Deletion of *SNF1* Affects the Nutrient Response of Yeast and Resembles Mutations Which Activate the Adenylate Cyclase Pathway

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ABSTRACT

We have isolated a *snf1/ccr1* mutant of *Saccharomyces cerevisiae* which loses viability upon starvation and fails to accumulate glycogen in response to abrupt depletion of phosphate or glucose. A *snf1* null mutant is sensitive to heat stress and starvation and fails to accumulate glycogen during growth in rich medium. The phenotypes of the *snf1* mutants are those commonly associated with an overactivation of the adenylate cyclase pathway. Mutations in adenylate cyclase or *RAS2* which decrease the level of cAMP in the cell moderate the *snf1* phenotype. In contrast, a mutation in RAS2 ($RAS2^{val19}$) which increases the level of cAMP or a mutation in the regulatory subunit (*BCY1*) of cAMP-dependent protein kinase which results in unregulated cAMP-dependent protein kinase activity accentuates the *snf1* phenotype. However, the action of SNF1 in the stress response appears at least partly independent of cAMP-dependent protein kinase because a *snf1* phenotype is observed in a strain that lacks all three of the genes that encode the catalytic subunits of cAMP-dependent protein kinase. SNF1 therefore acts at least in part through a cAMP-independent pathway.

NUMEROUS mutations which interfere with the proper response to nutrient limitation or depletion of glucose have been isolated in *Saccharomyces cerevisiae*. Nonetheless, the regulatory mechanisms governing these responses are not yet well understood. The *SNF1* (*CAT1/CCR1*) gene (CIRIACY 1977; ZIMMERMANN *et al.* 1977; CARLSON, OSMOND and BOTSTEIN 1981) encodes a protein kinase which is required for the derepression of many glucose repressible enzymes (CELENZA and CARLSON 1986, 1989; WRIGHT and POYTON 1990). Although several proteins have been identified which are possible targets or modulators of SNF1 activity (SCHULTZ and CARLSON 1987; CELENZA and CARLSON 1989), the nature of the events between sensing of glucose concentration, the action of SNF1, and the accompanying metabolic changes remains unclear.

Several pieces of information implicate the cAMP-adenylate cyclase pathway in the nutrient sensing process. BOY-MARCOTTE, GARREAU and JACQUET (1987) have shown that cAMP controls the response of yeast to ammonium availability. Furthermore, yeast cells with mutations that reduce the activity of the cAMP pathway, such as *ras2* or $cdc35^{ts}$ (*CDC35/CYR1* encodes adenylate cyclase), respond as if nutrient-limited, even when grown in rich medium (MATSUMOTO, UNO and ISHIKAWA 1983; TATCHELL, ROBINSON and BREITENBACH 1985; TODA *et al.* 1985). In contrast, yeast which have mutations that lead to increased cAMP-dependent protein kinase (cAPK) activity (such as $RAS2^{val19}$ or *bcy1/sra1*) fail to respond to nutrient limitation (MATSUMOTO, UNO and ISHIKAWA 1983; CANNON and TATCHELL 1987). Strains with these mutations do not accumulate the storage carbohydrate glycogen, are heat shock sensitive when nutrient-limited, and fail to sporulate.

Recently, CAMERON *et al.* (1988) demonstrated that there is a cAMP-independent pathway involved in the nutrient sensing process. Yeast cells which lacked the regulatory subunit of the cAPK (*BCY1/SRA1*) and had only a functionally attenuated catalytic subunit of the cAPK were able to accumulate glycogen and became heat-shock resistant in response to nutrient depletion.

To gain more information about the pathway(s) involved in the sensing of nutrient levels, we generated mutants that were defective in their ability to accumulate glycogen when starved of a given nutrient. This particular phenotype was chosen because glycogen accumulation is one of the hallmarks of yeast cells that have been depleted of a nutrient (LILLIE and PRINGLE 1980). We report here that the phenotypes of mutations in SNF1 resemble those which result from high cAPK activity, and that SNF1 is required for several responses to nutrient depletion.

