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# Functional Analysis of the Yeast Glc7-Binding Protein Reg1 Identifies a Protein Phosphatase Type 1-Binding Motif as Essential for Repression of *ADH2* Expression

KENNETH M. DOMBEK,* VALENTINA VORONKOVA, ALEXA RANEY, AND ELTON T. YOUNG

*Department of Biochemistry, University of Washington, Seattle, Washington 98195-7350*

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**In *Saccharomyces cerevisiae*, the protein phosphatase type 1 (PP1)-binding protein Reg1 is required to maintain complete repression of *ADH2* expression during growth on glucose. Surprisingly, however, mutant forms of the yeast PP1 homologue Glc7, which are unable to repress expression of another glucose-regulated gene, *SUC2*, fully repressed *ADH2*. Constitutive *ADH2* expression in *reg1* mutant cells did require Snf1 protein kinase activity like constitutive *SUC2* expression and was inhibited by unregulated cyclic AMP-dependent protein kinase activity like *ADH2* expression in derepressed cells. To further elucidate the functional role of Reg1 in repressing *ADH2* expression, deletions scanning the entire length of the protein were analyzed. Only the central region of the protein containing the putative PP1-binding sequence RHIHF was found to be indispensable for repression. Introduction of the I466M F468A substitutions into this sequence rendered Reg1 almost nonfunctional. Deletion of the central region or the double substitution prevented Reg1 from significantly interacting with Glc7 in two-hybrid analyses. Previous experimental evidence had indicated that Reg1 might target Glc7 to nuclear substrates such as the Snf1 kinase complex. Subcellular localization of a fully functional Reg1-green fluorescent protein fusion, however, indicated that Reg1 is cytoplasmic and excluded from the nucleus independently of the carbon source. When the level of Adr1 was modestly elevated, *ADH2* expression was no longer fully repressed in *glc7* mutant cells, providing the first direct evidence that Glc7 can repress *ADH2* expression. These results suggest that the Reg1-Glc7 phosphatase is a cytoplasmic component of the machinery responsible for returning Snf1 kinase activity to its basal level and reestablishing glucose repression. This implies that the activated form of the Snf1 kinase complex must cycle between the nucleus and the cytoplasm.**

Protein phosphatase type 1 (PP1) plays a key role in regulating a diverse variety of processes in eukaryotic cells (3, 48). The amino acid sequences of the mammalian and yeast homologues of the PP1 catalytic subunit ($PP1_c$) are more than 80% identical, suggesting that their function and the regulatory mechanisms that control their activity have been conserved throughout evolution. The gene coding for the *Saccharomyces cerevisiae* homologue of $PP1_c$ is *GLC7*. Glc7 is required for the appropriate regulation of a number of cellular processes, including glycogen biosynthesis; translation; cell cycle progression; chromosome segregation, meiosis, and sporulation; and repression of many glucose-regulated genes (54). Unlike protein kinase catalytic subunits which can recognize a window of amino acid sequence surrounding the phosphorylation site (39), $PP1_c$ exhibits little inherent substrate specificity in vitro (11, 39). There is now a large body of evidence suggesting that specificity is conferred by regulatory subunits. When complexed with $PP1_c$, they target it to specific substrates (11, 22). In *S. cerevisiae*, a number of Glc7-binding proteins affecting specific cellular processes have been identified (54). These potential regulatory subunits include Gac1 and Pig1, which affect glycogen accumulation (9, 55); Reg2 and Sds22, which affect growth and cell cycle progression (24, 33, 41); Gip1, which is required for completion of meiosis and sporulation (59); Scd5, which affects the vesicular secretory pathway (59); Pig2, whose function is unknown (9); and Reg1, which affects glucose repression, growth, and glycogen accumulation (23, 24, 35, 42, 44).

In *S. cerevisiae*, glucose repression is the major mechanism through which the expression of genes involved in the utilization of alternative or fermentable carbon sources is coordinately regulated (25). In the presence of high concentrations of glucose, the expression of glucose-regulated genes is low or repressed. When the concentration of glucose drops below 0.2%, expression of these genes is activated or derepressed. For glucose-repressible genes like *SUC2*, *GAL1*, and *ADH2*, this change in the level of expression can be 200-fold or greater (7, 20, 27). A number of genes have been identified as playing integral roles in glucose repression (25). Among these are *REG1*, *GLC7*, and *SNF1*, the yeast homologue of the catalytic subunit of AMP-activated protein kinase (30). *SNF1* is required for derepression of gene expression in glucose-limited cells (4, 10, 67), while *REG1* and *GLC7* are required for the maintenance of the fully repressed state (23, 42, 44). A combination of genetic, two-hybrid, and coimmunoprecipitation experiments have indicated that Snf1 is complexed with Snf4 and one member of the Sip/Gal83 class of proteins (7, 65). Snf1 is thought to be anchored in the complex by its C-terminal regulatory domain to the centrally located KIS domain of the Sip/Gal83 protein (38). Snf4 is also anchored in the complex by interacting with the Sip/Gal83 protein; however, this interaction is with the C-terminal ACS domain. These interactions do not appear to be carbon source regulated. The interaction of Snf1 with Snf4, however, does appear to be carbon source regulated (37). In repressed cells, the N-terminal kinase domain of Snf1 appears to interact with its C-terminal regulatory

* Corresponding author. Mailing address: Department of Biochemistry, Box 357350, University of Washington, Seattle, WA 98195-7350. Phone: (206) 543-6035. Fax: (206) 685-9144. E-mail: kmd@u.washington.edu.

domain, which is thought to inhibit kinase activity. Upon depletion of glucose from the growth medium, Snf4 is thought to bind to the kinase domain, displacing the regulatory domain and, thereby, freeing the Snf1 kinase domain from autoinhibition. Two-hybrid and coimmunoprecipitation experiments have also suggested that Reg1 and Glc7 act together as a complex (59). Like interactions with the Sip/Gal83 component of the Snf1 complex, the interaction between Reg1 and Glc7 does not appear to be glucose regulated. Recently, evidence has been presented indicating that Reg1 interacts with the kinase domain of Snf1, altering protein-protein interactions within the kinase complex (40). Two-hybrid experiments have suggested that Reg1 interacts weakly with the kinase domain of Snf1 in repressed cells and strongly in derepressed cells. This interaction required amino acid T210 in the activation loop, which is essential for Snf1 kinase activity and for the interaction with Snf4. Based on these observations, it was proposed that Reg1 targets Glc7 to an active Snf1 complex by binding to the kinase domain. Once bound, Glc7 could then dephosphorylate Snf1, thereby releasing Snf4 from the kinase regulatory domain and returning the complex to an autoinhibited state.

Although the Reg1-Glc7 complex has been clearly implicated in the repression of *SUC2* expression, surprisingly, only Reg1 has been demonstrated to play a role in repressing *ADH2* expression (20). Even though *reg1* mutant cells growing under normally repressing conditions have up to 40-fold greater *ADH2* expression than wild-type cells, a *glc7-T152K* mutant, which has a constitutively high level of *SUC2* expression, is fully repressed for *ADH2* expression (20). The level of this constitutive *ADH2* expression is similar to that seen in cells with an *ADR1$^c$* allele. *ADR1$^c$* mutations fall within or near the cyclic AMP-dependent protein kinase (cAPK) phosphorylation site at serine 230 of *ADR1* (18), the major activator of *ADH2* transcription (17). Reg1 appears to act independently of this phosphorylation site, however, since *ADR1$^c$* alleles synergistically increase *ADH2* expression in *reg1* mutant cells under normally repressing growth conditions (20). Like activated *ADH2* expression in derepressed wild-type cells, constitutive *ADH2* expression in *reg1* mutant cells under normally repressing growth conditions requires *ADR1*. Cells with mutations in both *ADR1* and *REG1* have fully repressed *ADH2* expression during growth on a high-glucose medium. Also, a reporter gene with promoter sequences containing UAS1, the Adr1 binding site in the *ADH2* promoter (51), is constitutively expressed in *reg1* mutant cells (20). The level of Adr1 in *reg1* mutant cells under normally repressing growth conditions is nearly the same as that in derepressed wild-type cells. This 3-fold higher level of Adr1, however, cannot fully account for the 40-fold higher level of *ADH2* expression, since *ADH2* expression appears to increase linearly with the level of *ADR1* expression in repressed cells (15). Also, recent data from our lab has shown that repressed cells having the same level of Adr1 as derepressed cells do not constitutively express *ADH2* (53).

*ADH2* expression has the same requirement for Snf1 as *SUC2* expression (10), and repression of both *ADH2* and *SUC2* in a high-glucose medium has the same requirement for Reg1 (4, 20). This raised the question of how Reg1 could function in the apparent absence of a requirement for Glc7. In the study presented here, we provide the first evidence showing that even though repression of *ADH2* expression has an apparent differential requirement for Reg1 and Glc7, Glc7 may indeed play a role in repressing *ADH2* expression. A sequence similar to the mammalian PP1-binding motif (R/K)(V/I)XF (22) was identified as being essential for repression of both *ADH2* and *SUC2* expression and for the interaction of Reg1 with Glc7. We also show that the level of Adr1 in *glc7* mutant cells is limiting for constitutive *ADH2* expression under repressing growth conditions. Subcellular localization of Reg1-green fluorescent protein (GFP) suggested that Reg1 is cytoplasmic and excluded from the nucleus. These findings suggest that the Reg1-Glc7 phosphatase complex is part of the cytoplasmic machinery for resetting Snf1 kinase activity to a basal level and imply that the activated form of the Snf1 complex rapidly cycles between the nucleus and the cytoplasm.

