The REG2 Gene of *Saccharomyces cerevisiae* Encodes a Type 1 Protein Phosphatase-Binding Protein That Functions with Reg1p and the Snf1 Protein Kinase to Regulate Growth

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The GLC7 gene of *Saccharomyces cerevisiae* encodes the catalytic subunit of type 1 protein phosphatase (PP1) and is essential for cell growth. We have isolated a previously uncharacterized gene, REG2, on the basis of its ability to interact with Glc7p in the two-hybrid system. Reg2p interacts with Glc7p in vivo, and epitope-tagged derivatives of Reg2p and Glc7p coimmunoprecipitate from cell extracts. The predicted protein product of the REG2 gene is similar to Reg1p, a protein believed to direct PP1 activity in the glucose repression pathway. Mutants with a deletion of *reg1* display a mild slow-growth defect, while *reg2* mutants exhibit a wild-type phenotype. However, mutants with deletions of both *reg1* and *reg2* exhibit a severe growth defect. Overexpression of REG2 complements the slow-growth defect of a *reg1* mutant but does not complement defects in glycogen accumulation or glucose repression, two traits also associated with a *reg1* deletion. These results indicate that *REG1* has a unique role in the glucose repression pathway but acts together with *REG2* to regulate some as yet uncharacterized function important for growth. The growth defect of a *reg1 reg2* double mutant is alleviated by a loss-of-function mutation in the *SNF1*-encoded protein kinase. The *snf1* mutation also suppresses the glucose repression defects of *reg1*. Together, our data are consistent with a model in which Reg1p and Reg2p control the activity of PP1 toward substrates that are phosphorylated by the Snf1p kinase.

The reversible phosphorylation of proteins has long been recognized as a widespread mechanism of posttranslational regulation among eukaryotes. The phosphorylation state of a given protein is dependent on the relative activities of protein kinases and protein phosphatases. Early biochemical studies suggested that protein phosphatases might represent a much smaller group of enzymes than protein kinases. Whereas most kinases recognize specific motifs of five or six amino acids (46), phosphatases generally exhibit a fairly broad substrate specificity (17, 64). These data contributed to the idea that cellular signaling responses were largely determined by the activities of specific protein kinases whereas phosphatases functioned at a low constitutive level (64). Recent advances have underscored the importance of protein phosphatases in controlling physiological processes and have demonstrated that protein phosphatases are in fact highly regulated.

The serine/threonine protein phosphatases are among the most conserved proteins throughout evolution. The type 1 protein phosphatase (PP1) is >80% identical in mammals and in yeasts (24, 55) and has been demonstrated to play key roles in a variety of cellular processes. In mammalian cells, PP1 regulates glycogen metabolism, muscle contractility, and protein synthesis (4, 17, 64) and has been shown to interact with the product of the retinoblastoma tumor suppressor gene (20). Studies of *S. cerevisiae* have likewise demonstrated multiple physiological roles for PP1, including glycogen metabolism (10, 24), sporulation (10), cell cycle progression (36, 84), chromosome segregation (27), protein synthesis (80), and glucose repression (74). The obvious question is, how does a single enzyme regulate so many diverse processes in so many different locations within the cell?

Several years ago, Cohen and colleagues introduced the idea of regulatory or targeting subunits in response to this question (18, 43). Their hypothesis proposed that both the subcellular location and the catalytic activity of PP1 were governed by a variety of regulatory subunits that directed the phosphatase to particular locations and/or altered its activity toward particular substrates. Several examples of PP1 regulatory subunits have now been described, and the list is likely to grow. PP1 isolated from mammalian skeletal muscle is found in association with a glycogen-targeting subunit that when bound to the catalytic subunit increases the activity of PP1 toward glycogen-associated substrates (41, 42). The smooth muscle form of PP1 is a heterotrimer composed of the catalytic subunit and a regulatory complex that enhances the rate at which the smooth muscle form of PP1 dephosphorylates smooth muscle myosin (1, 19).

Studies of *S. cerevisiae* by combined molecular and genetic approaches have led to the identification of new PP1 regulatory subunits. The *GAC1* gene is required for normal glycogen accumulation in *S. cerevisiae*. Gac1p is most similar to the mammalian glycogen-targeting subunit from skeletal muscle (28, 69), and it physically interacts with PP1 (67). A gene in the fission yeast *Schizosaccharomyces pombe* (sds22+) and an *S. cerevisiae* homolog (SDS22/EGP1), encode essential PP1-binding proteins believed to be required for the metaphase-to-anaphase transition during mitosis (35, 48, 56, 66).