MATERIALS AND METHODS

Yeast strains, genetic techniques, and measurement of cell numbers: *S. cerevisiae* strains used are listed in Table 1. Standard procedures for crosses, sporulation and tetrad analysis were followed (MORTIMER and HAWTHORNE 1969). *S. cerevisiae* strains were transformed by the spheroplast

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TABLE 1

***Saccharomyces cerevisiae* strains used in this study**

Strain	Genotype	Source
CH100	*MATα ura3 ade2 lys2 tyr1 trp1Δ his3Δ cap2-Δ1::HIS3*	AMATRUDA *et al.* (1990)
EG281.2-4A	*MATα ura3 his3 ade2-1 leu2 bcy1::HIS3*	This study
EG283-8A	*MATa ura3 his3 can1 leu2 Δsnf1::URA3*	This study
JC482[a]	*MATα leu2 ura3 his4*	CANNON and TATCHELL (1987)
JG11a1	*MATa ade2 ade8 leu2 ura3 his3 trp1 CTT::lacZ*	This study
JG228D[b]	*MATa ade8 leu2 ura3 his3 trp1 bcy1::LEU2 tpk1^{w} tpk2::HIS3 tpk3::TRP1 CTT::lacZ*	This study
JG228D-*snf1*	*MATa ade8 leu2 ura3 his3 trp1 bcy1::LEU2 tpk1^{w} tpk2::HIS3 tpk3::TRP1 CTT::lacZ Δsnf1::URA3*	This study
KT591[a]	*MATα leu2 ura3 his4 ras2::LEU2*	K. TATCHELL
KT685[a]	*MATα leu2 ura3 his4 bcy1-20::URA3*	K. TATCHELL
LRA84[a]	*MATa leu2 ura3 his4 cdc35-10*	L. ROBINSON
MCY1845	*MATα ade2 ura3 snf1-Δ10*	M. CARLSON
SGP406	*MATa leu2 trp1 ura3 his3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 ade8*	S. GARRETT
ST*Δsnf1*[a]	*MATα leu2 ura3 his4 Δsnf1::URA3*	This study
ST172-11[a]	*MATα leu2 ura3 his4 snf1-172*	This study
ST172-15[a]	*MATa leu2 ura3 his4 snf1-172*	This study
ST13-3B[a]	*MATa leu2 ura3 his4 snf1-172 ras2::LEU2*	This study
ST13-3D[a]	*MATα leu2 ura3 his4 snf1-172 ras2::LEU2*	This study
ST17-1[a]	*MATa leu2 ura3 his4 Δsnf1::URA3 ras2::LEU2*	This study
ST17-2[a]	*MATα leu2 ura3 his4 Δsnf1::URA3*	This study
STΔtpkΔ*snf1*	*MATa leu2 trp1 ura3 his3 tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2 ade8 Δsnf1::ADE8*	This study

[a] These strains are congenic.
[b] Derived from strain RS13-58A-1[h] (CAMERON *et al.* 1988).

method of HINNEN, HICKS and FINK (1978). Bacterial transformations were done according to the method of HANAHAN (1983). Total cell numbers and sizes were determined with the ZM Coulter Counter and Coulter Channelyzer 256.

Media and culture conditions: YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. For YPA, YPE and YPG, glucose was substituted by 2% potassium acetate, 2% ethanol and 3% glycerol, respectively. Complete synthetic media (CM) contained 0.67% yeast nitrogen base, 2% glucose, and amino acids as needed. Starvation media were made essentially as described by JOHNSTON, PRINGLE and HARTWELL (1977), except that succinate (disodium salt) was used rather than succinic acid and NaOH. The pH was approximately 6.5. Media designated as −N, −P, −S and −G were missing ammonium, phosphate, sulfate, and glucose, respectively. Cells were grown at 30° unless stated otherwise.