## MATERIALS AND METHODS

**Yeast strains, plasmids, media, and growth conditions.** The yeast strains used in this study are listed in Table 1. Strains created for this study were constructed by using standard genetic methods (29). Strains KDY80, KDY82, KDY88, KDY90, and KDY92 have the S288C genetic background. KDY80 and KDY82 are congenic segregants derived from a *REG1/reg1-1966::URA3 ADH2/ADH2::YIp24ADH2-lacZ* diploid that was otherwise homozygous at other loci. The *snf1::URA3* and *bcy1::HIS3* alleles were introduced into these strains by using the one-step gene disruption technique described by Rothstein (47). Their presence was confirmed as described previously (21). Strains KDY18, KDY37, and KDY38 are isogenic with MC71-18Bα. Strain KDY107 was created by changing the *URA3* gene marking the *(lexA$_{op}$)$_8$-lacZ* reporter to *TRP1* by transforming strain L40 (34) with *Sma*I-digested pUT11 DNA as described by Cross (13). The *glc7-127* allele was introduced into cells carrying four copies of the *ADR1* gene by first transforming KT1640 with the *ADH2-lacZ* reporter plasmid pLG*ADH2-lacZ*. One transformant was then crossed with JSY14, which had been constructed previously by integrating three copies of the *ADR1* gene into the genome of strain HHY10 (53). The resulting diploid was sporulated, and segregants were screened for growth on leucine-deficient raffinose–2-deoxyglucose medium. Some of the desired segregants were auxotrophic for uracil, indicating that they did not contain the pLG*ADH2-lacZ* plasmid, so they were then transformed with the *ADH2-lacZ* reporter centromeric plasmid pBGM18, creating strains VBY1, VBY2, and VBY3. Yeast cells were transformed by using a modified version of the lithium acetate procedure as described by Gietz et al. (26). The plasmids used in the present study are listed in Table 2. Their construction is described in subsequent sections.

Media and culture conditions were essentially as described by Sherman (49). Yeast cells were grown at 30°C in yeast extract-peptone (YEP) medium or, when appropriate, selective synthetic medium (SM), which lacks amino acids and uracil and contains either 0.4% Casamino Acids and tryptophan to select for *URA3* plasmids or amino acid dropout solution lacking leucine to simultaneously select for *URA3* and *LEU2* plasmids. Unless otherwise indicated, repressed and derepressed cells were prepared essentially as described previously (21).

**Construction of *CEN* plasmids carrying the wild-type and mutant HA-tagged *REG1* genes.** To make Reg1 easily detectable by Western blotting, its amino terminus was tagged with the hemagglutinin (HA) epitope. First, an *Eco*RI-*Sal*I fragment containing the *REG1* promoter, open reading frame, and 3′ noncoding sequences from pUCSRN1 (61) was cloned between the *Eco*RI and *Sal*I sites of pRS316 (52) to create plasmid pKD63. Next, sequence coding for a single HA tag was introduced immediately after the ATG start codon by using the recombinant PCR strategy described by Higuchi (32). Primers 62-A, 62-B, 62-C, and 62-D (Table 3) were used to simultaneously insert the HA tag and amplify the *REG1* sequence from pUCSRN1 with Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.). The sequence amplified starts just 5′ of the *Nhe*I site in the promoter and ends just past the *Cla*I site in the *REG1* open reading frame. The resulting DNA fragment was treated with Klenow, digested with *Cla*I, and then ligated between the *Sma*I and *Cla*I sites of pGEM7ZF(+) (Promega Corporation, Madison, Wis.) to create plasmid pKD88. The insert was sequenced on both strands and shown to be error-free. Finally, a 0.3-kb *Nhe*I-*Cla*I fragment from pKD88, which has the HA-tagged 5′ end of the *REG1* open reading frame and some proximal promoter sequence; a 3.3-kb *Cla*I-*Sal*I fragment from pUCSRN1, which has the remainder of the open reading frame and 3′ noncoding sequence; and a 5.7-kb vector fragment from pKD63 were assembled in a three-way ligation. This created plasmid pKD89, which has the complete *REG1* gene, including the HA-tagged open reading frame carried on a pRS316 backbone.

Mutations were introduced into the *REG1* open reading frame of pKD89 by using two complementary strategies. All primers used in these constructions are listed in Table 3. Deletions Δ1, Δ3, Δ4, Δ5, Δ8, and Δ9 were introduced with primer pairs 89-1-3′ and 89-1-5′, 89-3-3′ and 89-3-5′, 89-4-3′ and 89-4-5′, 89-5-3′ and 89-5-5′, 89-(455-475)-3′ and 89-(445-475)-5′, and 89-1-5′ and 89-2-3′-N, respectively, by the Quick-Change method (Stratagene, La Jolla, Calif.) with the high fidelity thermostable DNA polymerase *Pfu*. These primer pairs created a *Bgl*II site at the deletion point. The remaining mutant constructs were created by using a combination of preparative PCR and traditional cloning. For deletion Δ2, sequences 5′ to the deletion point were amplified from pKD89 by *Pfu* polymerase with the primer combination of pRS-5′ and 89-2-5′-N. The resulting DNA fragment was then digested with *Eco*RI and *Bgl*II. Sequences 3′ to the deletion point were created in a similar way with the primer combination of pRS-3′ and

TABLE 1. Yeast strains used in this study

| Strain | Genotype | Reference or source |
|---|---|---|
| SB90 | *MATα glc7::LEU2 his4 leu2 trp1-1 ura3-52* pNC160(*TRP1*)-*GLC7* | 1 |
| SB214 | *MATα glc7::LEU2 his4 leu2 trp1-1 ura3-52* pNC160(*TRP1*)-*glc7-127* | 1 |
| SB241(HA) | *MATα glc7::LEU2 his4 leu2 trp1-1 ura3-52* pNC160(*TRP1*)-*glc7-131* | 1 |
| SB259 | *MATα glc7::LEU2 his4 leu2 trp1-1 ura3-52* pNC160(*TRP1*)-*glc7-133* | 1 |
| SB219 | *MATα glc7::LEU2 his4 leu2 trp1-1 ura3-52* pNC160(*TRP1*)-*glc7-134* | 1 |
| KDY80 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ his3Δ200 leu2 lys2-801$^a$ ura3-52 trp1Δ1* | 21 |
| KDY82 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ his3Δ200 leu2 lys2-801$^a$ reg1-1966::LEU2 ura3-52 trp1Δ1* | This study |
| KDY88 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ his3Δ200 leu2 lys2-801$^a$ snf1::URA3 ura3-52 trp1Δ1* | This study |
| KDY89 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ his3Δ200 leu2 lys2-801$^a$ reg1-1966::LEU2 snf1::URA3 ura3-52 trp1Δ1* | This study |
| KDY90 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ bcy1::HIS3 his3Δ200 leu2 lys2-801$^a$ ura3-52 trp1Δ1* | 21 |
| KDY92 | *MAT***a** *ade2-101$^0$ ADH2::YIp23ADH2-lacZ bcy1::HIS3 his3Δ200 leu2 lys2-801$^a$ reg1-1966::LEU2 ura3-52 trp1Δ1* | This study |
| MC71-18Bα | *MATα adh1Δ adh3 leu2 trp1 ura3* | 20 |
| KDY18 | *MATα adh1Δ adh3 leu2 reg1-1966::LEU2 trp1 ura3* | 20 |
| KDY37 | *MATα adh1Δ adh3 leu2 snf1::URA3 trp1 ura3* | This study |
| KDY38 | *MATα adh1Δ adh3 leu2 reg1-1966::LEU2 snf1::URA3 trp1 ura3* | This study |
| KDY107 | *MAT***a** *ade2 his3Δ200 leu2-3,112 LYS2::($lexA_{op}$)$_4$-HIS3 trp1-901 ura3::TRP1::($lexA_{op}$)$_8$-lacZ* | This study |
| BV370 | *MAT***a** *glc7-133 his3 leu2 ura3* | K. Tatchell |
| KT1591 | *MATα leu2 ppz1::LEU2 ppz2::URA3 ura3* | K. Tatchell |
| x209-5d | *MATα glc7-133 his3 leu2 ppz1::URA3 ppz2::LEU2 ura3* | K. Tatchell |
| KT1640 | *MATα glc7-127 his3 leu2 ura3* | K. Tatchell |
| JSY14 | *MAT***a** *adh3 LEU2::(pRS305-ADR1)$_3$ trp1 ura3* | This lab |
| HHY10 | *MAT***a** *adh3 leu2 trp1 ura3* | This lab |
| VBY1 | Leu$^-$ Ura$^+$ 2-DOG$^r$ segregant from KT1640(pBMG18) × JSY14 | This study |
| VBY2 | Leu$^+$ Ura$^+$ 2-DOG$^s$ segregant from KT1640(pBMG18) × JSY14 | This study |
| VBY3 | Leu$^+$ Ura$^+$ 2-DOG$^r$ segregant from KT1640(pBMG18) × JSY14 | This study |

