Relationship of the cAMP-Dependent Protein Kinase Pathway to the SNF1 Protein Kinase and Invertase Expression in Saccharomyces cerevisiae

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ABSTRACT

The SNF1 protein kinase and the associated SNF4 protein are required for release of glucose repression in Saccharomyces cerevisiae. To identify functionally related proteins, we selected genes that in multicopy suppress the raffinose growth defect of snf4 mutants. Among the nine genes recovered were two genes from the cAMP-dependent protein kinase (cAPK) pathway, MSII and PDE2. Increased dosage of these genes partially compensates for defects in nutrient utilization and sporulation in snf1 and snf4 null mutants, but does not restore invertase expression. These results suggest that SNF1 and cAPK affect some of the same cellular responses to nutrients. To examine the role of the cAPK pathway in regulation of invertase, we assayed mutants in which the cAPK is not modulated by cAMP. Expression of invertase was regulated in response to glucose and was dependent on SNF1 function. Thus, a cAMP-responsive cAPK is dispensable for regulation of invertase.

The yeast Saccharomyces cerevisiae prefers to utilize glucose as a carbon source. When glucose is plentiful, the cell represses the expression of genes that are involved in utilization of alternate carbon sources, a phenomenon known as glucose repression. This regulatory response is important to the cell and apparently involves many regulatory genes; however, the signaling pathway is not yet understood [for review see Entian (1986), Gancedo and Gancedo (1986) and Carlson (1987)].

One of the key genes is SNF1, which encodes a protein-serine/threonine kinase that is required for release of gene expression from glucose repression (Celenza and Carlson 1986). SNF1 is the same gene as CAT1 and CCR1 (Ciriacy 1977; Denis 1984; Entian and Zimmermann 1982; Schuller and Entian 1987). Mutations in SNF1 cause defects in growth on carbon sources that are less preferred than glucose, general unhealthiness, and defects in sporulation of homozygous diploids (Carlson, Osmond and Botstein 1981). While the SNF1 kinase is clearly essential for the regulatory response to glucose, its exact role remains unclear. The SNF1 kinase activity detected in vitro is not affected by the availability of glucose in the culture medium (Celenza and Carlson 1989); however, the physiologically relevant targets are as yet unidentified and it remains possible that their phosphorylation is regulated.

Previously, we showed that the SNF4 gene (also known as CAT3; Entian and Zimmermann 1982; Schuller and Entian 1988) encodes a protein that is functionally related to the SNF1 kinase. The SNF4 protein is physically associated with the SNF1 protein kinase and is required for maximal activity of the kinase in vitro, but SNF4 does not appear to convey regulatory signals (Celenza and Carlson 1989; Celenza, Eng and Carlson 1989; Fields and Song 1989). Mutations in snf4 cause the same array of phenotypes as snf1, but are slightly less severe (Neigeborn and Carlson 1984; Celenza, Eng and Carlson 1989).

In an effort to further our understanding of the regulatory pathway for the glucose response, we sought to identify other genes that encode proteins that are functionally related to the SNF1 protein kinase. We selected for genes that in multicopy suppress the raffinose growth defect of a snf4 mutant. Although SNF1 kinase activity is greatly reduced in a snf4 mutant, genetic and biochemical evidence indicates that some residual SNF1 kinase activity remains (Celenza and Carlson 1989). We selected for suppression of reduced kinase activity, rather than no kinase activity, because we anticipated that a broader range of suppressor genes would be recovered. There are various possible mechanisms by which increased dosage of a gene could compensate for the snf4 defect. For example, the suppressor genes could encode additional activators of the kinase, substrates of the kinase, activators of a parallel or partially redundant kinase pathway, or repressors of an antagonistic pathway.

We report here that among the nine genes recovered using this strategy were two genes from the cAMP-dependent protein kinase (cAPK) pathway, MSII and PDE2. MSII (also called JUNI) was previously isolated as a multicopy suppressor of the heat...
shock sensitive phenotype of *ira1* and *RAS2* mutants (NIKAWA, SASS and WIGLER 1987; RUGGIERI et al. 1989), and PDE2 encodes a high-affinity cAMP phosphodiesterase (SASS et al. 1986; WILSON and TATCHELL 1988).

The role of cAMP and cAPK in glucose repression in *S. cerevisiae* is problematic. cAMP does not function as a direct effector by a mechanism analogous to that in *Escherichia coli* (MATSUMOTO et al. 1982, 1983; ERASO and GANCEDO 1984). However, a RAS-dependent transient elevation in cAMP levels upon addition of glucose to glucose-starved cells has been documented (MBONYI et al. 1990, and references therein).