Mutagenesis and determination of glycogen: Ethyl methylsulfonate (EMS) mutagenesis of JC482 was done as follows: approximately 10^8 cells from an overnight culture in YPD were resuspended in 1 ml of 0.1 M KH_2PO_4 (pH 8.0), and 25 μl of EMS was added. The cells were incubated at 30° for 60 min, then 100 μl of this mixture was mixed with 4 ml of 5% sodium thiosulfate to inactivate residual EMS (SHERMAN, FINK and HICKS 1986). Cells were plated at a density of about 200 colonies per YPD plate. The survival rate with this protocol was about 75%.

Surviving yeast cells were replicated onto the four different types of nutrient-depletion plates (−N, −P, −S, −G). Because normal yeast accumulate glycogen when starved (LILLIE and PRINGLE 1980), this phenotype was used as an initial screen to identify yeast which failed to respond to nutrient starvation. Yeast colonies on the respective nutrient-depleted plates were exposed to iodine vapor in a closed glass chamber. Yeast which did not contain glycogen remained unstained. After screening approximately 20,000 colonies, several were obtained which did not stain under one or more of the starvation conditions. To narrow the number of interesting mutants, candidates chosen from the first screening were tested for loss of viability when starved. Glycogen accumulation mutants and the wild type parent strain were patched onto YPD plates and allowed to grow for 2 days. From the YPD plates the cell patches were replica-plated onto the −N, −P, −S and −G plates and incubated for 48 hr at 24°, 30°, or 37°. These nutrient-depleted plates were replica-plated back onto YPD plates and incubated for 26–30 hr; the density of mutant cell patches compared to the wild-type patch allowed us to estimate the loss of viability.

To quantitate glycogen levels, cells were incubated in 1 M Na_2CO_3 for 2 hr at 90° and the glycogen content was determined as described by FRANÇOIS, ERASO and GANCEDO (1987). Protein concentrations in the alkaline supernatant were determined with the BCA Protein Assay Reagents (Pierce) with bovine serum albumin as the standard.

Construction of the *SNF1* deletion: The *SNF1* gene was isolated from a YCp50 yeast genomic library, kindly provided by MARK ROSE. Transformants of ST172–11 in uracil-deficient selective top agar were scraped from agar plates, injected through a plastic syringe into glucose-deficient media (JOHNSTON, PRINGLE and HARTWELL 1977), and incubated for 48 hr at 37° to select for transformants that complemented the lethal phenotype of ST172–11 when grown at 37° in the absence of glucose. Plasmid pST-A complemented the defects of ST172–11 and contained yeast genomic DNA sequence with a restriction map that matched that of *SNF1* (CELENZA and CARLSON 1984a).

Nucleotides −3 to +1884 of the plasmid-carried *SNF1*

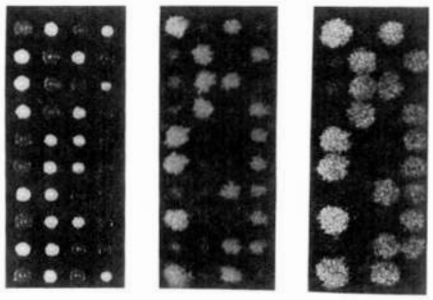

FIGURE 1.—The pleiotropic phenotype of ST172 is due to a single mutant allele. Meiotic progeny from a cross between ST172 (*snf1–172*) and JC482 (wild type) were replica-plated onto rich medium (YPD). After incubation for 36h, the YPD plate was stained with iodine vapor to estimate glycogen content (YPD/I_2, left panel). To test for growth on a nonfermentable carbon source, the tetrads were replica-plated onto 2% ethanol (YPE, center panel). To test viability, the tetrads were replica-plated onto phosphate-deficient medium, incubated for 3 days at 37° and then replica-plated back onto rich medium and incubated for 24 hr at 30° ($-PO_4^{2-}$, left panel).