Most recently, the *REG1* gene product of *S. cerevisiae* has been demonstrated to bind PP1 and is proposed to direct the activity of PP1 in glucose repression (75). Glucose repression is the phenomenon in yeast cells whereby a large number of genes required for metabolism of alternate carbon sources are repressed in the presence of glucose. A previous study has reported that PP1, encoded by *GLC7*, is required for mainte-
Strains were grown in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose), or synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids. Diploid strains were induced to sporulate on medium containing 1% yeast extract, 2% peptone, and 2% acetate. For assays of galactose-inducible gene expression, strains were grown in minimal media containing 2% galactose and the appropriate amino acid supplements. 2-Deoxyglucose (2-DG) resistance was tested on synthetic media containing 2% sucrose and 200 μg of 2-DG (Sigma) per ml under anaerobic conditions with GasPaks (Difco Laboratories). Control plates lacked 2-DG.

### Materials and Methods

**Strains, media, and growth conditions.** The genotypes of the *S. cerevisiae* strains used in this work are listed in Table 1. New alleles were introduced into the JC482 background by yeast transformation or by performing a minimum of six serial backcrosses. Yeast transformation was performed by the lithium acetate method of Gietz et al. (29). Standard yeast genetic procedures for diploid construction, tetrad analysis, and medium preparation were as described by Rose et al. (61). *Escherichia coli* DH5α was used for propagation of plasmids. Yeast strains were grown in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose), or synthetic medium (0.67% yeast nitrogen base, 2% glucose) supplemented with amino acids. Diploid strains were induced to sporulate on medium containing 1% yeast extract, 2% peptone, and 2% acetate. For assays of galactose-inducible gene expression, strains were grown in minimal media containing 2% galactose and the appropriate amino acid supplements. 2-Deoxyglucose (2-DG) resistance was tested on synthetic media containing 2% sucrose and 200 μg of 2-DG (Sigma) per ml under anaerobic conditions with GasPaks (Difco Laboratories). Control plates lacked 2-DG.

#### TABLE 1. *S. cerevisiae* strains used

<table>
<thead>
<tr>
<th>Yeast strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype</th>
<th>Source or reference</th>
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<td>S. J. Elledge</td>
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<sup>a</sup> All strains excluding Y190 and Y187 are congenic to JC482 (11).  
<sup>b</sup> UAS, upstream activation sequence.
no color development when mated to Y187 harboring pAS1-SNF1, pAS1-Lamin, pAS1-p35, or pAS1-CDK2. Strains containing pACT-GAC1 and pAS1-GLC7 or pACT-SNF4 and pAS1-SNF1 served as positive controls for measuring β-galactosidase activity. Plasmids containing potential interacting cDNAs were rescued from strain Y190 and transformed into E. coli DH5α. Restriction enzyme analysis revealed two different plasmid species among 10 E. coli transformants analyzed. Following transformation into strain Y190 carrying pAS1-GLC7, clone 21-4 showed strong β-galactosidase activity. This plasmid was chosen for further analysis.

**DNA sequencing of REG2**. Restriction fragments from both the original REG2 cDNA isolated in the two-hybrid screen (clone 21-4) and a genomic clone encoding REG2 were subcloned into pBluescript SK (Stratagene Cloning Systems). Both the template and complementary strands of the REG2 open reading frame were sequenced by using a double-stranded template and Sequenase enzyme and reagents (Sequenase version 2.0, United States Biochemical Corp.). Sequencing reactions used the M13-20 and reverse primers. Custom-made oligonucleotides were used to fill internal gaps in the sequence. Synthesis of custom primers and some sequencing reactions were carried out by the Iowa State University DNA Sequencing and Synthesis Facility. A primer homologous to the Galp transcriptional activation domain in pACT was used to sequence through the GALA-REG2 cDNA gene fusion, confirming that the REG2 coding sequence was in the correct reading frame.

**Generation of reg1::LEU2, reg2::LEU2 and rnl1-1 alleles.** A HindIII-Apa1 fragment containing the REG2 coding sequence was excised from genomic clone QJ2, a gift of Jonathan Warner (45). This fragment was subcloned into the HindIII-Apa1 sites of pBluescript SK to generate plasmid pBS-REG2. A blunt end was created by digestion of BSF-REG2 with BstI followed by treatment with the Klenow fragment of DNA polymerase. Plasmid YEp24 (Stratagene) was digested with AccI, and the coding the URA3 selectable marker, was similarly blunt ended by digestion with BstD1 and subsequent treatment with Klenow enzyme. Both blunt-ended plasmids were then digested with XbaI. The 1.5-kb XbaI-BstD1 URA3 fragment was ligated into the XbaI-AccI sites of pBS-REG2 to generate pBS-URG2, creating a double-stranded template and Sequenase enzyme and reagents (Sequenase version 2.0, United States Biochemical Corp.). Sequencing reactions used the M13-20 and reverse primers. Custom-made oligonucleotides were used to fill internal gaps in the sequence. Synthesis of custom primers and some sequencing reactions were carried out by the Iowa State University DNA Sequencing and Synthesis Facility. A primer homologous to the Galp transcriptional activation domain in pACT was used to sequence through the GALA-REG2 cDNA gene fusion, confirming that the REG2 coding sequence was in the correct reading frame.