Mutations in *CYR1*, the gene encoding adenylate cyclase, reduce expression of invertase and α-1-glucosidase (MATSUMOTO, UNO and ISHIKAWA 1984; SCHULTZ and CARLSON 1987). In contrast, mutants defective in *BCY1*, which encodes the cAMP-responsive negative regulatory subunit of cAPK, express invertase, galactokinase, and α-1-glucosidase at wild-type levels or a fewfold higher, and expression is still subject to glucose repression (MATSUMOTO et al. 1983; J. SCHULTZ and M. CARLSON, unpublished results). The cAPK pathway is known to affect expression of the glucose repressible gene *ADH2* via phosphorylation of the transcriptional activator ADR1 (BEMIS and DENIS 1988; CHERRY et al. 1989; TAYLOR and YOUNG 1990; THURRAL et al. 1989); however, the data do not establish that this phosphorylation regulates glucose repression of *ADH2*.

We have examined the ability of increased *MSI1* and *PDE2* gene dosage to suppress various defects in both snf4 and snf1 null mutants. We found that these multicopy suppressor genes did not restore invertase expression in response to glucose deprivation, but rather seemed to compensate for defects in nutrient utilization and sporulation. We also examined the effects of the cAPK pathway on regulation of invertase expression by using mutant strains in which cAPK activity is no longer responsive to the levels of cAMP; these strains lack *BCY1* and carry attenuating mutations in the genes encoding the catalytic subunits of cAPK (CAMERON et al. 1988). In these strains, expression of invertase was regulated in response to glucose and still dependent on *SNF1* function.

**MATERIALS AND METHODS**

**Strains and general genetic methods:** Strains of *S. cerevisiae* used in this study and their sources are listed in Table 1. MCY strains have the S288C genetic background except where other derivation is noted. Standard methods were used for genetic analysis (SHERMAN, FINK and LAWRENCE 1978) and transformation (Ito et al. 1983). Media contained 2% of the carbon source unless otherwise noted. Anaerobic growth was scored after incubation in a GasPak Disposable Anaerobic System (BBL).

**Isolation of multicopy suppressor plasmids:** Strain MCY1853 (*snf4-Δ2 ura3*) was transformed with a genomic library on the multicopy vector YEP24 (CARLSON and BOTSTEIN 1982). Approximately 24,000 *Ura* colonies were replica-plated onto supplemented synthetic medium (SHERMAN, FINK and LAWRENCE 1978) containing raffinose and lacking uracil (SR-Ura). The 476 *Raf* transformants were retested by spotting cell suspensions onto SR-Ura, and 170 colonies again scored *Raf*+. We selected for further study 90 colonies that grew nearly as well as or better than MCY1853 carrying *SNF1* on a multicopy plasmid (pCE9; CELENZA and CARLSON 1989). Plasmid DNAs were recovered by passage through *E. coli* (HOFFMAN and WINSTON 1987). Five plasmids carrying *SNF4* and 35 carrying *SNF1* were identified by diagnostic restriction digests and Southern blot analysis. Nine different plasmids, which conferred significant suppression upon retransformation of MCY1853, accounted for 37 of the remaining 52 plasmids.

**Plasmid constructions:** DNA was manipulated and analyzed using standard methods (MANIATIS, FRITSCH and SAMBROOK 1982). pJH10 contains the *SMX1* gene cloned into pUC19 (YANISCH-PERRON, VIEIRA and MESSING 1985). pJH39 carries the 1.4-kb *Nru*I fragment of pB37 cloned into the *Smal* site of YEp24 (BOTSTEIN et al. 1979). To construct pJH44, pB37 was digested with *XhoI* plus *BglII*, the ends were filled in with Klenow fragment, and the vector-containing fragment was gel-puriﬁed and ligated. pJH46 and pJH49 contain the *Ca1/Smal* and *SalI/XhoI* fragments of pB37 cloned into the *BamHI/Smal* and *SalI* sites of YEp24, respectively. pXY1 contains the *SalI* to *XbaI* fragment of pB88 ligated to the large *SalI/NheI* fragment of YEp24. pXY2 was constructed by cloning the *XbaI/Smal* fragment of pB88 into the *NheI/Smal* site of YEp24. pXY7 and pXY8 were constructed by cloning the *SpH1/Scal* and *BamH1/NheI* fragments of pB88 between the *SpH1/Smal* and *BamH1/NheI* sites of YEp24, respectively.

**Sequence analysis and computer methods:** Restriction fragments were cloned into M13 mp18 or M15 mp19 (NORRIS, KEMPE and MESSING 1983) and sequenced by the method of SANGER, NICKLEN and COULSON (1977) using Sequenase (U.S. Biochemical) and a 17-mer sequencing primer. Sequences were analyzed using DNA Strider (Commissariat a l’Energie Atomique—France) and the GCG program TFASTA (PEARSON and LIPMAN 1988). Searches of reported sequences were made in the GenBank (BIOLISYK et al. 1986) and EMBL (HAMM and CAMERON 1986) databases using the GCG program TFASTA (PEARSON and LIPMAN 1988).