89-2-3′-N. The resulting DNA fragment was then digested with *Bgl*II and *Xho*I. Sequences 5′ to the deletion point were combined with sequences 3′ to the deletion point and cloned between the *Eco*RI and *Sal*I sites of pRS316. Deletions Δ6 and Δ10 were similarly created, except that either the 5′ or 3′ side of the deletion was prepared from other constructs and not by PCR. Deletion Δ6 was created by ligating a 3.6-kb PCR fragment, which was created with primers 89-6-5′-N and pRS-5′, to the 5.3-kb *Eco*RI-*Bgl*II vector fragment of pKD112. For deletion Δ10, a 3-kb *Eco*RI-*Bgl*II fragment from pKD92 was combined with a

TABLE 2. Plasmids used in this study

| Plasmid | Vector | Description | Reference or source |
|---|---|---|---|
| pBGM18 | pRS316 | *URA3-CEN ADH2-lacZ* reporter plasmid (codon 109 of *ADH2* fused to *lacZ*) | This lab |
| pLG*ADH2-lacZ* | pLG669Z | 2μm-*URA3 ADH2-lacZ* reporter plasmid (codon 23 of *ADH2* fused to *lacZ*) | This lab |
| pHDY10 | pLG669Z | 2μm-*URA3* UAS1 reporter plasmid | This lab |
| YIp*23ADH2-lacZ* | YIp5 | Integrating *TRP1 ADH2-lacZ* reporter plasmid | 21 |
| pKD63 | pRS316 | *URA3-CEN* plasmid with *REG1* | This study |
| pKD89 | pRS316 | *URA3-CEN* plasmid with *HA-REG1* | This study |
| pKD93 | pRS316 | pKD89 with the Δ1 deletion | This study |
| pKD94 | pRS316 | pKD89 with the Δ2 deletion | This study |
| pKD95 | pRS316 | pKD89 with the Δ3 deletion | This study |
| pKD96 | pRS316 | pKD89 with the Δ4 deletion | This study |
| pKD92 | pRS316 | pKD89 with the Δ5 deletion | This study |
| pKD97 | pRS316 | pKD89 with the Δ6 deletion | This study |
| pKD98 | pRS316 | pKD89 with the Δ7 deletion | This study |
| pKD104 | pRS316 | pKD89 with the Δ8 deletion | This study |
| pKD111 | pRS316 | pKD89 with the Δ9 deletion | This study |
| pKD112 | pRS316 | pKD89 with the Δ10 deletion | This study |
| pKD114 | pRS316 | pKD89 with the I466M F468A substitutions | This study |
| pKD115 | pRS316 | pKD95 with the deleted region restored | This study |
| pKD109 | pRS316 | pKD63 with *REG1* fused in frame at codon 1002 to *GFP* | This study |
| pKD110 | pRS316 | pKD89 with *HA-REG1* fused in frame at codon 1002 to *GFP* | This study |
| pRSM306 | pRS306 | pRS306 with the 2μm origin from YEp24 | T. Davis |
| pKD123 | pRSM306 | *URA3* 2-hybrid *lexA-REG1* expression plasmid | This study |
| pKD125 | pRSM306 | *URA3* 2-hybrid *lexA-REG1* plasmid with the Δ3 deletion | This study |
| pKD126 | pRSM306 | *URA3* 2-hybrid *lexA-REG1* plasmid with the Δ8 deletion | This study |
| pKD127 | pRSM306 | *URA3* 2-hybrid *lexA-REG1* plasmid with the I466M F468A substitutions | This study |
| pGAD-GLC7 | pGAD | *LEU2* 2-hybrid *GAD-GLC7* plasmid | 57 |
| pTT49 | pGAD | *LEU2* 2-hybrid *GAD-ORC1* plasmid | R. Sternglanz |

TABLE 3. Oligonucleotide primers used in plasmid constructions

| Oligonucleotide primer | Sequence (5′→3′) |
|---|---|
| 62-A | ACCACCTCCTGAAAGAGAAC |
| 62-B | AGCGTAGTCTGGCACGTCATATGGGTACATTTTTGGATTTTTCTTATCTCGTCTTCG |
| 62-C | ATGTACCCATATGACGTGCCAGACTACGCTTCAACAAATCTAGCAAATTACTTCGCCG |
| 62-D | CAATATATTCATCAAGAAGGCCC |
| 89-1-3′ | GTGCCAGACTACGCTAGATCTTGGAGAACATGGGC |
| 89-1-5′ | GCCCATGTTCTCCAAGATCTAGCGTAGTCTGGCAC |
| 89-2-5′-N | CTTCTCCCTTGTCACCAGATCTCCATTCATGTGACAG |
| 89-2-3′-N | GCCAGAAAGACATGTTAGATCTAATTCAAATGGTGGCGG |
| 89-3-3′ | CGAACAGTAGCGTTAGATCTGAAGAACACGGCGG |
| 89-3-5′ | CCGCCGTGTTCTTCAGATCTAACGCTACTGTTCG |
| 89-4-3′ | CCATTGCTAGCCATTCAGAGATCTTCATCAGATAGCG |
| 89-4-5′ | CGCTATCTGATGAAGATCTCTGAATGGCTAGCAATGG |
| 89-5-3′ | GAAAAAAGATCTAGTGATGTTGCCATAGAGGG |
| 89-5-5′ | ATCACTAGATCTTTTTTCCTTGGATTCTACCGC |
| 89-6-3′ | CACCAGCAAACAGATCTTAGAAGAAAGAATTTTGAAGTCAAC |
| 89-6-5′-N | ATACGCTAGATCTAACCAGCTGACTGTTAGCGGGTAATGGTC |
| 89-(455-475)-3′ | CCTCAAACCCAAGTGAAAGATCTTGTATGGCACTACGATATCC |
| 89-(455-475)-5′ | GGATATCGTAGTGCCATACAAGATCTTTCACTTGGGTTTGAGG |
| 89-(349-554)-3′ | CCGTGAGATCTATCATCAGAATGTGATGAAGATGATGATTGTG |
| 89-(349-554)-5′ | AACAGTGGATCCAGTTTGAAGAGTCAACACTCTGAC |
| pRS-5′ | GCAACTGTTGGGAAGGGCGATCGGTGCGGG |
| pRS-3′ | CCATGATTACGCCAAGCTCGGAATTAACCC |
| 89-I466M, F468A-3′ | CCTACTAAAAATAGACATATGCATGCTAATGACAGGGTGG |
| 89-I466M, F468A-5′ | CCACCCTGTCATTAGCATGCATATGTCTATTTTTAGTAGG |
| *Xho*I-GFP-3′ | CACTATCTCGAGAATTGGAGCTCGGTACCAG |
| *Aat*II-GFP-5′ | TCTAGGACGTCCGCAGGCGCTGGAGCCGGTG |

0.4-kb *Bgl*II-*Xho*I PCR fragment, which was created with primers 89-6-3′ and pRS-3′, and an *Eco*RI-*Sal*I vector fragment of pRS316 in a three-way ligation. Deletion Δ7 was created by cloning a 0.98-kb *Eco*RI-*Bgl*II fragment from pKD93 into pKD92. The I466M F468A double substitution was also introduced by using a similar strategy. Sequences 5′ to the substitution were amplified from pKD89 with the primer combination of pRS-5′ and 89-I466M F468A-5′. The resulting DNA fragment was then digested with *Eco*RI and *Sph*I. Sequences 3′ to the substitution were created with the primer combination pRS-3′ and 89-I466M F468A-3′. This DNA fragment was then digested with *Sph*I and *Xho*I. The 5′ and 3′ fragments were then cloned between the *Eco*RI and *Sal*I sites of pRS316 in a three-way ligation. The Δ3+ reconstruction of the *REG1* gene was prepared by cloning a 0.64-kb *Bam*HI-*Bgl*II PCR fragment, which was created with primers 89-(349-554)-5′ and 89-(349-554)-3′, into the *Bgl*II site of pKD95.

**Construction of wild-type and mutant *URA3*-selectable *LexA-REG1* expression plasmids.** To create a *LexA-REG1* expression plasmid into which the *REG1* mutations could be easily transferred, a 5.4-kb *Nar*I-*Sal*I fragment from plasmid pLexA-REG1, which had the *Nar*I end blunted with T4 polymerase, was ligated between the *Sma*I and *Sal*I sites in the polylinker of pRSM306, a 2μm derivative of pRS316, which was kindly provided by the lab of Trisha Davis. The resulting plasmid, pKD123, has a unique *Cla*I site in the *REG1* open reading frame 0.16 kb from the fusion junction with *lexA*. Mutations could then be introduced by cloning a *Cla*I-*Sal*I fragment from one of the mutant *REG1* centromeric plasmids into pKD123. Plasmids pKD125, pKD126, and pKD127 were created in this way.