**Finding of reg2::LEU2 null allele, pBM1966 (a gift from Mark Johnston) was digested with PstI and then transformed into the diploid strain DF104. pBM1966 carries a LEU2 disruption of the reg2 coding sequence. Tetrad dissection of meiotic progeny from LEU1+ transformants yielded the expected 2:2 segregation of the reg1 slow-growth phenotype. In subsequent studies, the reg1::LEU2 allele behaved identically to a reg2::URA3 disruption strain (a generous gift from Marian Carlson).

**Isogenic rnl1-1 strains were generated by transforming strain DF104 with XbaI-linearized plasmid pRU6 (rnl1-1 allele) or pRU53 (rnl1b allele).** These plasmids were a gift of Anna Hopper and have been described previously (72).

**Coimmunoprecipitation of Reg2p and PP1.** The HindIII-Xba1 fragment containing the REG2 open reading frame was subcloned into the HindIII-Xba1 sites of YEp351 (33) to generate Yep-REG2.

**PCR amplification was used to place REG2 under control of the GAL1 promoter.** One pair of primers was designed to introduce BamHI sites (underlined) at the 5′ ‘5′-AAAGACATGTGAGTGAACATTTCTG’ 3′ and 5′ ‘5′-AAATGATCCATTATAGAAGCAAGGTGATGATT’ 3′ ends of the REG2 open reading frame, using standard PCR conditions and Pfu polymerase (Stratagene). The BamHI-REG2 fragment was ligated into the BamHI site of YEpC15 (26) to produce YcpHo-REG2. In addition to being placed under GAL1 control, protein coding sequences cloned into YcpC15 are fused to the hemagglutinin (Ha) epitope which is recognized by monoclonal antibody 12CA5.

**Analysis of reg1::LEU2, reg2::LEU2 and rnl1-1 alleles.** A HindIII-Apa1 fragment containing the REG2 coding sequence was excised from genomic clone QJ2, a gift of Jonathan Warner (45). This fragment was subcloned into the HindIII-Apa1 sites of pBluescript SK to generate plasmid pBS-REG2. A blunt end was created by digestion of BSF-REG2 with BstI followed by treatment with the Klenow fragment of DNA polymerase. Plasmid YEp24 (Stratagene) was digested with AccI, and the coding the URA3 selectable marker, was similarly blunt ended by digestion with BstD1 and subsequent treatment with Klenow enzyme. Both blunt-ended plasmids were then digested with XbaI. The 1.5-kb XbaI-BstD1 URA3 fragment was ligated into the XbaI-AccI sites of pBS-REG2 to generate pBS-URG2, creating a double-stranded template and Sequenase enzyme and reagents (Sequenase version 2.0, United States Biochemical Corp.). Sequencing reactions used the M13-20 and reverse primers. Custom-made oligonucleotides were used to fill internal gaps in the sequence. Synthesis of custom primers and some sequencing reactions were carried out by the Iowa State University DNA Sequencing and Synthesis Facility. A primer homologous to the Galp transcriptional activation domain in pACT was used to sequence through the GALA-REG2 cDNA gene fusion, confirming that the REG2 coding sequence was in the correct reading frame.

**Identification of probable interacting cDNAs.** A cDNA library fused to sequences encoding the Gal4p activation domain (amino acids 768 to 881) was coexpressed with a plasmid (pAS1-GLC7) containing the GLC7 gene encoding FPI1 fused to the Gal4p DNA binding domain (amino acids 1 to 147). Positive interacting clones were identified by the ability to induce transcription of the GAL-regulated lacZ reporter gene. Clone 21-4, which we have since renamed pACT-REG2, repeatedly showed high β-galactosidase activity. Induction of β-galactosidase was dependent on the presence of pAS1-GLC7, as no color development was observed in the presence of plasmids containing nonspecific gene fusions. The GAC1-encoded glycosyl regulatory subunit interacts strongly with wild-type GLC7 but is defective in its interaction with the glycosogen-deficient allele, glc7-1 (67). In contrast, pACT-REG2 interacted strongly with both pAS1-GLC7 and pAS1-glcn-7.1. Partial DNA sequence analysis followed by a BLAST search revealed that the cDNA insert in clone 21-4 was identical to a previously uncharacterized open reading frame located 366 nucleotides upstream of the REB1 locus on chromosome II. The complete gene sequence was obtained from a genomic clone (a gift of Jonathan Warner) corresponding to a 5.8-kb region of chromosome II containing REB1 (45). This gene, REG2, consists of an uninterrupted open reading frame 1,015 nucleotides in length encoding a protein product of 338 amino acids (Fig. 1). The original cDNA isolated in the two-hybrid screen was missing only the first 16 nucleotides of the REG2 coding sequence. During the course of this work, our sequence data and location of REG2 were confirmed in the published sequence of chromosome II (23) available in the databases.