**Construction of the *msi1-Δ1::URA3* allele:** First, the plasmid pJH51 was constructed by ligating the *KpnI/SpH1* fragment of pB37 to the *KpnI/SpH1* fragment of pUC19. pJH51 was then cut with *Ca1* and *BglII*, filled in with Klenow, isolated, and ligated to a 1.1-kb *SalI* fragment containing *URA3* to obtain pJH52. The *KpnI/SpH1* fragment of pJH52 was then used to transform (BOTSTEIN et al. 1983) the diploid MCY1095 × MCY1094 to uracil prototrophy. The presence of the *msi1-Δ1::URA3* allele on one homologue of the diploid was confirmed by Southern blot analysis using probes prepared from pJH51.

**Construction of the *snf1-15::LEU2* allele:** pJH18 was constructed by inserting the *BglII* fragment containing the LEU2 gene into the *BglII* site of the *SNF1* gene in pCC107 (J. CELENZA and M. CARLSON, unpublished results). pCC107 contains *SNF1* on a *HincII-BamH1* partial fragment cloned in pUC18. The *BamH1/SpH1* fragment of pJH18 was used to transform SP1, TFI.5PrF™, TFI.5PrC™, and MCY2372. This results in a disruption of *SNF1* at codon 175 (CELENZA and CARLSON 1986).

**Southern blot and Northern blot analysis:** Standard methods were used for preparation and analysis of genomic...
DNA (Hoffman and Winston 1987; Maniatis, Fritsch and Sambrook 1982). Poly(A)-containing RNAs were isolated from glucose-repressed and derepressed cell cultures as described previously (Sarokin and Carlson 1985).

Invertase assays: Glucose-repressed and derepressed cells were prepared and assayed as described previously (Neige-born and Carlson 1984). Repressed cultures were grown to mid-log phase in 2% glucose, and cells were derepressed by shifting to 0.05% glucose for 2.5 hr when prepared in rich media and 3 hr when synthetic media were used. Cells carrying plasmids were grown in supplemented synthetic medium with selection to maintain the plasmid. Secreted invertase activity was assayed as previously described (Goldstein and Lampen 1975; Celenza and Carlson 1984).

Analysis of sporulation efficiency: Transformants of MCY1855 carrying pB37, pXY8 or YEP24 were crossed to MCY1647 to generate diploids homozygous for snf4. MCY1845 transformants carrying pB37, pXY8 or YEP24 were crossed to MCY1846 to generate diploids homozygous for snf1. MCY1094 carrying YEP24 was crossed to MCY1093 to generate the wild-type diploid. Single colonies were isolated on supplemented synthetic medium with selection for the plasmid and then transferred to solid sporulation medium (Sherman, Fink and Lawrence 1978). After incubation for 8–9 days (6 days for the wild type) at room temperature, at least 500 cells from each colony were examined microscopically.

RESULTS

Isolation of multicopy suppressor plasmids: The snf4-Δ2 mutant strain MCY1853 was transformed with a yeast genomic library in a multicopy vector, and transformants able to grow on raffinose were identified (see MATERIALS AND METHODS). The snf4 mutant fails to derepress invertase, which catalyzes the extracellular hydrolysis of raffinose. In addition to plasmids carrying SNF4 and SNF1, we recovered nine different plasmids that conferred a range of growth phenotypes (see MATERIALS AND METHODS). One of the plasmids contained the gene MSN1, which was previously isolated as a multicopy suppressor of a snf1-ts mutation (Estruch and Carlson 1990). Two other plasmids, which allowed slow growth on raffinose, are described here.

Identification of MSN1 as a multicopy suppressor of snf4: A plasmid designated pB37 (Figure 1) was recovered from 19 independent transformants. In the first experiment to characterize the insert, the labeled plasmid pHJ10 was hybridized to a Northern blot of poly(A)-containing RNA prepared from glucose-repressed and derepressed cells. A 1.7-kb RNA was detected, which was highly abundant in glucose-repressed cells and present only at low levels in derepressed cells. Sequence analysis starting at the poly(A) site (Figure 1) revealed an open reading frame. The partial sequence of 82 codons was compared with sequences in the database (see MATERIALS AND METHODS). The sequence showed complete identity with the PGI1 gene of S. cerevisiae (Tekamp-Olson, Najarian and Burke 1988). Comparison of the restriction maps indicated that pB37 contains only the 3' end of PGI1.

