° for 4 hr with glutathione sepharose, washed three times, and then boiled and separated on SDS-PAGE and analyzed by Western blot. GSTGAL3 and *GAL80* were expressed under the *ADH2* promoter in pMPW60 and pMPW82, respectively. (B) Constitutive *in vivo* expression of the integrated *P_{GAL}HIS3* reporter gene determined by a colony-growth assay. Strain Sc781 carrying the indicated plasmid was grown in liquid culture to late log phase and

adjusted to the same number of cells. Dilutions (10-fold) were spotted on selective agar plates and incubated for 3–6 days. Wild-type and *GAL3* mutants were expressed under their native promoter in pTEB16. (C) Constitutive expression of the endogenous *MEL1* gene determined by an α -galactosidase enzymatic-activity assay. Strain Sc781 carrying the indicated plasmid was grown in liquid culture to an OD₆₀₀ of 0.5, and the cell-free culture media was removed and assayed for α -galactosidase activity. Three independent transformants were assayed, and the average and standard deviation were reported. Wild-type and *GAL3* mutants were expressed under their native promoter in pTEB16.

bond with T291. In the *L. lactis* galactokinase, the positions of these residues were reciprocally exchanged to T57 and Q388 such that they are equivalent to T291 and E31, respectively, of HSK. This type of exchange is known as “concerted substitution.” Curiously, we have isolated *GAL3^c* mutations that closely correspond to the positions described above. The V69E mutation is next to D68, which corresponds to E31 of HSK in a sequence alignment, while the S509P/L mutations correspond to T291 of HSK (alignment not shown). We wondered if a concerted substitution could also occur in Gal3 by a reciprocal exchange of D68 and S509, as observed for the *L. lactis* galactokinase.

To investigate this further, we created two new mutations to produce a reciprocal exchange in side chain characteristics: D68S and S509D. The *GAL* gene switch phenotype of cells carrying the single and double mutations was determined by evaluating *P_{GAL}HIS3*-dependent cell growth of Sc781. Cells carrying the D68S mutation had a phenotype similar to wild-type *GAL3* and grew on histidine-deficient selective agar only in the presence of galactose (see below in Figure 3A), whereas cells with the S509D mutation grew in the absence of galactose (Figure 2A). Therefore, the *GAL3*-S509D is a new *GAL3^c* allele. In contrast to the robust growth of cells carrying *GAL3*-S509D, cells carrying *GAL3*-S509D/D68S grew poorly in the absence of galactose (Figure 2A). In

the presence of galactose, the growth of cells carrying the *GAL3*-D68S, the *GAL3*-S509D, or the *GAL3*-S509D/D68S was similar to that of wild-type *GAL3*, indicating that the suppressed growth of the double mutant was not due to a loss of Gal3 function.

We next quantified the suppression using the α -galactosidase enzymatic-activity assay for *MEL1* gene expression. Cells with the S509D mutation expressed *MEL1* in the absence of galactose (Figure 2B), confirming that it is a constitutive allele. In contrast, cells with the S509D/D68S mutation expressed *MEL1* at a much lower level, verifying the suppression observed above. Cells with either the S509D or S509D/D68S mutations showed fully induced *MEL1* expression in response to galactose similar to wild-type *GAL3*.

The suppression that was evident from the *P_{GAL}HIS3* and *MEL1* reporter assays was most likely due to suppression of Gal3^c binding to Gal80. To confirm this we performed GSTGal3 pull-down assays. The GSTGal3-S509D protein showed galactose-independent interaction with ScGal80 (Figure 2C). The GSTGal3-S509D/D68S protein showed notably less interaction with ScGal80 in the absence of galactose. However, in the presence of galactose, both proteins exhibited levels of binding to ScGal80 equivalent to the wild-type GSTGal3.

The results of all the above assays taken together firmly establish D68S as an intragenic suppressor of

FIGURE 2.—The suppression of constitutivity of *GAL3*-S509D by *GAL3*-D68S. (A) Expression of the integrated $P_{GAL}HIS3$ reporter gene in the yeast strain Sc781 determined by a colony-growth assay. (B) Expression of the endogenous *MEL1* gene in the yeast strain Sc781 determined by an α -galactosidase enzymatic-activity assay. (C) Interaction between Gal3 and Gal80 *in vitro* as determined by a pull-down assay.