**Results**. A BLAST search revealed that the REG2 coding sequence was most similar to that of the REG1 gene from S. cerevisiae. REG1 encodes a 1,014-amino-acid protein reported to be a negative regulator of glucose-repressible genes (21, 51). In addition to its role in glucose repression, REG1 was isolated as an extragenic suppressor of maI mutants which are defective in both RNA processing and transport of RNA from the nucleus to the cytoplasm (57, 79). The predicted protein products of REG1 and REG2 are 29% identical and 48% similar. The similarity extends throughout the length of Reg2p but is limited to the central half of Reg1p (residues 217 to 763) (Fig. 1). Alignment by using the Bestfit program (Genetics Computer Group, Madison, Wis.) introduced 13 gaps into the Reg1p amino acid sequence (Fig. 1A) but only 3 small gaps in the Reg2p sequence. Despite the relatively low level of similarity, we were encouraged that Reg2p was in fact a regulator of PPI activity by a recent report demonstrating that Reg1p also associates with PPI and may direct its role in the glucose repression pathway (75).

**Immunoprecipitation of Reg2p and PPI.** To substantiate our hypothesis that REG2 encodes a PPI-binding protein, we tested the association of Reg2p and PPI1 in vitro with an immunoprecipitation assay. The REG2 coding sequence was amplified by PCR and subcloned into the BamHI site of plasmid YcpC15 (Fig. 2). Insertion of a protein-coding sequence into
themultiplecloningsiteofYCpIF15placesproteinsynthesis undercontrolofthe\textit{GAL1}promoter,whichtodrivetranscrip-
tionwhencellsaregrownongalactose-containingmediumbut
notwhentheyaregrownonglucose-containingmedium. In
addition,cloningofa protein-codingsequenceintoYCpIF15
resultsinthefusionoftheHaepitopetagtotheaminoter-
minaloftheprotein.FusionoftheHaepitopetoReg2pdid
notimpairfunction(seebelow).Cellularextractsfromstrains
carryingYCp-Ha-REG2grownunderevokingconditions
wereanalyzedbyimmunoblotting. Figure3AshowsthatHa-
Reg2migratesatapproximately48kDa.Ha-Reg2pis ex-
pressedatalevelthatisbarelydetectableineitherawild-type
ora\textit{reg2}strainbutisup-regulatedsignificantlyinboththe\textit{reg1}
and\textit{reg1 reg2}mutants(Fig.3A;comparelanes2and3with
lanes4and5).Becauseofthisobservation,immunoprecipita-
tionexperimentswereperformedina\textit{reg1::URA3}
background.

Epitope-taggedderivativesofReg2p(Ha-Reg2p)andPP1
(Myc-Glc7p)wereprecipitatedwithmonoclonalantibodies,
12CA5and9E10,respectively.Constructionofepitope-tagged
Glc7phasbeendescribedpreviously(68).Immunoprecipitates
wereelectrophoresedonsodiumdodecylsulfate(SDS)-poly-
acrylamidegels,transferredtonitrocellulose,andevidencedby
immunoblotting.WhenHa-Reg2pisdirectlyprecipitatedwith
ananti-Haantibody,asmallamountofMyc-Glc7pmi-
gratingat36kDaiscoprecipitated(Fig.3B,lane3).Similarly,
when anti-Myc antibody is used to precipitate Myc-Glc7p, a
bandcorrespondingtoraReg2piscoprecipitated(Fig.3B,
lane4).Abandof48kDaissubjectedinimmunoprecipi-
tatiorsofMyc-Glc7pintheabsenceofYCp-
Ha-REG2(data
notshown)andcannotbeprecipitatedwiththeanti-Mycan-
tibodyfromstrainslacking\textit{myc-GLC7}(Fig.3,lane2).From
thesesresults,weconcludethatHa-Reg2pandMyc-Glc7p
are
physicallyassociatedinourcellextracts.

\textit{REG1}and\textit{REG2}arefunctionallyredundant.A\textit{REG2}
null
allele was constructed to determine whether cells require
\textit{REG2}function. A diploid yeast strain was transformed with a
linearized plasmid in which all but the carboxy-terminal 19
nucleotides of the\textit{REG2}codingsequencewere replaced with
the\textit{URA3}selectablemarker(Fig.2).Theintegrityofthe
\textit{reg2::URA3}disruption was confirmed by genomic Southern