To identify the region in pB37 responsible for the suppression phenotype, we constructed several subclones in a multicopy vector (pJH39, pJH44, pJH46, and pHJ49; Figure 1) and tested their ability to suppress the Raf- phenotype of a snf4 mutant. These data showed that the complementing region lies 3' to PGI1. We therefore inspected the sequence 3' to PGI1 reported by Tekamp-Olson, Najarian and Burke (1988) for the presence of another open reading frame (ORF). An ORF extending more than 900 bp was identified, and comparison to the sequences in the database (see Methods) revealed identity to the gene MSN1. The coding sequences for MSN1 and PGI1 are separated by 356 bp. The two genes were both mapped genetically to the right arm of chromosome II: MSN1 lies ~4 cM from tyr1 and 33 cM from lys2 (Ruggieri et al. 1989), and PGI1 lies 15 cM from tyr1 and 31 cM from lys2 (Maitra and Lobo 1977).
MSIl (also called JUN1) was isolated as a multicopy suppressor of the heat shock-sensitive phenotype of ira1 and \textit{RAS2} \textsuperscript{2619} mutants and also restores a low level of sporulation to the homozygous diploids (Nikawa, Sass and Wigler 1987; Ruggieri \textit{et al.} 1989). The exact relationship of this gene to the cAPK pathway is not clear, although Ruggieri \textit{et al.} suggest that MSIl is a negative regulator of the RAS-mediated induction of CAMP. The predicted protein has repeated sequences homologous to a repeat motif present in the \( \beta \) subunit of transducin, the yeast genes \textit{TUP1}, \textit{CDC4}, \textit{STE4}, \textit{PRP4} and the Drosophila gene \textit{Enhancer of split} (Ruggieri \textit{et al.} 1989; for review, see Simon, Strathmann and Gautam 1991 and Williams and Trumbly 1990).

\textit{pB37} contains the entire \textit{MSIl} gene, and \textit{MSIl} corresponds to the functional region of \textit{pB37} (Figure 1). Thus, \textit{MSIl} is also a multicopy suppressor of \textit{snf4}.

\textbf{Multicopy MSIl suppresses pleiotropic defects of \textit{snf4} mutants:} The \textit{snf4} mutation causes a variety of defects in addition to the defect in growth on raffinose (Neigborn and Carlson 1984; Celezna, Eng and Carlson 1989). To determine whether multicopy \textit{MSIl} suppresses the other defects of \textit{snf4} mutants, we tested strain MCY1853 (\textit{snf4-\textit{A}2}) carrying \textit{pB37} or \textit{pJH49} for growth on synthetic medium lacking uracil and containing glucose, sucrose, raffinose, galactose, or glycerol as the carbon source. Plates were incubated anaerobically except for those containing glycerol. Because copy number can vary, four different transformants were tested. The \textit{MSIl} multicopy plasmid improved growth of the \textit{snf4} mutant on sucrose, raffinose, galactose, and glycerol relative to the control strain carrying the \textit{YEpl2} vector, but did not improve growth nearly as well as a multicopy plasmid carrying \textit{SNF1} (data not shown; see Figure 3).

Diploid strains homozygous for \textit{snf4} are also defective in sporulation. To determine whether increased dosage of \textit{MSIl} suppresses the sporulation defect, we tested \textit{snf4-\textit{A}2} homozygous diploids carrying \textit{pB37} or \textit{YEpl2}. Two diploid colonies carrying each plasmid were induced to sporulate, as described in Materials and Methods. A slight increase in the percentage of sporulating cells was observed in diploids carrying multicopy \textit{MSIl} relative to diploids carrying the vector (Table 2). In addition, some mature asci were observed in the strains carrying \textit{MSIl} in multicopy while no mature ascis were observed in strains carrying the vector.

\textbf{Disruption of the \textit{MSIl} gene:} Previous studies showed that deletion of \textit{MSIl} did not affect growth rate, heat shock sensitivity, or sporulation of homozygous diploids (Ruggieri \textit{et al.} 1989). Because \textit{MSIl} was here isolated as a suppressor of \textit{snf4}, we constructed a new disruption and tested for other phenotypes previously associated with \textit{snf4} mutations. Diploids heterozygous for a deletion/substitution mutation in the \textit{MSIl} gene, designated \textit{msil-\textit{\Delta}1::URA3} (Figure 1), were constructed as described in Materials and Methods. Tetrad analysis of two diploids yielded four viable spore clones from each ascus. Seven complete tetrads (four from one diploid and three from the other) were tested for growth on glucose, raffinose, galactose, and glycerol at 30° and on glucose and raffinose at 37°. All spore clones showed normal growth phenotypes. Invertase activity was also assayed in two complete tetrads, and the mutants were indistinguishable from the wild-type spore clones with respect to regulation of invertase activity (average de-repressed values 380 and 350, respectively). Thus no new phenotype was identified.