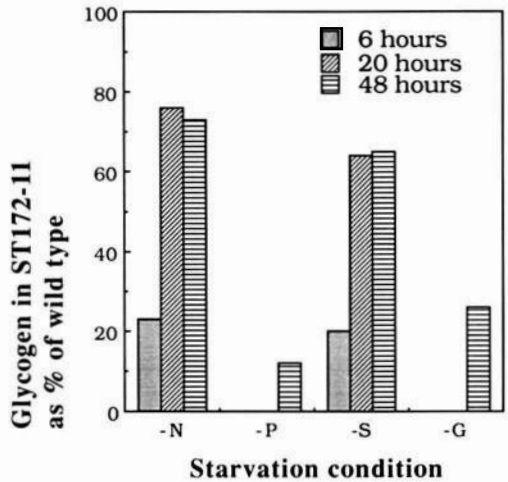

FIGURE 2.—Glycogen accumulation of JC482 and ST172–11 after 6, 20 and 48 hr of incubation in starvation media at 30°. JC482 (wild type) and ST172–11 (*snf1–172*) cells were grown in rich medium to a density of 1.7×10^7 cells/ml. Aliquots of 10 ml were pelleted, the pellets washed and then taken up in 10 ml starvation media, pH 6.5, as indicated. After 6, 20 and 48 hr of incubation at 30° the respective aliquots were pelleted and glycogen accumulation was measured. Glycogen in ST172–11 is expressed as % of the value found for JC482. (−N, −P, −S and −G designate media deplete of ammonium, phosphate, sulfate, or glucose, respectively).

coding sequence were deleted by digesting with *Hinc*II as described by CELENZA and CARLSON (1989) and were replaced with either a 1.1-kb *Xba*I fragment containing the *URA3* gene or a 2-kb *Bgl*II-*Eco*RI fragment containing the *ADE8* gene. The resulting plasmids, pST70 and pST80, respectively, were digested with *Hin*dIII and *Kpn*I to excise the marker gene and flanking *SNF1* sequences. The pST70-derived fragment was transformed into strains JC482 and JG228D, and the pST80-derived fragment was transformed into SGP406, by one-step gene replacement (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). Southern blot analysis was done on Ura^+ and Ade^+ transformants to confirm that the *SNF1* coding region had been deleted in each of the three strains.

RESULTS

Isolation of a *SNF1* mutant aberrant in its response to nutrient depletion: EMS mutagenesis of strain JC482 and screening of mutants unable to accumulate glycogen was done as described in MATERIALS AND METHODS. One mutant, ST172, was chosen for further study because it accumulated less glycogen than the wild-type strain when incubated on phosphate- or glucose-depleted solid medium. This strain also appeared to lose viability after 3 days of ammonium, phosphate, sulfate or glucose starvation at 37°. Lack of glycogen accumulation and loss of viability segregated 2:2 through two backcrosses to the parental wild type strain JC482 (Figure 1). The mutation in ST172 was recessive, as a heterozygous diploid was indistinguishable from the wild type. Further experiments were done with meiotic segregants from the second backcross to JC482, either ST172–11 (*MAT*α) or ST172–15 (*MAT***a**).

Glycogen concentrations were determined after incubation of ST172–11 and JC482 at 30° for 6, 20 or 48 hr under the four types (ammonium, phosphate, sulfate or glucose) of starvation. Results in Figure 2 show that the effect of the mutation in ST172–11 on glycogen levels was dependent on the starvation condition. The defect was most severe when cells were starved of phosphate or glucose and less severe when cells were starved of ammonium or sulfate. Glycogen also normally accumulates as cells approach stationary phase during growth in rich medium (YPD); when ST172–11 was grown in rich medium, however, glycogen was at or below the level of detection (see STΔsnf1 in Figure 4).

Quantitative analysis of viability loss in nutrient-depleted liquid media at 30° for 3 days showed that ST172–11 lost viability only during glucose starvation. However, ST172–11 suffered a 100% loss of viability when incubated in media depleted of ammonium, phosphate, sulfate or glucose at 37° for three days. ST172–11 did not lose viability when grown to saturation at 37°C in rich medium (YPD) for four days.

