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, *MSI1* 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, *MSI1* and *PDE2*. *MSI1* (also called *JUN1*) was previously isolated as a multicopy suppressor of the heat

shock sensitive phenotype of *ira1* and *RAS2^{Val19}* 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-D-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-D-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; THUKRAL *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 33 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 *SalI/EcoRI* fragment of pB37 cloned into pUC19 (YANISCH-PERRON, VIEIRA and MESSING 1985). pJH39 carries the 1.4-kb *NruI* fragment of pB37 cloned into the *SmaI* 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 *ClaI/SmaI* and *SalI/XhoI* fragments of pB37 cloned into the *BamHI/SmaI* 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/SmaI* fragment of pB88 into the *NheI/SmaI* site of YEp24. pXY7 and pXY8 were constructed by cloning the *SphI/ScaI* and *BamHI/NheI* fragments of pB88 between the *SphI/SmaI* and *BamHI/NheI* sites of YEp24, respectively.

Sequence analysis and computer methods: Restriction fragments were cloned into M13 mp18 or M13 mp19 (NORRANDER, 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 package (DEVEREUX, HAEBERLI and SMITHIES 1984). Searches of reported sequences were made in the GenBank (BILOFSKY *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/SphI* fragment of pB37 to the *KpnI/SphI* fragment of pUC19. pJH51 was then cut with *ClaI* and *BglII*, filled in with Klenow, isolated, and ligated to a 1.1-kb *SmaI* fragment containing *URA3* to obtain pJH52. The *KpnI/SphI* fragment of pJH52 was used to transform (ROTHSTEIN 1983) the diploid MCY1093 × 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: pJH80 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- BamHI* partial fragment cloned in pUC18. The *BamHI/SphI* fragment of pJH80 was used to transform SP1, TF1.5prF^{HR}, TF1.5prC^{HS} 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

TABLE 1

List of *S. cerevisiae* strains

Strain	Genotype	Source
MCY1093	*MATa his4-539 lys2-801 ura3-52 SUC2*	This laboratory
MCY1094	*MATα ade2-101 ura3-52 SUC2*	This laboratory
MCY1647	*MATα snf4-Δ1 lys2-801 ura3-52 leu2::HIS3 (his3-Δ200?) SUC2*	This laboratory
MCY1845	*MATα snf1-Δ10 ade2-101 ura3-52 SUC2*	This laboratory
MCY1846	*MATa snf1-Δ10 lys2-801 ura3-52 SUC2*	This laboratory
MCY1853	*MATα snf4-Δ2 his4-539 lys2-801 ura3-52 SUC2*	This laboratory
MCY2171	*MATa msi1-Δ1::URA3 his4-539 lys2-801 ura3-52 SUC2*	This work
TF1.5prF^{HR}	*MATα tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::URA3 his3 leu2 ura3 trp1 ade8 (can1?) SUC*	M. WIGLER
TF1.5prC^{HS}	*MATα tpk1^{w} tpk2::HIS3 tpk3::TRP1 bcy1::URA3 his3 leu2 ura3 trp1 ade8 (can1?) SUC*	M. WIGLER
SP1	*MATa his3 leu2 ura3 trp1 ade8 can1 SUC*	M. WIGLER
MCY2344	Derivative of TF1.5prC^{HS} with *snf1-15::LEU2*	This work
MCY2346	Derivative of TF1.5prF^{HR} with *snf1-15::LEU2*	This work
MCY2367	Derivative of SP1 with *snf1-15::LEU2*	This work
MCY2369	*MATa snf1-15::LEU2 leu2-3,112 his3-Δ200 ura3-52 SUC2*	This work
MCY2372	*MATa his3-Δ200 leu2-3,112 ura3-52 SUC2*	This laboratory

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 (NEIGEBORN 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 MCY1853 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 *MSI1* 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 pJH10 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 *Kpn*I 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 pJH49; 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 *MSI1*. The coding sequences for *MSI1* and *PGI1* are separated by 356 bp. The two genes were both mapped genetically to the right arm of chromosome II3: *MSI1* 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).