\textbf{Identification of \textit{PDE2} as a suppressor gene:} An-
TABLE 2
Multicopy MS1 and PDE2 improve sporulation of snf1 and snf4 homozygous diploids

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Relevant genotype</th>
<th>Plasmid-borne gene</th>
<th>Sporulation (%) of colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>snf4/snf4 (pB37)</td>
<td>MS1</td>
<td>A 7.0 B 3.4</td>
</tr>
<tr>
<td></td>
<td>snf4/snf4 (YEp24)</td>
<td>None</td>
<td>A 1.4 B 1.4</td>
</tr>
<tr>
<td>2</td>
<td>snf1/snf1 (pB37)</td>
<td>MS1</td>
<td>A 1.6 B 2.6</td>
</tr>
<tr>
<td></td>
<td>snf1/snf1 (YEp24)</td>
<td>None</td>
<td>A 0.4 B 0.2</td>
</tr>
<tr>
<td>3</td>
<td>snf4/snf4 (pXY8)</td>
<td>PDE2</td>
<td>A 15.3 B 9.6</td>
</tr>
<tr>
<td></td>
<td>snf4/snf4 (YEp24)</td>
<td>None</td>
<td>A 4.1 B 6.0</td>
</tr>
<tr>
<td></td>
<td>snf1/snf1 (pXY8)</td>
<td>PDE2</td>
<td>A 5.6 B 8.0</td>
</tr>
<tr>
<td></td>
<td>snf1/snf1 (YEp24)</td>
<td>None</td>
<td>A 1.0 B 0.2</td>
</tr>
<tr>
<td></td>
<td>Wild type (YEp24)</td>
<td>None</td>
<td>A 42 B 43</td>
</tr>
</tbody>
</table>

* Strains in a single experiment were grown and sporulated at the same time under identical conditions. Room temperature was generally slightly lower during Experiment 3, and the requirement for SNF4 is known to be less stringent at 23°C than at 30°C (CELENZA, ENG and CARLSON 1989).

Percent of sporulating cells was determined as described in Materials and Methods.

Other plasmid, designated pB88, was recovered once in this study (Figure 2). pB88 contains a ~7.5 kb insert and carries a duplication of part of the 2-μm sequence from the vector. Sequence analysis from the XbaI site yielded a partial sequence of 71 codons with complete identity to the sequence of the PRT1 gene of S. cerevisiae (HANIC-JOYCE, SINGER and JOHNSTON 1987). As judged by its restriction map, pB88 contains the entire PRT1 gene and also the adjacent gene PDE2 (also called SRA5; WILSON and TATCHELL 1988). To identify the region responsible for the suppression, we constructed and tested a set of subclones (pXY1, pXY2, pXY7, pXY8; Figure 2). The results show that PDE2 is the relevant gene. PDE2 encodes a high affinity cAMP phosphodiesterase. Increased dosage of PDE2 suppresses defects caused by the RAS2Δ119 mutation, and mutations in PDE2 suppress a ras2 mutation (CANNON, GIBB and TATCHELL 1986; SASS et al. 1986; WILSON and TATCHELL 1988).

Four transformants of MCY1853 (snf4-Δ2) carrying PDE2 on pXY8 were also tested for growth on synthetic media lacking uracil and containing sucrose, raffinose, galactose, or glycerol, as above. The plasmid improved growth on all these carbon sources relative to the control strain carrying YEpl4 but was not as effective a suppressor as a multicopy plasmid carrying SNF1 (data not shown; see Figure 3). Sporulation efficiency was also assessed in snf4/snf4 diploids carrying multicopy PDE2. An increase in the percentage of sporulating cells was observed in diploids carrying PDE2 relative to diploids carrying the vector (Table 2). In addition, multicopy PDE2, like MS1, markedly improved the formation of mature ascis.

**Increased dosage of MS1 and PDE2 suppresses defects of snf1Δ mutants:** Previous studies indicated that the SNF4 protein is physically associated with the SNF1 protein kinase and functions as an activator of the kinase (CELENZA and CARLSON 1989; CELENZA, ENG and CARLSON 1989). One possible mechanism by which increased MS1 or PDE2 dosage could suppress snf4 mutant defects is by causing compensatory activation of the SNF1 kinase. If this were the mechanism of suppression, we would not expect multicopy MS1 or PDE2 to be able to suppress defects resulting from the complete loss of SNF1 function.