S509D, a mutation that we show confers to Gal3 the capacity to bind to ScGal80 independently of the normally required ligand, galactose. We call the D68S allele *GAL3^{soc}* (suppressor of constitutivity). These results demonstrate for the first time that a single amino acid substitution within the N-terminal domain of Gal3 can alter a new property of the protein that is conferred by a single amino acid substitution within its C terminus.

Suppression of constitutivity by *GAL3^{soc}* is *GAL3^c* allele specific: The discovery of the *GAL3^{soc}* allele led us to test its ability to suppress other *GAL3^c* mutations that showed appreciable constitutivity. These were L50P, F237Y, D368V, V396A, S509P, and S509L. Mutations V203I, F414L, and K510E were not included in the suppression analysis because their levels of constitutivity in the *MEL1* (α -galactosidase) and the pull-down assays were too low for reliable suppression testing.

Each mutation was combined with D68S to produce a double mutation allele. Suppression of each was evaluated using the following assays: $P_{GAL}HIS3$ reporter-based colony growth (Figure 3A), *MEL1*-expressed α -galactosidase activity (Figure 3B), and the GSTGal3-ScGal80 pull-down assay (Figure 3C). As mentioned above, the *GAL3*-D68S allele yielded similar results to those of wild-type *GAL3* in all three assays. All double mutants except L50P/D68S retained full galactose-responsive induction as indicated by all three assays. D68S suppression of constitutivity was evident in all three assays for the S509P and S509L alleles. The F237Y allele was not suppressed in the $P_{GAL}HIS3$ -based growth assay, weakly suppressed in the *MEL1* α -galactosidase assay, and moderately suppressed in the pull-down assay. V396A was

moderately suppressed by D68S in both the $P_{GAL}HIS3$ and *MEL1* assays. In repeated attempts, the GSTGal3-V396A/D68S protein was not detectable. However, the untagged protein was detectable (Figure 3D), consistent with its ability to fully induce the *GAL* switch in the presence of galactose in the $P_{GAL}HIS3$ and *MEL1* assays. In contrast to the four suppressible alleles described above, D368V/D68S showed no suppression in all three assays.

We note that the magnitude of constitutivity determined for some *GAL3^c* alleles was different, depending on the assay used. For example, the S509P allele showed moderate *GAL* gene expression *in vivo*, but only weak interaction with ScGal80 in the pull-down assay. We do not know the reason for this, but perhaps it reflects mechanistic aspects of the Gal3-ScGal80 interplay that are not well understood. For example, recent evidence points to ScGal80 nucleocytoplasmic shuttling, monomer-dimer equilibrium, and monomer *vs.* dimer preferences for binding Gal3 and ScGal4 as key features of the *GAL* gene switch (PILAURI *et al.* 2005).

Despite the discrepancies between the *in vivo* and *in vitro* assays, the results presented here show that the *GAL3^{soc}* allele suppressed the *GAL3^c* alleles at positions 237, 396, and 509, but not at position 368.

Homology model of Gal3: Gal3 and ScGal1 belong to the GHMP kinase superfamily, but they contain an ~ 100 amino acid insertion domain relative to other members (Figure 4). All family members contain conserved motifs for the binding of ATP and specific substrates. The structures of several members have been determined and found to have a shared core fold (ZHOU *et al.* 2000; BONANNO *et al.* 2001; ROMANOWSKI *et al.* 2002; YANG *et al.*

FIGURE 3.—Specificity in suppression of *GAL3^C* alleles. (A) Expression of the integrated *P_{GAL}HIS3* reporter gene in the yeast strain Sc781 determined by a colony-growth assay. (B) Expression of the endogenous *MEL1* gene in the yeast strain Sc781 determined by an α -galactosidase enzymatic-activity assay. (C) Interaction between Gal3 and Gal80 *in vitro* as determined by a pull-down assay. (D) Protein levels of the Gal3-V396A and Gal3-V396A/D68S mutant proteins not tagged with GST, as determined by a Western blot.