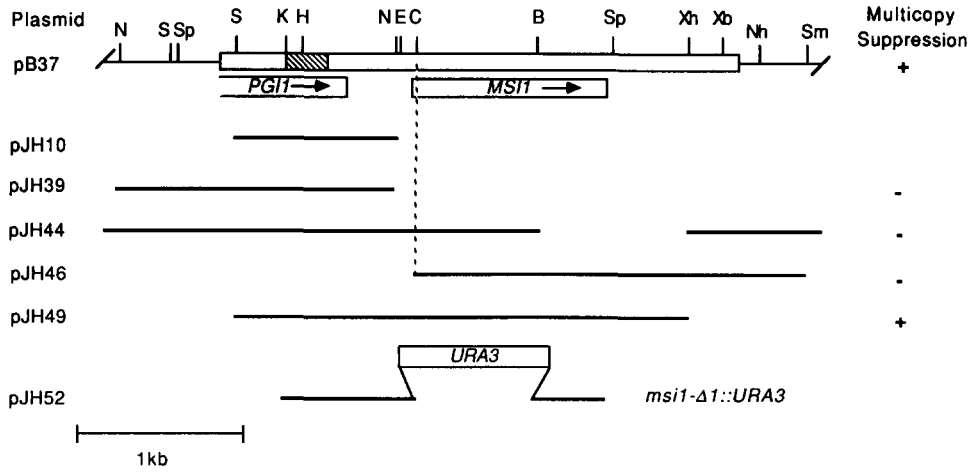

FIGURE 1.—Restriction map of plasmid pB37 and derivatives. Plasmids are described in the text. The open bar indicates the genomic yeast DNA sequence. The shaded area indicates the region that was sequenced. Part of the vector DNA is represented by the thin line. Heavy line indicates the segment of pB37 included in each derivative. Suppression of the *snf4*Δ defect in growth on raffinose is shown for each of the multicopy plasmids. Arrows indicate the 5′ to 3′ direction of the ORFs. Restriction sites: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; Nh, *Nhe*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I. pJH52 was used to construct the chromosomal allele *msi1-Δ1::URA3*.

MSI1 (also called *JUN1*) was isolated as a multicopy suppressor of the heat shock-sensitive phenotype of *ira1* and $RAS2^{Val19}$ mutants and also restores a low level of sporulation to the homozygous diploids (NIKAWA, SASS and WIGLER 1987; RUGGIERI *et al.* 1989). The exact relationship of this gene to the cAPK pathway is not clear, although RUGGIERI *et al.* suggest that *MSI1* 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 β subunit of transducin, the yeast genes *TUP1*, *CDC4*, *STE4*, *PRP4* and the *Drosophila* gene *Enhancer of split* (RUGGIERI *et al.* 1989; for review, see SIMON, STRATHMANN and GAUTAM 1991 and WILLIAMS and TRUMBLY 1990).

pB37 contains the entire *MSI1* gene, and *MSI1* corresponds to the functional region of pB37 (Figure 1). Thus, *MSI1* is also a multicopy suppressor of *snf4*.

Multicopy *MSI1* suppresses pleiotropic defects of *snf4* mutants: The *snf4* mutation causes a variety of defects in addition to the defect in growth on raffinose (NEIGEBORN and CARLSON 1984; CELENZA, ENG and CARLSON 1989). To determine whether multicopy *MSI1* suppresses the other defects of *snf4* mutants, we tested strain MCY1853 (*snf4-Δ2*) carrying pB37 or 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 *MSI1* multicopy plasmid improved growth of the *snf4* mutant on sucrose, raffinose, galactose, and glycerol relative to the control strain carrying the YEp24 vector, but did not improve growth nearly as well as a multicopy plasmid carrying *SNF1* (data not shown; see Figure 3).

Diploid strains homozygous for *snf4* are also defective in sporulation. To determine whether increased dosage of *MSI1* suppresses the sporulation defect, we tested *snf4-Δ2* homozygous diploids carrying pB37 or YEp24. 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 *MSI1* relative to diploids carrying the vector (Table 2). In addition, some mature asci were observed in the strains carrying *MSI1* in multicopy while no mature asci were observed in strains carrying the vector.

Disruption of the *MSI1* gene: Previous studies showed that deletion of *MSI1* did not affect growth rate, heat shock sensitivity, or sporulation of homozygous diploids (RUGGIERI *et al.* 1989). Because *MSI1* was here isolated as a suppressor of *snf4*, we constructed a new disruption and tested for other phenotypes previously associated with *snf4* mutations. Diploids heterozygous for a deletion/substitution mutation in the *MSI1* gene, designated *msi1-Δ1::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 derepressed values 380 and 350, respectively). Thus no new phenotype was identified.