**Subcellular localization of Reg1-GFP by fluorescence microscopy.** For this study, a *CEN* plasmid carrying the *REG1* promoter and coding sequence fused in-frame at codon 1002 to *GFP* was constructed. A 0.9-kb fragment encoding the F64L,S65T enhanced version of GFP (12) with an *Aat*II site introduced immediately 5′ to sequences coding for an amino terminal alanine-glycine flexible linker was created by PCR with plasmid pLI2000, which was generously supplied by Eric Muller, as the template by using primers *Xho*I-GFP-3′ and *Aat*II-GFP-5′ (Table 3). The resulting PCR fragment was digested with *Aat*II and *Xho*I, gel purified, and then ligated to the 8.8-kb *Aat*II-*Xho*I vector fragment of pKD89 to create pKD106. Next, this plasmid was digested with *Aat*II, treated with T4 polymerase to blunt the ends, and religated to place the *REG1* coding sequence in frame with GFP to create plasmid pKD109.

In preparation for fluorescence microscopy, cells were grown at 30°C in SM broth lacking uracil with 5% glucose as the carbon source and 1.5 mM adenine to suppress the endogenous vacuolar fluorescence of these *ade2* mutant cells. At a density of $10^7$ cells/ml, an aliquot of cells was prepared for viewing. Another aliquot was washed once with cold SM lacking amino acids, adenine, uracil, or glucose. Then the cell pellet was suspended in prewarmed selective SM with 0.05% glucose as the carbon source and 1.5 mM adenine and incubated at 30°C. At 2, 4, 8, and 12 h after shifting to derepressing medium, aliquots of cells were prepared for viewing. Additional aliquots were saved for invertase and β-galactosidase assays at each time point to monitor the course of *SUC2* and *ADH2* derepression. To prepare cells for viewing, they were stained in culture with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co., St. Louis, Mo.), as described by Shero et al. (50). Then an 18-μl aliquot of each DAPI-stained culture was mixed with 6 μl of melted 0.6% agarose containing fresh growth medium and mounted on a microscope slide. Cells were viewed as described by Moser et al. (43) with a Zeiss Axioplan microscope fitted with the appropriate filters for discriminating between DAPI and GFP fluorescence. Images were processed by using Adobe Photoshop, version 4.0 (Adobe Systems, Inc., San Jose, Calif.), and prepared for publication by using Microsoft PowerPoint, version 4.0 (Microsoft Corp., Redmond, Wash.).

**Western blot analyses.** HA-tagged Reg1 and Reg1 fusion proteins were analyzed in native whole-cell extracts. At a density of between $1 \times 10^7$ and $4 \times 10^7$ cells/ml, 40 ml of each culture was centrifuged and the resulting cell pellets were washed once with 5 ml of cold buffer A (25 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 0.01 mM EDTA, 10% glycerol) supplemented with 50 mM KCl, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Then each washed cell pellet was suspended at a density of $9 \times 10^7$ cells/ml in buffer A supplemented with 200 mM KCl, 1 mM dithiothreitol, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM EGTA, 10 mM sodium fluoride, 1 mM PMSF, and 1 μg (each) of pepstatin, aprotinin, and leupeptin per ml. Approximately 110 mg of 500-μm-diameter acid-washed glass beads was added for every 100 μl of cell suspension, and the suspensions were vortexed six times at high speed for 2 min each, with the addition of extra PMSF and 2 min of cooling on ice between each round of vortexing. To clarify the extracts, they were spun at high speed in a microcentrifuge two to four times for 15 min. The resulting clarified extracts were quick-frozen in powdered dry ice and stored at −80°C until needed. Separation of proteins by denaturing polyacrylamide gel electrophoresis and the subsequent transfer of proteins from the gel to nitrocellulose membrane were carried out by using the Mini-Protean II gel system from Bio-Rad Laboratories (Hercules, Calif.) according to the manufacturer's instructions. Blots were probed with either anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim, Indianapolis, Ind.) at a concentration of 5 μg/ml, anti-LexA monoclonal antibody 2-12 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) at a concentration of 2 μg/ml, or polyclonal anti-GFP antibodies, which were kindly provided by T. Davis's lab. Adr1 was analyzed in denatured whole-cell extracts as previously described (20, 21).

**Enzyme and protein assays.** β-Galactosidase activities were determined in permeabilized yeast cells as described by Guarente (28). Invertase activities were measured in whole cells essentially as described by Celenza and Carlson (5). ADH enzyme activity was analyzed in yeast extracts either by directly assaying enzyme activity for *adh1 adh3* mutant cells as described by Denis et al. (17) or by nondenaturing polyacrylamide gel electrophoresis followed by chromogenic staining as described by Williamson et al. (63) for *ADH* wild-type cells. The

TABLE 4. Glc7 proteins defective in repressing *SUC2* expression fully repress *ADH2* expression

| Strain | Relevant genotype | β-Galactosidase activity[a] (Miller units) | | Invertase activity[b] (nmol) | |
|---|---|---|---|---|---|
| | | R | DR | R | DR |
| SB90 | *GLC7* | 3 | 450 | 3 | 200 |
| SB241(HA) | *glc7-131* | 6 | 320 | 54 | 170 |

[a] *ADH2* expression was assayed as β-galactosidase activity expressed by the *ADH2* reporter plasmid pLG*ADH2-lacZ*. These values are the means for at least two independent transformants assayed in duplicate and had a maximum standard deviation of 30%. Repressed cells (R) had 5% glucose and derepressed cells (DR) had 2% glycerol, 2% lactate, 2% ethanol, and 0.1% glucose as the carbon sources, respectively.

[b] *SUC2* expression was assayed as invertase activity in nanomoles of sucrose hydrolyzed per min per unit of optical density at 600 nm of culture used. Each assay was performed in duplicate and had an average range of 30%. R had 5% glucose and DR had 0.1% glucose as the carbon source, respectively.

TABLE 5. Deletion of *snf1* or *bcy1* suppresses constitutive *ADH2* expression

| Strain | Relevant genotype | β-Galactosidase activity[a] (Miller units) | |
|---|---|---|---|
| | | R | DR |
| KDY80 | *BCY1 REG1 SNF1* | 9.5 | 1,140 |
| KDY88 | *BCY1 REG1 snf1* | 6.0 | 6.0 |
| KDY90 | *bcy1 REG1 SNF1* | 4.8 | 9.0 |
| KDY82 | *BCY1 reg1 SNF1* | 220 | 2,740 |
| KDY89 | *BCY1 reg1 snf1* | 4.9 | 4.8 |
| KDY92 | *bcy1 reg1 SNF1* | 12 | 22 |

[a] *ADH2* expression was assayed as β-galactosidase activity expressed from the integrated reporter YIp*23ADH2-lacZ*. The values for KDY80 and KDY82 are means of three independent assays performed in duplicate. All other values are means for three independent disruptants derived from either KDY80 or KDY82 and assayed in duplicate. Each measurement had a standard deviation of less than 20%. Repressed cells (R) had 5% glucose and derepressed cells (DR) had 3% ethanol as the carbon source, respectively.

protein concentration of native cell extracts was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories). To measure the protein concentration of denatured cell extracts, a 5-μl aliquot of each extract was diluted to 125 μl with 100 mM potassium phosphate (pH 7.5), incubated at room temperature for 10 min, and centrifuged in a microcentrifuge at high speed for 10 min to precipitate excess sodium dodecyl sulfate (SDS) (66). Then the Bio-Rad reagent was used to determine the protein concentration in the supernatant.

## RESULTS

**Mutant alleles of *GLC7* that are defective in repressing *SUC2* expression fully repress *ADH2* expression.** Previously, we had reported that *ADH2* expression is undetectable in cells carrying the *glc7-T152K* allele under repressing growth conditions (20). Speculating that this lack of expression might be allele specific, four different mutants, each with a different glucose repression-defective allele of *glc7*, were assayed for *ADH2* expression by ADH native gel analysis (data not shown). ADHII enzyme activity was undetectable in repressed cells expressing either the *glc7-127*, *glc7-131*, *glc7-133*, or *glc7-134* allele (1). Additionally, an episomal *ADH2-lacZ* reporter plasmid, pLG*ADH2-lacZ*, was introduced into cells having the *glc7-131* allele, and β-galactosidase was assayed as a more sensitive measure of *ADH2* expression (Table 4). Invertase activity was also assayed as a measure of *SUC2* expression. β-Galactosidase activity in *glc7-131* mutant cells under normally repressing growth conditions was only 2-fold higher than that in wild-type cells, while invertase activity was 18-fold higher. Therefore, unlike for *SUC2* expression, *ADH2* expression is not significantly affected by mutations in *GLC7*. Similar observations were obtained with the *UAS1-lacZ* reporter pHDY10 (data not shown), suggesting that, unlike mutations in *REG1*, mutations in *GLC7* do not significantly affect *ADR1*-dependent expression. These results confirmed our earlier observation and caused us to question whether Reg1 is repressing *ADH2* expression via the same mechanism as *SUC2* expression.