To test whether MS1 or PDE2 can suppress defects of a snf1 deletion mutation, plasmids pJH49 and pXY8 were used to transform the strain MCY1845 (snf1-Δ10). Transformants were tested for growth on raffinose, galactose and glycerol, and both genes improved growth on these carbon sources relative to growth of control transformants carrying the vector (Figure 3, A and B, and data not shown). Greater variation in growth phenotype was observed in strains carrying PDE2 plasmids, suggesting that suppression by PDE2 is more dependent on copy number than is suppression by MS1. Furthermore, plasmids pB37 (MS1) and pXY8 (PDE2) partially suppressed the sporulation defect of snf1-Δ10 homozygous diploids (Table 2). These results indicate that the observed suppression does not require functional SNF1 protein and therefore occurs by a mechanism other than activation of the SNF1 kinase.
Suppression of the Raf phenotype is not due to restoration of invertase expression: The snf1 and snf4 mutants are defective in growth on raffinose at least in part because they fail to derepress expression of secreted invertase from the SUC2 gene in response to glucose deprivation. To determine whether suppression of the raffinose defect results from restoration of invertase expression, we assayed invertase activity in snf4 and snf1 mutant strains carrying MSII or PDE2 plasmids (Table 3). Neither gene in multicopy significantly increased the derepression of invertase in response to a shift to low glucose. The snf4 strain carrying pB37 was also assayed after growth to mid-log phase in 2% raffinose (nonrepressing conditions) with similar results (data not shown). In addition, multicopy MSII and PDE2 do not affect expression of invertase in wild-type cells (data not shown).

Increased dosage of MSII and PDE2 improves nutrient utilization of snf1 mutants: An alternate explanation for the restoration of growth on raffinose is that MSII and PDE2 in multicopy confer to the mutants increased ability to utilize effectively low lev-

### Table 3

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Plasmid-borne gene</th>
<th>Invertase activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>snf4 (pLN132)</td>
<td>SNC4</td>
<td>Repressed</td>
</tr>
<tr>
<td>snf4 (pCE9)</td>
<td>SNC1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf4 (pB37)</td>
<td>MSII</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf4 (pB88)</td>
<td>PDE2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf4 (YEp24)</td>
<td>None</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf1 (pCE9)</td>
<td>SNC1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf1 (pJH49)</td>
<td>MSII</td>
<td>&lt;1</td>
</tr>
<tr>
<td>snf1 (pJY8)</td>
<td>PDE2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a Strains were MCY1855 and MCY1845 carrying the indicated plasmids.  

Micrograms of glucose released/minute/100 mg (dry weight) of cells. Cultures were grown in SC-Ura to select for plasmid maintenance. Values are the average of 2 assays for the snf4 strains and single assays for the snf1 strains. Derepressed values for strains carrying multicopy MSII, PDE2 or vector were all ≤5.
els of hexoses and/or other nutrients present in the media. To test this idea, we first streaked snf1-Δ10 mutants carrying MSI1 or PDE2 plasmids for single colonies on rich medium (YEP) containing 0.1% glucose as the carbon source. Figure 3C shows that both MSI1 and PDE2 in multicopy improved colony formation relative to the control strain carrying the vecto.

Colony size was highly variable relative to the control carrying the wild-type SNF1 plasmid, and similar variation in colony size was also observed on selective synthetic medium (data not shown); variation in plasmid copy number may be responsible. We also spotted cell suspensions onto YEP medium with no added carbon source, and both MSI1 and PDE2 in multicopy improved the growth of the snf1-Δ10 mutant strain (Figure 3B). Thus, the weak restoration of growth on raffinose and other carbon sources may be attributable to improved ability to utilize low levels of nutrients rather than to suppression of the defects in derepression of specific enzymes such as invertase.

CAMP-responsive CAPK is dispensable for regulation of invertase by glucose: The recovery of two genes from the CAMP-dependent protein kinase (CAPK) pathway as suppressors of snf4 prompted us to explore further the relationship of CAPK to glucose regulation. We tested whether this pathway is required for regulation of invertase expression in response to glucose availability by using two mutant strains, TF1.5prC<sup>HS</sup> and TF1.5prF<sup>HR</sup> (Table 1), in which CAPK activity is not regulated by CAMP (M. Wigler, personal communication). These strains carry a bcyl null allele and mutations in the three genes encoding the catalytic subunit of CAPK (Toda et al. 1987b): null mutations in TPK2 and TPK3 and an attenuating mutation in TPK1 (tpk<sup>l</sup>). These strains, referred to as bcyl<sup>tpk<sup>l</sup></sup> strains, have CAPK activity that is attenuated and independent of the level of CAMP in the cell (see Cameron et al. 1988). TF1.5prC<sup>HS</sup> is heat shock sensitive and TF1.5prF<sup>HR</sup> is heat shock resistant due to different degrees of attenuation of the CAPK activity encoded by the tpk<sup>l</sup> allele (M. Wigler, personal communication). We confirmed that our isolates of these strains displayed the expected heat shock phenotypes. We then assayed invertase activity in the two mutant strains and their parent SP1 after growth under glucose repressing conditions (2% glucose) and after a shift to derepressing conditions (0.05% glucose). All three strains showed high level, glucose-repressible invertase activity. Thus, regulation of invertase activity in response to glucose does not require modulation of CAPK activity by CAMP.