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{FIG. 1. The predicted \textit{REG2}proteinproductishomologoustoReg1p.The
BestFitprogram(GeneticsComputerGroup)wasusedtogeneratedanalignment
oftheReg1pandReg2paminoacid(aa)sequences.Overall,thetwoproteinsare
29%identicaland48%similar.(A)Reg1pandReg2paminoacidsequenceare
alignedtothemiddleconservedregionofReg1p.Verticalinesaredrawnbetweenidenticalresidues,andeconservative
changesareindicatedbydots.GapsinsertedwithinReg1paresrepresentedby
dottedlines.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{FIG. 2. \textit{REG2}plasmidconstructs. The restriction maps of genomic DNA contained in the various plasmids used for this study are depicted. The open bar
correspondstothelogcodingsequence,withsurroundingchromosomalDNArepresentedbyboldlines.Thepositionofthe\textit{URA3}-tagged\textit{reg2}deletion
(pBS-reg2::\textit{URA3})isindicatedbytheshadedbar. ThepositionoftheHaepitopetaginYCp-Ha-REG2isindicatedbyshading.}
\end{figure}
hybridization analysis (data not shown). Sporulation and tetrad analysis of meiotic progeny from the resulting \textit{reg2::URA3/REG2} heterozygous diploid revealed no observable traits associated with loss of \textit{REG2} function. The \textit{reg2} null mutants grew normally at temperatures ranging from 11 to 37°C and on a variety of growth media, including 2% ethanol and glycerol, 0.9 M NaCl, 8 mM caffeine, or 10 μg of benomyl per ml. Additionally, the \textit{reg2::URA3} mutants were normal with respect to glycogen accumulation, glucose repression, and sporulation. However, when a \textit{reg2::URA3} haploid strain was mated to a strain carrying a \textit{reg1::LEU2} null allele, the resulting Ura⁺ Leu⁺ double mutants exhibited a severe growth defect (Fig. 4A). While \textit{reg1} single mutants have a slightly smaller colony size on plates in our strain background, the \textit{reg1::URA3 reg2::LEU2} double mutants formed pin-sized colonies not visible in Fig. 4A. The same result was seen in experiments in which both \textit{reg1} and \textit{reg2} were disrupted with \textit{URA3}. Sporulation of a \textit{reg1::URA3/REG1 reg2::URA3/REG2} heterozygous diploid yielded tetratypes consisting of one normal-sized Ura⁺ colony (\textit{REG1 REG2}), one normal-sized Ura⁺ colony (\textit{REG1 reg2}), one slowly growing Ura⁺ colony (\textit{reg1 REG2}), and one pin-sized Ura⁺ colony (\textit{reg1 reg2}). In every case in which there were two Ura⁺ and two Ura⁻ colonies, the Ura⁻ (\textit{REG1 REG2}) spores showed normal growth whereas the Ura⁺ spores (\textit{reg1 reg2}) exhibited a severe growth defect (data not shown). The \textit{reg1 reg2} double mutants were not, however, growth arrested. These cells continued to grow and divide, albeit at a much lower rate than either wild-type or \textit{reg1} single-mutant strains. Microscopic examination of \textit{reg1 reg2} double mutants revealed no gross cell morphology defects and no accumulation of cells in a specific phase of the cell cycle.

To test whether overexpression of \textit{REG2} could compensate for loss of \textit{reg1}, plasmid YCp-Ha-REG2 was transformed into both \textit{reg1} and \textit{reg1 reg2} mutant strains. This construct, containing \textit{REG2} under control of the \textit{GAL1} promoter, complemented the growth defect of \textit{reg1::URA3} and \textit{reg1::URA3 reg2::URA3} strains when grown on galactose but not glucose (Fig. 5A). We conclude that overexpression of \textit{REG2} complements the slow-growth phenotype of both \textit{reg1::URA3} single mutants and \textit{reg1::URA3 reg2::URA3} double mutants, suggesting that \textit{REG1} and \textit{REG2} are functionally related.

**Overexpression of \textit{REG2} has no effect on the glucose derepression, glycogen hyperaccumulation, and \textit{RNA1-1} suppression phenotypes of a \textit{reg1} mutant.** \textit{REG1} was originally identified on the basis of its role in carbohydrate metabolism and is thought to encode a negative regulator of glucose-repressible genes. Strains harboring a \textit{reg1::URA3} disruption allele express...
invertase, a glucose-repressible enzyme, at a constitutively high level (54). Wild-type strains, on the other hand, express invertase poorly when grown on glucose. To determine if overexpression of \textit{REG2} could complement the glucose-derepressed phenotype of \textit{reg1} mutants, invertase activity was measured in a \textit{reg1::URA3} strain carrying either a high-copy-number \textit{REG2} plasmid (YEp-\textit{REG2}) or a control vector (YEp351). Qualitative analysis of glucose derepression was also carried out by streaking the identical strains onto sucrose plates containing 2-DG. 2-DG is a glucose analog which stimulates glucose repression but cannot be metabolized. As a result, only strains that if defect in glucose derepression accumulate 2-DG. 2-DG is a glucose analog which stimulates glucose repression but cannot be metabolized. As a result, only strains that accumulate 2-DG are able to utilize sucrose in the presence of 2-DG. From these experiments, we conclude that overexpression of \textit{REG2} does not abrogate the glucose-derepressed phenotype of a \textit{reg1} mutant. Even in the presence of a high-copy-number \textit{REG2} plasmid, a \textit{reg1} deletion strain maintains high invertase activity (Table 2) and remains 2-DG resistant (data not shown).