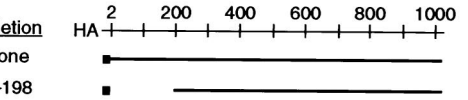
***SNF1* and *BCY1* are required for constitutive *ADH2* expression in *reg1* mutant cells.** If Reg1 represses *ADH2* expression via the same mechanism that it uses to repress *SUC2*, then *SNF1*, which is required for constitutive expression of *SUC2* in *reg1* mutant cells under normally repressing growth conditions (44), as well as for derepression of both *ADH2* and *SUC2* in wild-type cells (4, 10), should also be required for constitutive expression of *ADH2*. To test this argument, strains with an integrated *ADH2-lacZ* reporter and various combinations of *reg1* and *snf1* deletions were prepared and β-galactosidase activity was assayed as a measure of *ADH2* expression (Table 5). Deletion of *REG1* increased the expression of the reporter gene by 23-fold in repressed cells, while deletion of *SNF1* completely abolished derepression. When combined, the *SNF1* deletion was epistatic to the *REG1* deletion, completely preventing constitutive expression in repressed cells as well as derepression of the reporter. Similar results were obtained for the expression of the native *ADH2* gene in a different strain background, MC71-18Bα (20), indicating that expression of the *ADH2-lacZ* reporter was faithfully mimicking that of the endogenous gene and that these results were not strain specific (data not shown). Therefore, constitutive *ADH2* expression in *reg1* mutant cells has the same requirement for *SNF1* as constitutive *SUC2* expression and derepressed *ADH2* expression.

Constitutive *ADH2* expression in *reg1* mutant cells is also *ADR1* dependent (20). Since unregulated cAPK activity inhibits *ADR1*-dependent gene expression, it was of interest to determine whether deletion of *BCY1*, the gene coding for the regulatory subunit of cAPK in *S. cerevisiae*, was also epistatic to the *REG1* deletion. To address this issue, *BCY1* was deleted in the wild-type and *reg1* mutant strains containing the *ADH2-lacZ* reporter and β-galactosidase activity was assayed (Table 5). Deletion of *BCY1* prevented the reporter from significantly derepressing. When combined with the *REG1* deletion, no constitutive activity of the reporter was observed in repressed cells and, as for the single mutant, the double mutant did not significantly derepress reporter gene expression. Thus, constitutive *ADH2* expression in *reg1* mutant cells under normally repressing growth conditions has the same requirement for *BCY1* as *ADH2* expression in derepressed wild-type cells.

**Delineation of the regions of Reg1 required for function by deletion analysis.** Constitutive *ADH2* expression in *reg1* mutant cells required at least one factor needed for *SUC2* expression and appeared to be under the same controls as derepressed *ADH2* expression in wild-type cells. To further elucidate the role of Reg1 in repressing *ADH2* expression, we have delineated the regions of Reg1 required for function. We hoped to determine whether the same regions needed to repress *ADH2* expression were also needed to repress *SUC2* expression. Reg1 was tagged with a single HA epitope at its amino terminus to allow for sensitive and specific detection of the wild-type and mutant proteins in Western blot analyses. When expressed in *reg1* mutant cells from either a *CEN* plasmid or an integrating plasmid, the HA-tagged and untagged versions of Reg1 behaved identically, fully suppressing constitutive *ADH2* expression and the slow growth phenotype (data not shown), which is typical of *reg1* mutant cells having an

A.

| Construct | Deletion | ADH2 | SUC2 | Growth |
|---|---|---|---|---|
| WT | None | 11 | 12 | WT |
| Δ1 | 2–198 | 23 | 130 | WT |
| Δ2 | 166–369 | 20 | 52 | WT |
| Δ3 | 349–554 | 200 | 320 | S |
| Δ4 | 521–724 | 39 | 34 | WT |
| Δ5 | 694–904 | 12 | 13 | WT |
| Δ6 | 869–1014 | 16 | 18 | WT |
| Δ10 | 694–1014 | 14 | 16 | WT |
| Δ9 | 2–369 | 20 | 120 | WT |
| Δ7 | 2–904 | 300 | 480 | S |
| pRS316 | — | 290 | 430 | S |




B.

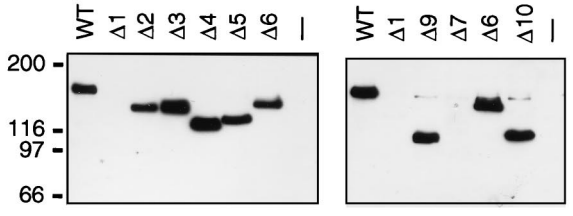



FIG. 1. Deletion analysis of Reg1. (A) Phenotypic analyses of *REG1* deletion constructs. Two independently derived clones of each deletion construct were transformed into strain KDY82. Transformants were grown under repressing conditions in SM broth lacking uracil with 5% glucose as the carbon source. Aliquots of each culture were assayed for *ADH2* and *SUC2* expression. Values for *ADH2* expression measured as *ADH2-lacZ* reporter gene expression are β-galactosidase activities (in Miller units). Values for *SUC2* expression are invertase activities in nanomoles of sucrose hydrolyzed per min per $10^7$ cells. Three transformants were analyzed for each clone, and each value presented represents an average for three independent transformants of the two independent clones. Standard deviations for these values were less than 20%. Growth was assayed by streaking individual transformants to single colonies on SM agar lacking uracil with 2% glucose as the carbon source and assessing colony size after 2 days. WT, wild-type colony size; S, small colony size. (B) Western blot analysis of *REG1* deletion constructs. Proteins from 100 μg of each cell extract were separated on SDS–5.5% acrylamide protein gels. After transfer from each gel to nitrocellulose, HA-Reg1 proteins were identified by chemiluminescence with anti-HA monoclonal antibody as the probe. To the left of the blots are marked the positions of standard molecular weight markers. The right panel was exposed to X-ray film for a fivefold longer period of time than the left panel.

otherwise wild-type genetic background (24). A series of six deletions of approximately 200 amino acids each, scanning the entire length of the protein, were constructed (Fig. 1A). Several larger N- and C-terminal deletions were also prepared. *ADH2-lacZ* and *SUC2* expression were assessed in *reg1* mutant cells containing the wild-type or deletion constructs on *CEN* plasmids. The levels of *ADH2-lacZ* expression fell into two main groups: those with a high mutant level of β-galactosidase activity, deletion constructs Δ3 and Δ7, and those with a low wild-type level of β-galactosidase activity, most of the remaining constructs. This indicates that one region of Reg1 required for repressing *ADH2* expression lies between amino acids 349 and 554. The same region was also required for repressing *SUC2* expression. However, cells carrying constructs Δ1 and Δ9, each of which had wild-type low levels of β-galactosidase activity, exhibited partially constitutive *SUC2* expression, having more than 25% of the constitutive level of invertase activity in *reg1* mutant cells. This suggests that an additional region N terminal to amino acid 198 also plays a role in mediating repression of *SUC2*.

In addition to assessing the effect of each deletion on constitutive *ADH2* and *SUC2* expression, the slow growth phenotype was examined. The growth rate of cells expressing each deletion was assessed as colony size on selective medium after incubation at 30°C for 3 days. In general, the deletions that were unable to suppress constitutive *ADH2* expression, Reg1Δ3 and Reg1Δ7, were also unable to fully suppress the slow growth phenotype (Fig. 1A). Therefore, the same region of Reg1, from amino acids 349 to 554, was required to suppress all three mutant phenotypes, constitutive *ADH2* expression, constitutive *SUC2* expression, and slow growth.

Western blot analysis showed that most of the HA-Reg1 deletion proteins were expressed at approximately the same level as the wild-type protein (Fig. 1B). The Reg1Δ7 protein was not detectable under the experimental conditions used because it was only 13 kDa and migrated off the polyacrylamide protein gels used in the analysis. Only a faint band was detected for Reg1Δ1. This was very surprising, since cells expressing it were almost fully repressed for *ADH2-lacZ* expression. A quantitative comparison by Western blot analysis indicated that this protein was indeed being expressed, however, at a five- to eightfold lower level than the other proteins and also appeared to be less stable (data not shown). Reg1Δ9 was equally defective in repressing *SUC2* expression but it was present at the same level as wild-type Reg1, lending further support for a functional role of the N terminus in repressing *SUC2* expression. Therefore, the region from amino acids 349 to 554 is required for repression of both *ADH2* and *SUC2* and for a wild-type growth rate, while the first 198 amino acids appear to be required mainly for repression of *SUC2*.