To determine if derepression of invertase requires SNF1 function in the bcyl<sup>tpk<sup>l</sup></sup> strains, we introduced the snf1-15::LEU2 mutation into these strains (see MATERIALS AND METHODS). The snf1 mutant derivatives failed to derepress invertase in response to glu-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Invertase activity&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Repressed</td>
</tr>
<tr>
<td>SP1</td>
<td>Wild type</td>
<td>1</td>
</tr>
<tr>
<td>TF1.5prC&lt;sup&gt;HS&lt;/sup&gt;</td>
<td>tpk&lt;sup&gt;l&lt;/sup&gt; tpk2 tpk3 bcyl</td>
<td>1</td>
</tr>
<tr>
<td>TF1.5prF&lt;sup&gt;HR&lt;/sup&gt;</td>
<td>tpk&lt;sup&gt;l&lt;/sup&gt; tpk2 tpk3 bcyl</td>
<td>1</td>
</tr>
<tr>
<td>MGY2367</td>
<td>snf1-15::LEU2</td>
<td>1</td>
</tr>
<tr>
<td>MGY2344</td>
<td>snf1-15::LEU2 tpk&lt;sup&gt;l&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>MGY2346</td>
<td>snf1-15::LEU2 tpk&lt;sup&gt;l&lt;/sup&gt; tpk2 tpk3 bcyl</td>
<td>1</td>
</tr>
</tbody>
</table>

* Micromoles of glucose released/minute/100 mg (dry weight) of cells. Values are the average of 2-4 assays. For values >1, standard errors were <25%.

cose limitation (Table 4). Therefore, the regulated expression of invertase activity in the bcyl<sup>tpk<sup>l</sup></sup> mutant strains still requires SNF1 function.

DISCUSSION

We report here the isolation of two genes related to the CAPK pathway, MSI1 and PDE2, as weak multicopy suppressors of the raffinose growth defect of a snf4 null mutant. Increased dosage of MSI1 and PDE2 only partially compensates for defects caused by a snf4 mutation, improving nutrient utilization and sporulation (which is a response to nutrient limitation) but not restoring invertase expression. A similar pattern of suppression is observed in a snf1 null mutant, indicating that the effects of increased dosage of MSI1 and PDE2 result from a partial bypass of the requirement for SNF1 kinase activity.

The CAPK pathway is known to affect nutrient utilization and sporulation (see Cameron et al. 1988 for review), but its role in the regulation of invertase has been less certain. Previously, conditional-lethal cyr1 mutations were shown to impair derepression of invertase expression, but a bcyl mutation allowed high-level derepression and normal glucose repression (Matsumoto, Uno, and Ishikawa 1984; Matsumoto et al. 1983; Schultz and Carlson 1987; and J. Schultz and M. Carlson, unpublished results). Our studies of the bcyl<sup>tpk<sup>l</sup></sup> strains tested the idea that the CAPK pathway mediates regulatory signals that control invertase expression. These strains showed normal regulation of invertase activity in response to glucose deprivation, even though CAPK activity is not modulated in response to CAPK levels. Moreover, derepression of invertase in bcyl<sup>tpk<sup>l</sup></sup> mutant strains is dependent on SNF1 function, as is true in wild type. Thus, a CAPK-responsive CAPK is dispensable for regulation of invertase. We cannot exclude that the CAPK pathway contributes to regulation, but we can conclude that an independent regulatory mechanism exists and is sufficient.
It is worth noting that our experiment does not address the possibility that the cAPK pathway affects the kinetics of repression or derepression, because we examined steady-state levels of invertase activity. It also remains possible that glucose-induced changes in cAMP levels (see MBONYI et al. 1990) may be involved in regulation, independent of any modulation of cAPK activity; however, cAMP levels are dramatically elevated in *beyI tpk* strains relative to wild type (NIKAWA et al. 1987).