When ST172–11 was tested for growth and the ability to accumulate glycogen on alternative carbon sources, we found that ST172–11 could not grow on lactose, maltose, pyruvate, glycerol, ethanol (see Figure 1) or acetate, and grew poorly on galactose and sucrose, although the parent strain was able to utilize all these carbon sources. Because the inability of ST172–11 to grow on alternative carbon sources was reminiscent of *snf1* (*ccr1*/*cat1*) mutants (CIRIACY 1977; CARLSON, OSMOND and BOTSTEIN 1981; ENTIAN and ZIMMERMANN 1982; CELENZA and CARLSON 1984a; DENIS 1984; SCHULLER and ENTIAN 1987), we

tested for, and found, the expected linkage of the mutation in ST172–11 to the *rna3* gene, which is located 5.6 centimorgans proximal to *SNF1* on the right arm of chromosome IV (CELENZA and CARLSON 1984a). To confirm that ST172–11 contains a mutation in the *SNF1* gene, we crossed ST172–15 (the **a**-mating type equivalent of ST172–11) with a *snf1* deletion strain (MCY1845). The resulting diploid did not accumulate glycogen, was unable to grow on ethanol or acetate, and failed to sporulate, indicating that the mutation in ST172–11 was allelic to *SNF1*.

Since ST172–11 most likely contains a point mutation in the *snf1* gene, we constructed and tested a *snf1* null mutant in the same genetic background as ST172–11. From the wild-type JC482, we constructed a *snf1* deletion tagged with the *URA3* gene. The deletion was confirmed by Southern blot analysis (MATERIALS AND METHODS). Like ST172–11, this strain, ST*Δsnf1*, was defective in glycogen metabolism and was unable to grow on gluconeogenic substrates.

SNF1 is necessary for proper cell arrest and acquisition of thermotolerance upon depletion of external glucose: An interesting observation made originally with our *snf1–172* mutant was its higher incidence of budding and larger cell size in stationary cultures. When cultures grown in rich medium to saturation (about 12 hr after glucose exhaustion) were assayed for the fraction of budded cells, we found a higher number of budded cells in the saturated cultures of ST172–11 (21 ± 2%) than in JC482 (7.6 ± 0.1%), suggesting that the *snf1–172* mutation interfered with entrance of cells into a stationary or resting phase. However, in ST*Δsnf1* we did not observe a higher percentage of budded cells in stationary phase cultures. We did observe that both ST172–11 (*snf1–172*) and ST*Δsnf1* (*Δsnf1::URA3*) cells in stationary phase (about 12h after medium glucose exhaustion) contained about two-fold more protein than the wild type and were very large (see Figure 3). Logarithmic phase *snf1* cells, however, were the same size as wild type. Stationary phase cultures of *snf1* strains (ST172–11, ST*Δsnf1*) were reproducibly more heat shock sensitive than the isogenic wild type (JC482) (see Table 2).

Mutations which affect the adenylate cyclase pathway alter the phenotype of a *SNF1* deletion strain: ST172–11 and ST*Δsnf1* resemble strains with either defective regulatory subunits of the cAMP-dependent protein kinase (*bcy1/sra1*) (MATSUMOTO, UNO and ISHIKAWA 1983; CANNON and TATCHELL 1987; TODA *et al.* 1987a) or elevated levels of cAMP (as in a $RAS2^{val19}$ strain) (KATAOKA *et al.* 1984). All of these strains exhibit decreased tolerance to heat stress and starvation, fail to accumulate glycogen when grown in YPD medium, and fail to sporulate. Strains with *ras2* or *cdc35/cyr1* mutations, which cause a reduction