2002; LUZ *et al.* 2003; THODEN and HOLDEN 2003). In general, the polypeptide chain folds into two domains of approximately equal size. The active site is located at a cleft between the N- and C-terminal domains.

To understand the allele-specific suppression data in a structural context, we calculated a homology model for Gal3 (Figure 5) on the basis of the recently determined ligand-bound structure of ScGal1 (THODEN *et al.* 2005a), which shares 72% amino acid identity with Gal3. Analysis of the surface charge showed a negatively charged channel that housed the galactose and ANP. In general, the remainder of the surface was uncharged with the exception of the C terminus, which was more negatively charged. In this model, residues 285–381

compose the insertion domain. It forms a four-helix motif and folds on the edge of the C-terminal domain (Figures 4 and 5).

The *GAL3^{SOC}* and *GAL3^C* alleles that were included in the suppression analysis (F237Y, D368V, V396A, and S509P/L) were mapped on the Gal3 homology model, along with the Gal3 equivalent positions of *KIGAL1* mutants shown in Table 1. The mutants were found to localize predominantly to two regions. One region consists of the *GAL3^{SOC}* alleles (green in Figure 5) and the suppressible *GAL3^C* alleles (F237Y, V396A, and S509P/L, red in Figure 5). This region is localized around a previously designated “hinge region” (MENEZES *et al.* 2003). The second region is remote from the hinge

FIGURE 4.—Multiple sequence alignment of Gal3 and related proteins and the predicted secondary structure of the Gal3 homology model. The multiple sequence alignment was generated using the T-Coffee server. The predicted secondary structure of the Gal3 homology model is represented by arrows (β -sheets) and rectangles (α -helices). The N-terminal domain is blue while the C-terminal domain is red. The four-helical insertion domain is represented by vertical red lines. (●) *GAL3^c* alleles, (○) *KIGAL1* constitutive mutants, and (□) *Klgall1* interaction-defective mutants. MVK_Mj is mevalonate kinase of *Methanococcus jannaschii*, GAL1_Kl is GAL1 of *Kluyveromyces lactis*, GAL3_Sc and GAL1_Sc are GAL3 and GAL1, respectively, of *Saccharomyces cerevisiae*.

region and consists of the single nonsuppressible *GAL3^c* allele (D368V, red) along with *Klgall1* interaction-defective mutants (cyan in Figure 5). Also in the model, the *KIGAL1*-constitutive mutants are yellow and the *Klgall1* mutants defective in both regulation and kinase activity are blue. In light of the suppression analysis, the significance of these two regions is discussed below.

DISCUSSION

In this study, we have provided evidence for the involvement of allosterism in the activation of Gal3. Our evidence comes from intragenic suppression analyses of newly and previously isolated *GAL3^c* alleles and the localization of these alleles on a Gal3 homology model. We created a new allele *GAL3^{soC}* and showed that it suppressed four of five *GAL3^c* alleles. The four suppressible alleles colocalize with *GAL3^{soC}* to a region in the Gal3 homology model that correspond to a putative ligand-regulatable hinge region in a *KIGal1* homology model that was thought to modulate remote *KIGal80*-

binding determinants. The single nonsuppressible *GAL3^c* allele (*GAL3^c*-D368V) localized to a different region that also includes residues corresponding to *Klgall1* mutants that cause defects in binding to *KIGal80*.

GAL3^c-D368V localized to the so-called insertion domain. There is indirect evidence that the insertion domain plays an essential role in binding of Gal3 to *ScGal80*. The *E. coli* galactokinase is unable to complement the *gal3* deletion, and in common with bacterial galactokinases, lacks the insertion domain (BHAT *et al.* 1990). By contrast, the yeast galactokinase gene, *ScGAL1*, does encode the insertion domain and can complement the *gal3* deletion (BHAT *et al.* 1990; BHAT and HOPPER 1991). The fact that the *GAL3^c*-D368V allele is located within the insertion domain strengthens the notion that this domain is involved in the Gal3-*ScGal80* interaction. The *GAL3^c*-D368V protein is unique because in the absence of galactose it binds *ScGal80* as well as wild-type Gal3 in the presence of galactose (BLANK *et al.* 1997). However, the other *Gal3^c* proteins (V69E/D70V, F237Y, S509P, and S509L) that all localized to the putative ligand-regulated hinge region retained dependence on