The Western blot analysis also revealed that Reg1 migrates anomalously under denaturing conditions in a polyacrylamide protein gel (Fig. 1B). Its predicted molecular mass is 114 kDa, yet it migrated as a protein larger than 150 kDa. All of the

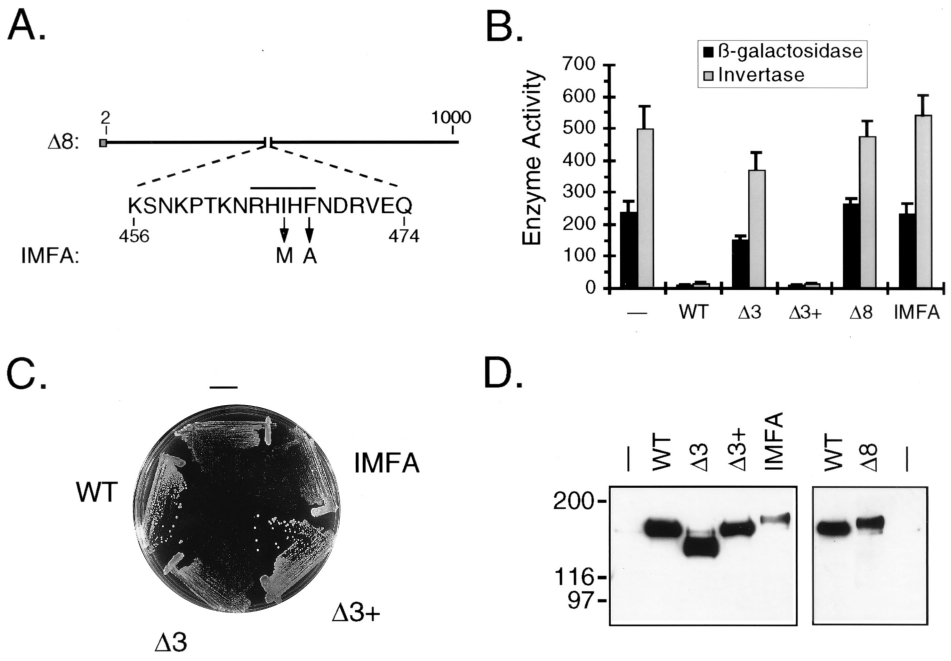



FIG. 2. Analysis of PP1-binding site mutants. (A) Diagram of the Δ8 and IMFA derivatives of Reg1. The amino acid sequence presented is that which is deleted in the Δ8 construct. The overline indicates the position of the putative PP1-binding sequence. Arrows indicate the amino acid substitutions introduced to create the IMFA construct. (B) Analysis of *ADH2* and *SUC2* expression. KDY82 cells transformed with either pRS316 (−), pKD89 (WT), pKD95 (Δ3), pKD115 (Δ3+), pKD104 (Δ8), or pKD114 (IMFA) were grown in synthetic selective broth containing 5% glucose. *ADH2* expression was assayed as β-galactosidase activity (in Miller units) expressed from the integrated *ADH2* reporter plasmid YIp*23ADH2-lacZ*. Each measurement represents the mean for six independent transformants, and error bars represent the standard deviation. *SUC2* expression was assayed as invertase activity (in nanomoles of sucrose hydrolyzed per min per $10^7$ cells). A single transformant having β-galactosidase activity nearest the average was assayed in triplicate. (C) Effect of PP1-binding site mutations on growth. Transformants were streaked to single colonies on SM agar lacking uracil and containing 2% glucose. The agar plate was incubated for 2 days before being photographed. (D) Western blot analysis of binding site mutants. Protein blots of KDY82 transformants were prepared and analyzed as described in the legend for Fig. 1B. The right panel was exposed to X-ray film for a fivefold longer period of time than the left panel. WT, wild type.

deletion derivatives of Reg1 also migrated at anomalously large molecular masses. If this anomalous migration is due to posttranslational modification of the protein, then the modifications must not be restricted to one portion of the protein. This anomalous migration also was not altered in derepressed cells (data not shown). Therefore, the major posttranslational modifications responsible for the anomaly are probably not subject to the controls of glucose repression.

**A PP1-binding motif is required both for Reg1 function and for the interaction with Glc7.** Egloff et al. (22) have pointed out that Reg1 contains the sequence RHIHF, which is homologous to the PP1-binding motif (R/K)(V/I)XF found in mammalian PP1-binding subunits. This putative PP1-binding sequence falls within the region that we have delineated as being required for most, if not all, Reg1 functions. In order to determine the importance of this sequence for function, several new derivatives of Reg1 were constructed and their effects on *ADH2-lacZ* expression, *SUC2* expression, and growth were assessed (Fig. 2). Reg1Δ3+p had amino acids 349 to 554 restored to Reg1Δ3. It was used as a control to determine if any mistakes which could make the protein nonfunctional had been inadvertently introduced into Reg1Δ3 during its construction. Cells expressing this protein had the same level of *ADH2-lacZ* and *SUC2* expression and the same growth rate as cells expressing the wild-type protein (Fig. 2B and C). Therefore, no crucial mistakes were present in the Reg1Δ3 sequence. Reg1Δ8 had amino acids 456 to 474, which contained the RHIHF sequence, deleted, and Reg1-IMFA contained the double amino acid substitution I466M F468A (Fig. 2A). If the RHIHF sequence functions in a manner analogous to that of the mammalian motif, then these two derivatives of Reg1 should be nonfunctional. Cells expressing either one of these proteins had the same levels of *ADH2-lacZ* and *SUC2* expression and the same growth rate as *reg1* mutant cells (Fig. 2B and C). Therefore, both the Δ8 and IMFA derivatives of Reg1 appeared to be nonfunctional. However, Western blot analysis complicated this interpretation because it showed that these two proteins were being expressed at a significantly lower level than the wild-type protein (Fig. 2D). Since Reg1Δ1 is expressed at an even lower level (data not shown) and yet is still functional, this suggests that the levels of expression of the Δ8 and IMFA derivatives should be high enough to suppress the phenotypes of *reg1* mutant cells. Therefore, both derivatives must be nonfunctional, suggesting that the RHIHF sequence is critical for the function of Reg1.

Western blot analysis of the new Reg1 derivatives also revealed that both Reg1Δ1 and Reg1-IMFA migrate more slowly than the wild-type protein in a denaturing polyacrylamide protein gel (Fig. 2D). This mobility shift, however, did not change when cells were derepressed (data not shown). Therefore, this altered mobility also does not appear to reflect a change in the state of glucose-regulated posttranslational modifications.

Since the RHIHF sequence was critical for function, it was of interest to determine whether the Δ3 and Δ8 deletions and the IMFA double substitution reduced or eliminated the interaction with Glc7. The two-hybrid assay with which the Reg1-Glc7 interaction had been first demonstrated was used to address this issue (58). The ability of GAD-Glc7 to specifically stimulate expression of a $lexA_{op}$-*lacZ* reporter gene in the presence of LexA-Reg1 versions containing these changes was as-

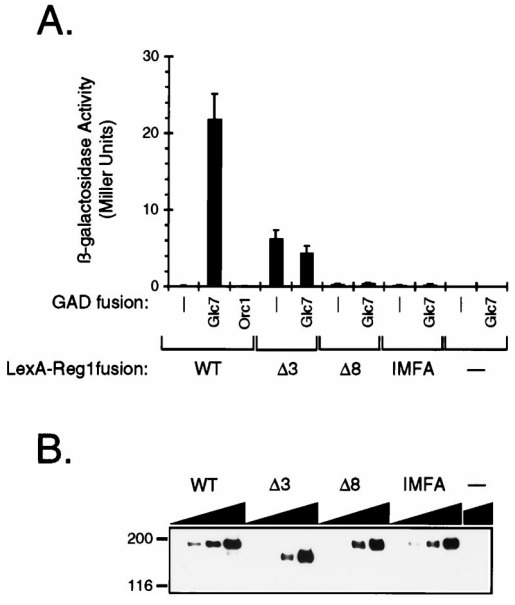



FIG. 3. Effect of PP1-binding site mutations on the two-hybrid interaction between Reg1 and Glc7. (A) Two-hybrid interaction as measured by $lexA_{op}$-*lacZ* reporter gene expression. Strain KDY107 was transformed with combinations of plasmids that allowed the simultaneous expression of wild-type (WT) or mutant versions of LexA-Reg1 and a GAD fusion protein. Transformants were grown in SM lacking leucine and uracil and containing 5% glucose. β-Galactosidase activity was assayed as a measure of $lexA_{op}$-*lacZ* reporter gene expression. Stimulation of reporter gene expression by GAD-Glc7 reflects an interaction with LexA-Reg1. Each measurement is the average of four to nine independent transformants. Each error bar represents the standard deviation of the measurement. (B) Western blot analysis of wild-type and mutant LexA-Reg1 proteins. Cell extracts were prepared from transformants expressing GAD-Glc7 and the various LexA-Reg1 proteins. Twofold serial dilutions, as represented by the dark triangles, starting at 50 μg of protein, were loaded in reverse order onto an SDS–5.5% acrylamide gel. Proteins were transferred to nitrocellulose, and LexA-Reg1 fusion proteins were detected with monoclonal antibodies directed against LexA.

sayed. The LexA-Reg1 fusion proteins behaved similarly to their nonfusion counterparts in their ability to suppress the slow growth phenotype of *reg1* mutant cells (data not shown). GAD-Glc7 stimulated reporter gene expression more than 100-fold in the presence of wild-type LexA-Reg1 (Fig. 3A). Neither GAD nor the nonspecific control GAD-Orc1 significantly stimulated expression. For the versions of LexA-Reg1 containing the Δ3 or Δ8 deletion as well as the IMFA double substitution, GAD-Glc7 was unable to significantly stimulate expression of the reporter gene. Western blot analysis of LexA-Reg1 expression showed that the binding-defective versions of the protein were expressed at a level no less than one-half that of the wild-type protein (Fig. 3B). Together, these results suggest that the RHIHF sequence is critical for the interaction of Reg1 with Glc7.