In addition to the slow-growth and glucose derepression phenotypes associated with \textit{reg1} null mutants, we and others (39, 75) observed that these strains hyperaccumulate glycogen. Qualitative glycogen measurements are made by inverting a petri plate over iodine crystals. Wild-type colonies stain medium brown within 1 to 2 min, while strains deficient in glycogen accumulation remain yellow. We consistently observed that \textit{reg1} disruption strains turn a dark brown color within 5 to 10 min after being inverted over iodine, while wild-type colonies remain unstained during the same time period. To test whether overexpression of \textit{REG2} was able to complement the glycogen hyperaccumulation phenotype of a \textit{reg1} null strain, a \textit{reg1::URA3} strain was transformed with either YEp-\textit{REG2} or a control vector (YEp351). Figure 5B shows the resulting transformants alongside wild-type strains carrying the identical plasmids following exposure to iodine crystals. The wild-type strain stains a yellow-light brown color irrespective of the \textit{REG2} plasmid, whereas the \textit{reg1::URA3} strain stains a very dark brown within the same time period (Fig. 5B). The \textit{reg1::URA3} strain harboring plasmid YEp-\textit{REG2} accumulates glycogen at levels comparable to those for the identical strain carrying an empty vector.

\textit{glc7-1} encodes an allele of the PP1 catalytic subunit, the distinguishing feature of which is a dramatic glycogen deficiency (10, 28, 58). The glycogen hyperaccumulation phenotype of \textit{reg1}, which encodes a predicted subunit of PP1, prompted us to assay glycogen levels in a \textit{reg1 glc7-1} double mutant. Crosses between \textit{reg1} and \textit{glc7-1} strains reveal that the double mutants accumulate an intermediate level of glycogen (data not shown). However, \textit{glc7-1} does not suppress the colony size defect of \textit{reg1}, and subsequent crosses to a \textit{reg2} strain reveal that \textit{glc7-1} does not suppress the severe growth defect of the \textit{reg1 reg2} double mutants (data not shown).

Along with the proposed role of \textit{REG1} as a negative regulator of glucose-repressible genes, \textit{reg1} mutants have been demonstrated to suppress RNA processing defects and temperature-sensitive growth of \textit{mal-1} and \textit{ppr} mutants (49, 57, 79). The \textit{RNA1} gene encodes a cytosolic protein (38) shown to affect pre-mRNA and pre-mRNA processing (37, 47) as well as transport of RNA from the nucleus to the cytoplasm (2, 44, 65). The \textit{PRP} genes affect pre-mRNA processing, and several have been found to encode splicing factors or components of spliceosomes (63). Given that \textit{Reg1p} and \textit{Reg2p} may perform redundant functions, we tested whether overexpression of \textit{REG2} might abolish \textit{reg1} suppression of \textit{mal-1}. We reasoned that if defects associated with \textit{mal-1} mutants were suppressed by loss of \textit{reg1} function, then overexpression of the functionally related \textit{REG2} gene might prevent suppression. Nonetheless, a \textit{reg1::URA3 mal-1} double mutant (strain DF205) carrying \textit{REG2} on a high-copy-number plasmid (YEp-\textit{REG2}) was able to maintain growth at the nonpermissive temperature of 34°C (data not shown).

Together, the genetic data suggest that while \textit{REG1} and \textit{REG2} appear to have overlapping functions with respect to regulation of cell growth, important distinctions remain. Only \textit{reg1} mutants are defective in glucose repression and glycogen hyperaccumulation.
metabolism. Overexpression of \textit{REG2} in a \textit{reg1} deletion strain complements the slow-growth defect but does not compensate for defects in glucose repression or glycogen accumulation. Furthermore, overexpression of \textit{REG2} has no effect on the ability of \textit{reg1} to suppress \textit{mal-1}.

The severe growth defect of \textit{reg1 reg2} double mutants is suppressed by a \textit{Snf1} protein kinase mutant. Mutants in the \textit{Snf1}-encoded protein kinase are unable to activate glucose-repressible genes (13, 14). In previous investigations, it was reported that \textit{snf1 reg1} double mutants exhibit the glucose-repressed phenotype of \textit{snf1} rather than the derepressed phenotype of \textit{reg1} (54, 78). To determine if the growth defect associated with \textit{reg1 reg2} was also suppressed by \textit{snf1}, we first crossed a \textit{snf1} mutant to a \textit{reg1::LEU2} mutant. For these experiments, we used \textit{snf1-172}, an allele previously shown to behave very similar to a null allele (70). As previously reported, \textit{snf1} was epistatic to \textit{reg1}. The \textit{snf1 reg1} double mutants had reduced glycogen levels and failed to grow on nonfermentable carbon sources, both hallmarks of a \textit{snf1} null mutant (70). A \textit{snf1 reg2::URA3} mutant was then crossed to a \textit{reg1::LEU2} strain. Tetrad analysis of meiotic progeny from the resulting diploid are represented in Fig. 4B. Tetrads were replica plated to medium lacking uracil and medium lacking leucine as well as to YPEG to score the \textit{snf1} genotype. Surprisingly, many of the Leu” Ura” progeny grew into normal-sized colonies. Tetrad analysis of 25 asci revealed that 14 \textit{reg1::LEU2 reg2::URA3} spore clones grew into normal-sized colonies. All 14 were \textit{snf1-172}, as determined by their failure to grow on YPEG and accumulate glycogen. Another 14 spore clones either failed to germinate or grew very slowly, and 12 of these were deduced unambiguously to be \textit{reg1 reg2 Snf1} mutants.