Identification of *PDE2* as a suppressor gene: An-

TABLE 2

Multicopy *MSI1* and *PDE2* improve sporulation of *snf1* and *snf4* homozygous diploids

Experiment[a]	Relevant genotype	Plasmid-borne gene	Sporulation (%)[b] of colony A	B
1	*snf4/snf4* (pB37)	*MSI1*	7.0	3.4
	snf4/snf4 (YEp24)	None	1.4	1.4
2	*snf1/snf1* (pB37)	*MSI1*	1.6	2.6
	snf1/snf1 (YEp24)	None	0.4	0.2
3	*snf4/snf4* (pXY8)	*PDE2*	15.3	9.6
	snf4/snf4 (YEp24)	None	4.1	6.0
	snf1/snf1 (pXY8)	*PDE2*	5.6	8.0
	snf1/snf1 (YEp24)	None	1.0	0.2
	Wild type (YEp24)	None	42	43

[a] 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° than at 30° (CELENZA, ENG and CARLSON 1989).

[b] 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 *Xba*I 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 $RAS2^{Val19}$ mutation, and mutations in *PDE2* suppress a *ras2* mutation (CANNON, GIBBS 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 YEp24 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 *MSI1*, markedly improved the formation of mature asci.

Increased dosage of *MSI1* 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 *MSI1* 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 *MSI1* or *PDE2* to be able to suppress defects resulting from the complete loss of SNF1 function.

To test whether *MSI1* 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 *MSI1*. Furthermore, plasmids pB37 (*MSI1*) 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.

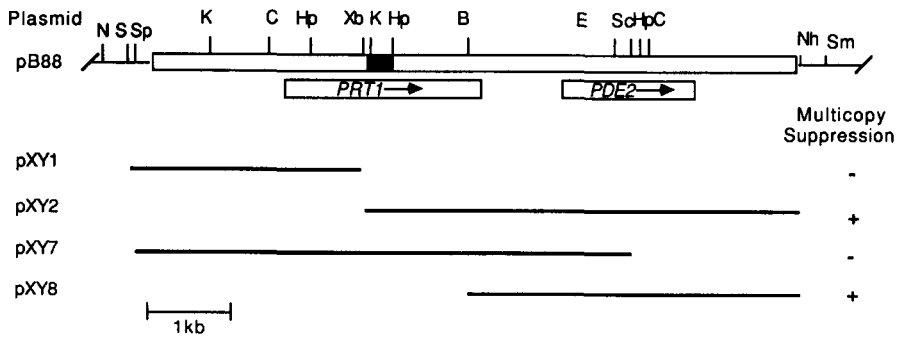

FIGURE 2.—Restriction map of plasmid pB88 and derivatives. Plasmids are described in the text. Symbols are as in Figure 1. The shaded area indicates the region that was sequenced. Restriction sites: as in Figure 1, plus B, *Bam*HI; Hp, *Hpa*I; Sc, *Sca*I.