**Reg1 is localized in the cytoplasm and excluded from the nucleus independently of the carbon source.** Niederacher and Entian have reported that Reg1 is likely to be nuclearly localized based on subcellular fractionation experiments with a Reg1–β-galactosidase fusion protein (46). Therefore, one possible explanation for the inability of the nonfunctional versions of Reg1 to suppress the mutant phenotypes was that they were unable to accumulate in the nucleus. To address this issue, a Reg1-GFP fusion and an HA-tagged version were constructed and their subcellular localization was determined by fluorescence microscopy. These GFP fusions were able to fully suppress constitutive *ADH2-lacZ* and *SUC2* expression, as well as the slow growth phenotype (data not shown). Contrary to our expectation, Reg1-GFP was localized in the cytoplasm and appeared to be excluded from the nucleus (Fig. 4A). Cytoplasmic localization was observed in both repressed and derepressed cells and did not change even up to 12 h after the start of derepression (data not shown). Identical results were obtained with the HA-tagged version of the fusion protein (data not shown). Western blot analysis of the Reg1-GFP fusion proteins showed that they were not being cleaved to release the GFP portion of the fusion protein (Fig. 4B), which then would be localized in the cytoplasm (45). Since wild-type Reg1-GFP was not nuclearly localized, it seemed reasonable to assume that the nonfunctional versions would not be either. Therefore, it seems unlikely that mislocalization to the cytoplasm can explain the defect in function of the mutant Reg1 proteins.

***ADH2* is constitutively expressed in *glc7-131* mutant cells when the level of Adr1 is elevated.** The critical role of the RHIHF sequence in Reg1 function suggests that Glc7 or another PP1-like protein phosphatase is required for full repression of *ADH2* expression. *S. cerevisiae* has three genes coding for protein phosphatases homologous to PP1: *PPZ1*, *PPZ2*, and *PPQ1* (8, 14). Strains containing several combinations of deletions of *PPZ1* and *PPZ2* and the *glc7-133* mutation were tested by ADH native gel analysis for constitutive *ADH2* expression. Strains having the *glc7-133* mutation alone or having deletions of both *PPZ1* and *PPZ2* or having a combination of *glc7-133* and both *PPZ* deletions showed no detectable ADHII enzyme activity when they were grown in glucose medium (data not shown). Therefore, the Ppz1 and Ppz2 phosphatases do not appear to act redundantly with Glc7 to keep *ADH2* expression repressed. *PPQ1* was not tested because it seemed unlikely that a phosphatase which plays a role in translational accuracy (62) would directly affect repression of *ADH2* expression which occurs at the level of transcription (2, 17).

Since other *GLC7* homologues either did not affect *ADH2* expression or seemed unlikely to have an effect, we decided to focus on the *glc7* mutant strains. Constitutive *ADH2* expression in *reg1* mutant cells requires *ADR1* (20), and the level of Adr1 is high in some strains and barely detectable in others. Therefore, we wondered whether the level of Adr1 in repressed *glc7* mutant cells was sufficient to allow constitutive *ADH2* expression. To address this issue, expression of an *ADH2-lacZ* reporter gene under repressing growth conditions was assayed in *glc7* mutant cells that express the same level of Adr1 as derepressed cells. Comparison of the Adr1 level in JSY14, which has three integrated copies of the *ADR1* gene in addition to the genomic copy, with that in HHY10, the strain from which it was created, showed that JSY14 grown under repressing conditions had approximately the same amount of Adr1 as derepressed HHY10 cells (53). When the *glc7-127* mutation was crossed into JSY14 containing an *ADH2-lacZ* reporter gene, the activity of the reporter gene in several segregants derived from two different crosses was always higher than in the *GLC7* wild-type counterpart, as indicated by blue color after growth on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates (data not shown). The level of reporter gene activity in one segregant of each type was quantitated by β-galactosidase assays (Fig. 5A). The *glc7* mutant strain with four copies of *ADR1* VBY3 had 13-fold more β-galactosidase activity than the *GLC7* wild-type counterpart VBY2. The *glc7* mutant strain with only one copy of *ADR1* VBY1 had only threefold more β-galactosidase activity than the *GLC7* wild-type counterpart HHY10. Similar results were obtained when expression of the chromosomal *ADH2* gene was monitored by ADH native gel analysis (data not shown). This indicated that reporter gene

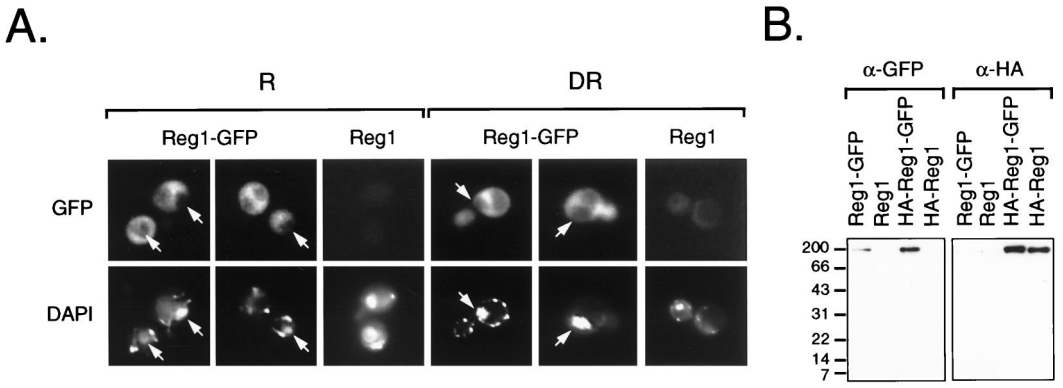



FIG. 4. Subcellular localization of Reg1-GFP. (A) Fluorescence micrographs of KDY82 cells expressing Reg1-GFP. Cells expressing Reg1-GFP as the sole source of functional Reg1 were created by transforming strain KDY82 with plasmid pKD109. KDY82 expressing HA-Reg1 from plasmid pKD89 served as the negative control for yeast cell autofluorescence. Repressed cells (R) were grown in medium containing 5% glucose. A portion of these cells was derepressed (DR) by shifting them to medium containing 0.05% glucose. The derepressed cells shown in these micrographs were prepared 4 h after the shift. By this time, *ADH2-lacZ* expression was beginning to derepress and *SUC2* expression had fully derepressed (data not shown). Cells examined at 2, 4, 8, and 12 h after the shift showed the same distribution of GFP fluorescence. Arrows in the micrographs mark the position of nuclei based on the location of DAPI fluorescence. (B) Western blot analysis of Reg1-GFP. Cell extracts were prepared from repressed KDY82 transformants expressing Reg1-GFP from plasmid pKD109, Reg1 from plasmid pKD63, HA-Reg1-GFP from plasmid pKD110, and HA-Reg1 from plasmid pKD89. Proteins from 100 μg of each cell extract were separated on an SDS–12% acrylamide gel and transferred to nitrocellulose. GFP-tagged proteins were detected with anti-GFP polyclonal antibodies (α-GFP), and HA-tagged proteins were detected with anti-HA monoclonal antibodies (α-HA).

expression was faithfully mimicking that of the chromosomal gene. Western blot analysis showed that the amount of Adr1 in each segregant with multiple copies of the *ADR1* gene did not correlate with the level of reporter gene expression. Both VBY2 and VBY3 had equally high levels of Adr1 (Fig. 5B). HHY10 had a very low level, while VBY1 had an intermediate level. This intermediate level of Adr1 may be responsible for the slightly higher level of reporter gene expression in VBY1 than in HHY10 (Fig. 5A). These results indicate that the level of Adr1 is limiting for constitutive *ADH2* expression in *glc7* mutant cells and also provide the first direct evidence that the Glc7 protein phosphatase can play a role in repressing *ADH2* expression.

## DISCUSSION

In this paper, we provide the first evidence suggesting that the yeast PP1 homologue Glc7 plays a role in repressing *ADH2* expression. It had previously been reported that the *glc7-T152K* allele, which is defective in glucose repression of *SUC2* expression, was able to fully repress *ADH2* expression (20). Here, we confirm this observation by using four other glucose repression-defective alleles of *GLC7*. We also provide evidence suggesting that, like *SUC2* expression, constitutive *ADH2* expression in *reg1* mutant cells requires the *SNF1* regulatory pathway. For *SUC2* expression, this pathway includes the Reg1-Glc7 PP1 complex as a member. Two independent lines of evidence are presented, suggesting that Glc7 does play a role in repressing *ADH2* expression. First, the region of Reg1 containing a PP1-binding motif was shown to be required for repressing *ADH2* expression. Additionally, this motif was shown to be essential for the interaction of Reg1 with Glc7. Second, *GLC7* was required to maintain full repression of *ADH2* expression in a strain expressing the derepressed level of Adr1. This suggests that the level of Adr1 in the original *glc7* mutant strains was probably too low to support constitutive *ADH2* expression. Consistent with this interpretation is the almost complete repression of *ADH2* expression seen for the *glc7* mutant strain expressing the lower than derepressed level of Adr1. *REG1* mutant cells express the fully derepressed level of Adr1 (20). Therefore, it is likely that the *glc7* alleles tested were not defective enough to allow the needed increase in Adr1 level for constitutive *ADH2* expression.