Mutations in \textit{snf1} as well as in components of the cyclic AMP (cAMP)-dependent protein kinase pathway have been found to affect some of the same physiological processes (31, 40, 70, 78). A \textit{snf1} null mutant is unable to grow on nonfermentable carbon sources, is sensitive to heat stress and starvation, and fails to accumulate glycogen in response to nutrient depletion (31, 70). These phenotypes resemble those commonly associated with a hyperactive adenylate cyclase pathway. Mutations in adenylate cyclase (\textit{CYR1/CDC35}), which decrease the level of cAMP in the cell, moderate the \textit{snf1} phenotype. In contrast, mutations in the regulatory subunit of cAMP-dependent protein kinase (\textit{BCY1}), which increase cAMP-dependent protein kinase levels, exacerbate the \textit{snf1} phenotype (70). Together, the glucose repression and cAMP-adenylate cyclase pathways control the cell’s response to glucose availability, ultimately affecting a large number of gene products involved in growth control (7, 60). Curiously, suppression of \textit{mal-1} by \textit{reg1} is mediated by both of these signaling pathways. Mutations in either \textit{snf1} or \textit{bcy1} abolish suppression of \textit{mal-1} by \textit{reg1} (78).

Given the relationship between \textit{REG1}, \textit{SNF1}, and the cAMP pathway, we were interested to see how mutations in either \textit{cdc35} or \textit{bcy1} might affect the growth of \textit{reg1 reg2} double mutants. A \textit{reg1::URA3} disruption strain was crossed to a temperature-sensitive \textit{cdc35-13} mutant. The \textit{cdc35-13} allele was chosen because it exhibits a partial growth defect at the semipermissive temperature of 30°C (59). Approximately one-quarter of the spores failed to grow into macroscopic colonies after 4 to 5 days of growth at 24°C. Tetrads were replica plated to synthetic medium and incubated at 37°C to score for temperature sensitivity and to medium lacking uracil to score the \textit{reg1::URA3} genotype. From over 50 tetrads analyzed, 95% of wild-type, \textit{cdc35-13}, or \textit{reg1::URA3} spore clones grew into colonies. However, \textit{cdc35-13 reg1::URA3} double-mutant spores either failed to germinate or germinated and divided a few times before arresting as microcolonies. No growth defect was observed when the \textit{cdc35-13} mutant was crossed to a \textit{reg2::URA3} strain.

The lethality of \textit{reg1 cdc35-13} mutants indicates that \textit{reg1} mutants are sensitive to reduced levels of intracellular cAMP. Since the major and possibly only physiological role of cAMP is to activate cAMP-dependent protein kinase (cAPK), it is likely that \textit{reg1} mutants are sensitive to reduced cAPK activity. One explanation for the synthetic lethality of \textit{cdc35-13} and \textit{reg1} is that \textit{reg1} and possibly \textit{reg2} are positive regulators of adenylate cyclase or cAPK. If this is so, we reasoned that traits associated with a \textit{reg1} and/or \textit{reg2} deficiency might be ameliorated by increased activity of cAPK. \textit{BCY1} encodes the regulatory subunit of cAPK, and loss-of-function mutations in \textit{bcy1} bypass the requirement for adenylate cyclase and its activators, resulting in constitutively active cAPK. \textit{BCY1} is consistent with our resultssuggesting that \textit{Reg1p} and \textit{Reg2p} activate cAMP, which is consistent with our results suggesting that \textit{Reg1p} may have other roles within the cell. This idea is consistent with our results suggesting that \textit{Reg1p} and \textit{Reg2p} function together to direct PP1 activity in an as yet undetermined pathway controlling cell growth. Given that the sequence similarity between \textit{Reg1p} and \textit{Reg2p} is confined to central half (amino acids 217 to 763) of \textit{Reg1p}, it seems likely that these sequences contain domains important for general growth regulation as well as the binding domain for PP1. The unconserved amino-
terminal and/or carboxy-terminal regions of Reg1p might comprise regulatory domains required for directing PP1 activity specifically to substrates in the glucose repression pathway.