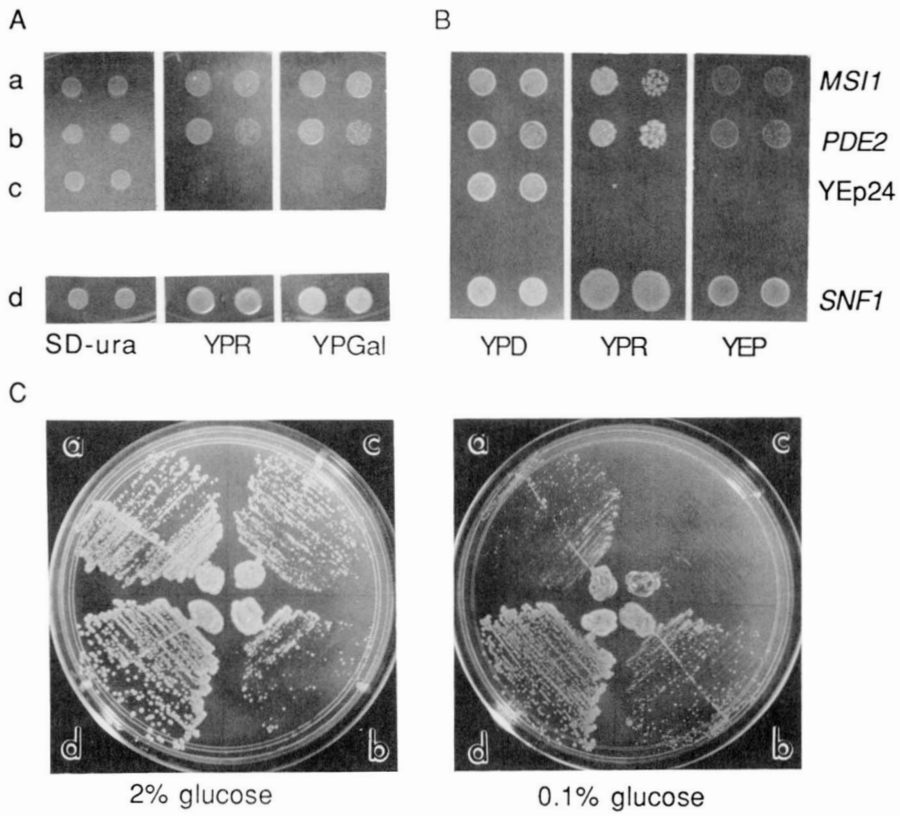

FIGURE 3.—Suppression of growth defects of a *snf1-Δ10* mutant by multicopy *MSI1* and *PDE2* genes. Strains are transformants of MCY1845 carrying: (a) pJH49 (*MSI1*); (b) pXY8 (*PDE2*); (c) vector YEp24; (d) pCE9 (*SNF1*). (A, B) Each row shows growth of two independent transformants. Cell suspensions were spotted onto the following media: supplemented synthetic medium lacking uracil and containing 2% glucose (SD-ura) or rich medium (YEP) containing raffinose (YPR), galactose (YPGal), glucose (YPD), or no added carbon source. (A) Plates were photographed after incubation at 30° in a GasPak for 14 days (YPR) and 12 days (YPGal), except that (d) was photographed after 3 days. (B) Plates were photographed after aerobic incubation at 30° for 24 hr (YPD), 5 days (YPR), and 5 days (YEP). (C) Cells were streaked onto YPD and YEP containing 0.1% glucose, and plates were incubated aerobically at 30° for 2 days (2% glucose) and 5 days (0.1% glucose).

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 *MSI1* 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 *MSI1* and *PDE2* do not affect expression of invertase in wild-type cells (data not shown).

Increased dosage of *MSI1* and *PDE2* improves nutrient utilization of *snf1* mutants: An alternate explanation for the restoration of growth on raffinose is that *MSI1* and *PDE2* in multicopy confer to the mutants increased ability to utilize effectively low lev-

TABLE 3

Multicopy *MSI1* and *PDE2* do not restore invertase expression in *snf* mutants

Relevant genotype[a]	Plasmid-borne gene	Invertase activity[b]	
		Repressed	Derepressed
snf4 (pLN132)	*SNF4*	1	135
snf4 (pCE9)	*SNF1*	<1	38
snf4 (pB37)	*MSI1*	<1	4
snf4 (pB88)	*PDE2*	1	3
snf4 (YEp24)	None	<1	2
snf1 (pCE9)	*SNF1*	<1	150
snf1 (pJH49)	*MSI1*	<1	<1
snf1 (pXY8)	*PDE2*	<1	<1

[a] Strains were MCY1853 and MCY1845 carrying the indicated plasmids.

[b] Micromoles 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 *MSI1*, *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 vector. 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^{HS} and TF1.5prF^{HR} (Table 1), in which cAPK activity is not regulated by cAMP (M. WIGLER, personal communication). These strains carry a *bcy1* 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* (*tpk1^w*). These strains, referred to as *bcy1 tpk^w* strains, have cAPK activity that is attenuated and independent of the level of cAMP in the cell (see CAMERON *et al.* 1988). TF1.5prC^{HS} is heat shock sensitive and TF1.5prF^{HR} is heat shock resistant due to different degrees of attenuation of the cAPK activity encoded by the *tpk1^w* 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 *bcy1 tpk^w* 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 glucose limitation (Table 4). Therefore, the regulated expression of invertase activity in the *bcy1 tpk^w* mutant strains still requires *SNF1* function.

TABLE 4

Regulation of invertase expression in *bcy1 tpk^w* strains

Strain	Relevant genotype	Invertase activity[a]	
		Repressed	Derepressed
SP1	Wild type	1	220
TF1.5prC^{HS}	*tpk1^w tpk2 tpk3 bcy1*	1	170
TF1.5prF^{HR}	*tpk1^w tpk2 tpk3 bcy1*	1	140
MCY2367	*snf1-15::LEU2*	1	1
MCY2344	*snf1-15::LEU2 tpk1^w tpk2 tpk3 bcy1*	1	<1
MCY2346	*snf1-15::LEU2 tpk1^w tpk2 tpk3 bcy1*	1	1

[a] 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%.

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 *bcy1* 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 *bcy1 tpk^w* 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 cAMP levels. Moreover, derepression of invertase in *bcy1 tpk^w* mutant strains is dependent on *SNF1* function, as is true in wild type. Thus, a cAMP-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 *bcy1 tpk*^{w} 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 ADR1 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 CIRILLO 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*, *bcy1*, and *RAS2*^{Val19} (TODA *et al.* 1985, 1987a; SASS *et al.* 1986; CANNON and TATCHELL 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 *snf1*Δ 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*^{Val19} 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. ROACH, 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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