Our deletion analysis has identified the RHIHF sequence starting at amino acid 464 as the only nonredundant region of Reg1 that is essential for function and for binding to the yeast

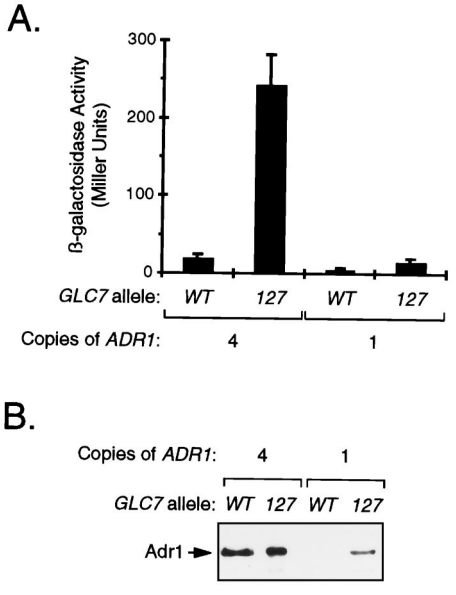



FIG. 5. Constitutive expression of *ADH2-lacZ* in repressed *glc7* mutant cells having a derepressed level of Adr1 protein. (A) Analysis of *ADH2-lacZ* expression. Strains HHY10 (*GLC7*; one copy of *ADR1*), VBY1 (*glc7-127*; one copy of *ADR1*), VBY2 (*GLC7*; four copies of *ADR1*), and VBY3 (*glc7-127*; four copies of *ADR1*), all containing the pBGM18 *ADH2-lacZ* reporter plasmid, were grown initially in SM lacking uracil and containing 8% glucose and then transferred to YEP broth containing 8% glucose to more fully repress expression of the reporter. After incubation overnight, β-galactosidase activity was assayed. Each bar represents the average of four to eight measurements, and each error bar represents the standard deviation of the measurement. (B) Western blot analysis of Adr1 levels. Denatured whole-cell extracts were prepared from repressed cells, and equal amounts of protein were loaded in each lane of an SDS–5.5% acrylamide gel. After transfer to nitrocellulose, Adr1 was detected by using polyclonal antibodies prepared against amino acids 335 to 740 of Adr1.

PP1 homologue Glc7. This is consistent with the previous observation that the N-terminal 317 amino acids are dispensable for function (46). A concurrent study in the Tatchell lab has shown that a related sequence, KNVRF, is required for the function of Gac1 as well as for binding to Glc7 (64). Cells expressing a Gac1 protein that is missing the N-terminal 130 amino acids which contain this sequence or that has the phenylalanine in the sequence replaced with alanine, F73A, are unable to accumulate normal levels of glycogen. The F73A mutant is also unable to interact with Glc7 in a two-hybrid assay. Both the RHIHF and KNVRF sequences conform to the motif (R/K)X(I/V)XF, which is present in a number of other putative Glc7 regulatory subunits (22). However, the importance of this motif for their function or for binding to Glc7 has not yet been reported.

The crystal structure of the mammalian PP1 catalytic subunit complexed with a peptide containing the binding motif from the muscle glycogen-targeting subunit shows that the peptide binds in an extended conformation to a hydrophobic channel on the surface of the protein (22). This channel is located at the junction of two β-sheets of the β-sandwich opposite the catalytic site. By analogy with the mammalian PP1 catalytic subunit, I466 and F468 of Reg1 would be expected to make extensive hydrophobic contacts with the β-strands comprising the channel. These contacts would lie primarily in β-strands or the loop between two strands. Of the known mutations in *GLC7* that are defective in glucose repression, none fall within this region of the protein. We have shown that the region of Reg1 containing the RHIHF sequence is the only contiguous part of the protein absolutely required for the interaction with Glc7. Since the *glc7-T152K* mutation appears to weaken but not abolish the interaction with Reg1 (58), the known *GLC7* mutations may be altering the position of one or more β-strands comprising the hydrophobic pocket rather than disrupting direct protein-protein contacts. Alternatively, the mutant residues may form weak secondary contacts that are not essential for maintaining the interaction.

Another region of Reg1, within the first 198 amino acids, appears to play a differential role in glucose repression. This region had a much stronger influence on *SUC2* repression than on *ADH2* repression. Part of this effect might be attributable to a decrease in protein stability because the level of Reg1 missing this region is much lower than that of the wild-type protein. If decreased stability does play a role, then the region of Reg1 from amino acids 166 to 369 must be responsible for destabilizing the protein. However, instability is probably not the entire picture. A version of Reg1 missing the first 369 amino acids was just as stable as full-length Reg1 and was just as defective in repressing *SUC2* expression as the protein missing the first 198 amino acids. There are at least two possible explanations for this behavior. In one scenario, the N terminus of Reg1 would play a role in stimulating the activity of Glc7 in the PP1 complex. The PP1 holoenzyme containing the N-terminal deletion would still have phosphatase activity; however, the activity would be at a lower level than that with the wild-type version of Reg1. This could account for the observed differential repression if *ADH2* expression required less PP1 activity to be fully repressed than *SUC2* expression. In a second scenario, a protein specifically required for full repression of *SUC2* expression would interact with the N terminus of Reg1. The implication of this protein not playing a major role in repressing *ADH2* expression would be that the Reg1-Glc7 complex which represses *SUC2* expression is different in composition from that which represses *ADH2* expression.

Constitutive *ADH2* expression in *reg1* mutant cells under normally repressing growth conditions is likely to be controlled by the same regulatory mechanisms as derepressed *ADH2* expression, because both were activated by the *SNF1* regulatory pathway and both were inhibited by the cAPK pathway. The *SNF1* requirement of constitutive *ADH2* expression fits nicely with the proposed role of Reg1 in regulating Snf1 activity (40) and suggests that the Reg1-Glc7-Snf1 regulatory mechanism is fundamentally the same as that for *SUC2* expression. In contrast to *ADH2* expression, however, *SUC2* expression is not inhibited by the cAPK regulatory pathway (36). This suggests that the inhibitory effect of cAPK on *ADH2* expression is not at the level of the Snf1 complex but rather at a downstream step. This also agrees with previous observations indicating that Snf1 acts independently of cAPK and Adr1 in controlling *ADH2* expression (16) and that cAPK acts in part by inhibiting expression of Adr1 (21). Other examples of glucose-regulated processes in yeast where the relationship between Reg1-Glc7-Snf1 and cAPK has been examined include RNA processing and glycogen accumulation (31, 35, 56, 60, 61). For RNA processing, cAPK acts as it did for constitutive *ADH2* expression by suppressing the mutant *reg1* phenotype (60, 61). cAPK and Reg1-Glc7-Snf1 also do not appear to affect glycogen accumulation by identical mechanisms because *bcy1* and *snf1* mutations have different effects on expression of *GSY2*, the gene coding for the predominant glycogen synthase activity in *S. cerevisiae* (31).

During the course of this study, we were surprised to find that the subcellular location of Reg1 was cytoplasmic and not nuclear. In fact, our data with a fully intact and functional Reg1-GFP fusion protein suggest that Reg1 may actually be excluded from the nucleus. Niederacher and Entian (46) had reported previously that a LacZ fusion protein containing the first 316 amino acids of Reg1 was nuclear and that the amino acid sequence of Reg1 contained several possible nuclear targeting signals. The Reg1-GFP fusion protein that we used in this study was not too large to enter the nucleus, and the GFP portion of the protein did not block nuclear entry because a substantially larger Adr1-GFP fusion protein was able to be accumulate in the nucleus (data not shown). This difference in results might be due to the artifactual accumulation of the Reg1-LacZ fusion in the nucleus when tetramers form through the association of the LacZ portion of the protein. GFP monomers are not known to associate into higher-ordered structures. Alternatively, a cryptic nuclear localization signal within the N-terminal segment of Reg1 may have been uncovered in the LacZ fusion protein used previously. The GFP fusion used in this study was missing only the C-terminal 12 amino acids of Reg1.

The cytoplasmic localization of Reg1 has an interesting implication for the regulation of Snf1 protein kinase activity and perhaps for glucose repression in general. One of the proposed roles for the Reg1-Glc7 protein phosphatase is its participation with the Snf1 kinase complex in a regulatory circuit that controls the subcellular localization of Mig1 (19), a glucose-regulated zinc finger protein involved in repressing expression of genes involved in the utilization of alternate carbon sources (25). In cells growing on glucose, Mig1 is nuclearly localized (19). Upon removal of glucose from the growth medium, Mig1 undergoes Snf1-dependent phosphorylation (57) and is rapidly translocated out of the nucleus (19). Two-hybrid and coimmunoprecipitation experiments have suggested that there is a direct interaction between Snf1 and Mig1 (57). Therefore, upon removal of glucose, the activated Snf1 complex is most likely located in the nucleus where it can phosphorylate Mig1. Since Reg1 is primarily cytoplasmically localized, any interaction between the Snf1 complex and the Reg1-Glc7 complex is likely to occur in the cytoplasm. This is consistent with the

results of indirect immunofluorescence studies indicating that both Snf1 and Snf4 are distributed throughout the cytoplasm as well as being localized in the nucleus (6, 7). However, since the activated Snf1 complex is probably nuclear, the activated form of Snf1 most likely cycles rapidly between the nucleus and the cytoplasm, where it can be inactivated by the Reg1-Glc7 form of PP1 when glucose is added to the growth medium.

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