Our data do not contradict the idea that cAMP or the cAPK activity may be necessary for invertase expression *per se*. Rather, these data specifically exclude the notion that modulation of cAPK activity by cAMP is required for regulation in response to glucose. The idea that proper functioning of the cAPK is necessary for normal levels of invertase expression is, in fact, consistent with the effect of a *cyr1* (conditional lethal) mutation. Moreover, the effects of the cAPK pathway on expression of another glucose-repressible gene, *ADH2*, could be explained similarly: phosphorylation by cAPK could reduce the intrinsic potency of the transcriptional activator ADR; rather than mediate regulatory signals regarding glucose availability (BEMIS and DENIS 1988; CHERRY et al. 1989; THUKRAL et al., 1989; TAYLOR and YOUNG 1990). We favor the view that the cAPK pathway affects the expression of invertase but not its regulation in response to glucose, whereas another pathway including the SNF1 protein kinase is responsible for signal transduction.

The invertase gene differs from many genes involved in utilization of nonpreferred carbon sources in that its expression is regulated solely by glucose repression, not by substrate induction. The cAPK pathway may indirectly affect the regulation of some genes that are both glucose repressible and inducible, such as the *GAL* genes, via catabolite inactivation of the cognate sugar transporters. Mutations in the cAPK pathway genes have been shown to alter the catabolite inactivation of the galactose and high affinity glucose transporters (RAMOS and GIRILLO 1989). Catabolite inactivation of the transporter could contribute to the glucose repression of *GAL* gene expression by reducing levels of the inducer.

The *snf1* and *snf4* mutations cause pleiotropic phenotypes that are also associated with mutations that stimulate the cAPK pathway, such as *ira1*, *beyl*, and *RAS2* (TODA et al. 1985, 1987a; SASS et al. 1986; CANNON and TATECHLL 1987; TANAKA, MATSUMOTO and TOH-E 1989). Among these are defects in general health, growth on carbon sources other than glucose, sporulation, thermotolerance and glycogen storage (THOMPSON-JAEGER et al. 1991). These phenotypes in *snf1* mutants may all be related to the failure to respond appropriately to glucose deprivation. [Heat shock sensitivity could reflect a failure to arrest properly in stationary phase, although *snf1A* mutants show the same percentage of unbudded cells during growth to saturation as wild type (THOMPSON-JAEGER et al. 1991; E. J. A. HUBBARD, unpublished results).]

WIGLER and his colleagues have previously suggested that responses such as sporulation, glycogen accumulation, and heat shock resistance are under multiple regulatory controls, including cAMP-independent regulatory mechanisms (CAMERON et al. 1988; TODA et al. 1988). Also, GRANOT and SNYDER (1991) showed that addition of glucose to stationary-phase cells induces cAMP-independent growth-related cellular events. It may be appropriate to consider the SNF1 kinase pathway as an important contributor to the general response to nutrient limitation.

THOMPSON-JAEGER et al. (1991) suggest that the SNF1 and cAPK kinases are antagonistic in their effects on thermotolerance and glycogen accumulation. Our recovery of *MSI1* and *PDE2* in this selection, and our finding that their increased dosage suppresses the *snf1* mutant defects in nutrient utilization and sporulation are consistent with the idea that the two kinase pathways function antagonistically with respect to these phenotypes. We attempted to assess suppression of the heat shock sensitivity of *snf1* mutants by *MSI1* and *PDE2*, but our efforts were hampered by the poor and variable survival of *snf1* mutant cells during growth to stationary phase in selective synthetic media. A similar loss of viability was reported for *RAS2* mutants (TODA et al. 1985).

Taken together, these data indicate that SNF1 and cAPK affect some of the same cellular functions, perhaps even sharing some common targets. However, these pathways clearly also have distinct functions, and we present evidence here that the cAPK pathway does not play an essential role in regulating invertase expression. DENIS and AUDINO (1991) have shown that SNF1 and cAPK act independently to control *ADH2* expression.

*MSI1* and *PDE2* are two of nine genes recovered by selecting for multicopy suppressors of a *snf4* mutant. Several of the other seven genes have also been characterized. Increased dosage of the *MSN1* gene restores high level, regulated invertase expression. *MSN1* encodes a nuclear protein that displays weak DNA-binding activity and may function in transcriptional activation; it is a candidate for a target of the SNF1 kinase (ESTRUCH and CARLSON 1990). Also recovered in this selection was the *YCK1* gene, which encodes a protein homologous to rabbit casein kinase I; *YCK1* and the highly similar gene *YCK2* together provide an essential function (L. ROBINSON, E. J. A. HUBBARD, P. ROAGH, M. CULBERTSON and M. CARLSON, personal communication). A third suppressor gene encodes a protein containing two zinc-finger motifs (F. ESTRUCH...
and M. CARLSON, unpublished results). The remaining four genes include two strong suppressors that restore substantial derepression of invertase (E. J. A. HUBBARD, unpublished results). Further study should elucidate the relationships of these multicopy suppressors to the SNF1 protein kinase and to the regulatory mechanism for glucose repression.

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