FIGURE 5.—Locations of residues corresponding to mutations affecting binding to *Sc/KGal80* on a Gal3 homology model. (A) Location of residues shown in space-filling representation. Residues colored cyan are the Gal3 equivalent residues of *Klgal1* reg⁺ kin⁺ mutations. The positions of *GAL3^C* mutations included in the suppression analyses are red. The two views are approximately orthogonal, due to a rotation around the y-axis in a counterclockwise direction. (B) Ribbon diagram in the same orientations as in A. In addition to the coloring adopted in A, residues in yellow are the Gal3-equivalent positions of *Klgal1* constitutive mutations, residues in blue are the Gal3-equivalent positions of *Klgal1* reg⁺ kin[−] mutations, and the position of *GAL3^{SC}* is green. Residues that are described in detail in the text have been labeled. For clarity, the labeling of other residues has been omitted.

galactose and ATP for wild-type levels of binding to *ScGal80*. We surmise that the constitutivity of these alleles is due to an allosteric effect that causes a local conformational change at the putative hinge region of Gal3 that partially mimics the galactose- and ATP-bound state. In contrast, we suggest that the constitutivity of the Gal3^C–D368V protein is most likely caused by a change either directly at the surface for *ScGal80* binding or at an intermediate site within the allosterism pathway between the hinge region and the *ScGal80* docking site(s). Strikingly, Gal3 residues corresponding to *Klgal1* mutants with defective *KGal80* interaction colocalize along a surface that is proximal to D368 in the Gal3 homology model. This surface is largely hydrophobic in nature and consists of several noncontiguous peptides. If this is the *ScGal80*-binding surface, it could perhaps explain why attempts to isolate a single Gal3 peptide showing *ScGal80* binding have been unsuccessful.

Allosteric activation of Gal3 binding to *ScGal80* caused by galactose and ATP may share a similar mechanistic feature with human glucokinase, an enzyme that dis-

plays a “hinge-regulated” activity. The binding of glucose and ATP to the active site of the glucokinase forms the ligand-bound active enzyme (KAMATA *et al.* 2004). Missense mutations that occur at positions distributed throughout the sequence but colocalize to a hinge region cause the protein to partially adopt the ligand-bound state with high enzymatic activities (GLASER *et al.* 1998; CHRISTENSEN *et al.* 2002; GLOYN 2003; CUESTA-MUNOZ *et al.* 2004; PEDELINI *et al.* 2005). The binding of a small molecule at this hinge region also activated the enzyme (GRIMSBY *et al.* 2003), further supporting the idea of allosterism via a hinge-regulated activity. We envision a somewhat similar scenario for Gal3 in accordance with the classic definition of allosterism as the binding of ligand(s) at one site controls the binding of ligand(s) at a second site. The *GAL3^C* alleles located at the hinge region could promote the active ligand-bound state (normally achieved by galactose and ATP) that allosterically effects presentation of the binding surface for the ligand, *ScGal80*. Introducing a second compensatory mutation (*GAL3^{SC}*) within the hinge region could alter the local structure and partially reverse the constitutive effect. This scenario would not apply to the *GAL3^C* allele at position D368 that is unaffected by the *GAL3^{SC}* allele, presumably due to its remote position from the hinge region.

In summary, the data presented here provide evidence for the involvement of allosterism in the mechanism for galactose and ATP regulation of Gal3. The results of the intragenic suppression analyses together with the locations of suppressible and nonsuppressible alleles on a Gal3 homology model, implicate the putative hinge region as the key ligand-responsive component for modulating the presentation of residues at the *ScGal80*-binding surface(s). This notion is similar to the overall mechanistic picture that has been suggested for *KGal1*, but is further supported by the juxtaposition of the *GAL3^C*–D368V allele and the *Klgal1* mutants defective in interaction with *KGal80*. Just how similar Gal3 and *KGal1* are in their mechanisms for galactose- and ATP-regulated binding to *ScGal80*/*KGal80* remains to be seen.

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