If the growth defect of the reg1 reg2 double mutant is the result of decreased PP1 activity, it seems logical that this defect could be compensated for by a mutation in an opposing protein kinase. Precedent for this type of antagonistic relationship has been reported for two other protein kinases and PPI in S. cerevisiae. GCN2 encodes a protein kinase that phosphorylates the α subunit of translation initiation factor 2 (eIF2α) in response to amino acid starvation. Phosphorylation of eIF2α leads to increased expression of Gcn4p, a transcription factor with a central role in the general amino acid control pathway (34). A dominant negative glc7 mutant, caused by deletion of the C-terminal 104 amino acid residues of Glc7p, restores GCN4 expression in strains containing a mutant gcn2-encoded kinase (80), consistent with the hypothesis that eIF2α is dephosphorylated by Glc7p. Another example involves the IPL1-encoded protein kinase that is required for chromosome segregation during mitosis. The temperature sensitivity of the ipi1-1 allele can be partially suppressed by expression of another C-terminal deletion of glc7, by glc7-1, or by a mutation in glc8, a potential modulator of Glc7p (27, 77). In contrast, hyperexpression of Glc7p in wild-type strains leads to a loss in fidelity in chromosome segregation (27). Together, these results indicate that PPI acts in opposition to Ip11p and suggests that the Ip11 kinase and PPI1 may act on the same substrate(s).

A similar scenario can be proposed for Reg1p and Reg2p in which the growth defect of reg1 reg2 mutants is caused by the failure of PPI to dephosphorylate a substrate or substrates important for growth regulation. The hyperphosphorylation of these putative substrates in reg1 reg2 mutants could be reversed by reducing the activity of the opposing protein kinase. Several lines of evidence implicate Snf1p as the kinase acting in opposition to Reg1p and Reg2p. First, snf1 mutants exhibit traits that are opposite those observed for reg1 mutants. snf1 mutants are unable to induce glucose-repressed genes and fail to accumulate glycogen. In contrast, reg1 mutants are constitutively glucose derepressed and hyperaccumulate glycogen. Second, a snf1 mutation is epistatic to reg1 and to the severe growth defect of reg1 reg2. The ability of increased expression of Reg2p to suppress the growth defect of reg1 but not its glucose derepression phenotype suggests that distinct substrates are involved in growth-related and glucose repression defects. In our model, Snf1p would be responsible for phosphorylating the target substrates for both Reg1p- and Reg2p-associated PPI.

The importance of the SNF1-encoded protein kinase in relieving glucose repression is well established (53, 60, 73). However, not unlike reg1 and glc7 mutants, snf1 mutants exhibit pleiotropic phenotypes including the inability to grow on non-glucose carbon sources, sensitivity to starvation and heat stress, failure to accumulate glycogen, and sporulation defects (70). These data are consistent with the idea that in addition to its role in glucose repression, Snf1p may regulate other cellular processes. Supporting this hypothesis are reports that Snf1p is present in both glucose-repressed and derepressed cells and appears to be distributed throughout the cell (14).

Recent evidence indicates that Snf1p and the related mammalian AMP-activated kinase (AMPK) may regulate a variety of metabolic pathways. Snf1p shows a remarkable degree of sequence identity (46%) to both mammalian AMPK and a protein kinase encoded by the RKN1 gene from rice (12). Expression of CRKIN1 in yeast snf1 mutants restores SNF1 function (12), indicating Snf1p, AMPK, and RKN1 form a conserved family of protein kinases. Mammalian AMPK functions in lipid metabolism, negatively regulating acetyl coenzyme A (acetyl-CoA) carboxylase (52), the enzyme catalyzing the first committed step in fatty acid synthesis. In a wild-type yeast strain, acetyl-CoA carboxylase undergoes a time-dependent decrease in activity when glucose is limiting (82). In snf1 mutants, acetyl-CoA carboxylase activity remains high (82), suggesting that like its mammalian homolog, Snf1p may inactive acetyl-CoA carboxylase in vivo. The proposed role of Snf1p in lipid metabolism is consistent with the idea that Snf1p may be involved in a wide range of metabolic pathways. It is of interest that mammalian acetyl-CoA carboxylase and hydroxymethylglutaryl-CoA reductase, both of which are in vivo substrates of AMPK (30, 52), are dephosphorylated by PPI in vitro (16, 32).

Alternative models for the antagonistic behavior of snf1 and reg1 reg2 cannot be ruled out at this time. Reg1p and Reg2p might negatively regulate Snf1p directly or could regulate a component of a Snf1p-protein complex such that the growth defect of the double mutant is the result of increased Snf1p kinase activity. Among the potential substrates for this type of regulation are members of a family of functionally related proteins involved in glucose repression. This group includes Sip1p, Sip2p, and Gai83p, which associate with Snf1p and are phosphorylated in a SNF1-dependent manner (83). gai83 mutants exhibit reduced Snf1p activity in vitro (83), and genetic evidence suggests that Reg1p and Gai83p act together (22). In this model, Reg1p and Reg2p would be predicted to have qualitatively different effects on Snf1p activity, since overexpression of Reg2p alleviates the growth defect of reg1 without influencing its glucose derepression phenotype.

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