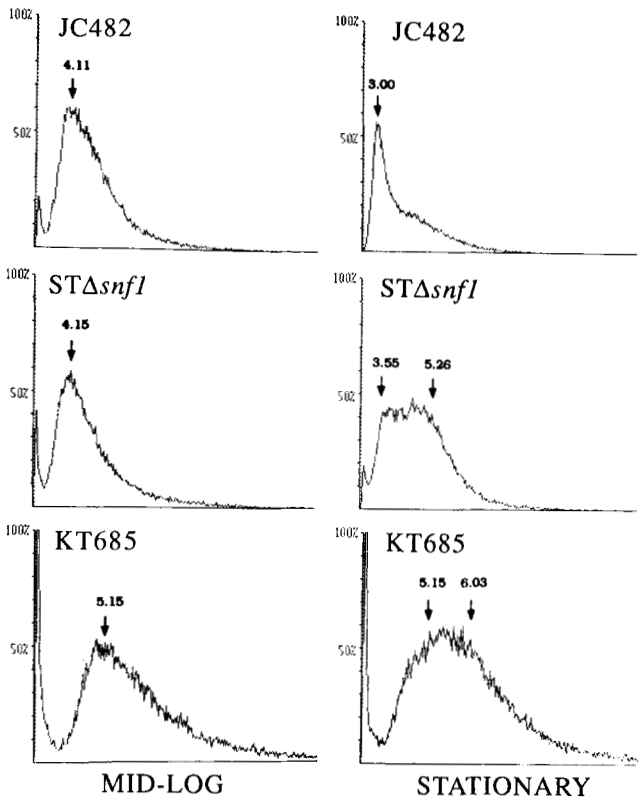

FIGURE 3.—Size distribution of JC482 (wild type), ST*Δsnf1* (*Δsnf1::URA3*) and KT685 (*bcy1::URA3*) cells. Logarithmic and stationary (about 12 hr after glucose depletion of the medium) YPD liquid cultures from each strain were sonicated, vortexed and the distribution of cell sizes was measured in a ZM Coulter counter channelyzer. Axis: cell size; ordinate: percent of cells in each size channel.

of cAMP, have the converse phenotype: *i.e.*, they hyperaccumulate glycogen, are heat shock resistant, and the homozygous diploids sporulate on rich media (IIDA and YAHARA 1984; BOUTELET, PETITJEAN and HILGER 1985; TATCHELL, ROBINSON and BREITENBACH 1985; PETITJEAN, HILGER and TATCHELL 1990). This prompted us to explore the effect of these mutations on the behavior of *snf1*.

ST*Δsnf1* was crossed with strain KT591 (*ras2::LEU2*) and the meiotic progeny were analyzed by tetrad analysis. Quantitative glycogen measurement performed on two *ras2::LEU2 Δsnf1::URA3* mutants (ST17–1 and ST17–2) which were grown in rich medium revealed values similar to those of the congenic wild type (intermediate between the *Δsnf1::URA3* and *ras2::LEU2* parents) (Figure 4). As shown in Table 2, the *ras2::LEU2 Δsnf1::URA3* double mutants were also more heat stress resistant than the *RAS2 Δsnf1::URA3* strain. In addition, diploids homozygous for both mutations could sporulate, although not as well as the diploid which was homozygous for *ras2::LEU2 SNF1*.

STΔsnf1 was also crossed with LR84 (*cdc35–10*, a temperature-sensitive *cdc35/cyr1* allele) and the progeny were analyzed by tetrad analysis. The ability of

TABLE 2

Quantitation of cell thermotolerance

Strain	Relevant genotype	Percent survival
Experiment 1[a]		(24/52 hr)
JC482	*SNF1 RAS2*	35/100
KT591	*SNF1 ras2::LEU2*	95/100
ST172-11	*snf1-172 RAS2*	0/0
ST127-15[b]	*snf1-172 RAS2*	0/9
ST13-3B	*snf1-172 ras2::LEU2*	33/70
ST13-3D[c]	*snf1-172 ras2::LEU2*	28/60
Experiment 2[d]		(20/24 hr)
JC482	*SNF1 RAS2*	89/87
KT591	*SNF1 ras2::LEU2*	97/100
STΔ*snf1*	Δ*snf1::URA3 RAS2*	28/22
ST17-1	Δ*snf1::URA3 ras2::LEU2*	79/93
ST17-2ke	Δ*snf1::URA3 ras2::LEU2*	93/94
JG228D	*bcy1::LEU2 tpk1^w tpk2::HIS3 tpk3::TRP1*	86/92
JG228D-*snf1*	*bcy1::LEU2 tpk1^w tpk2::HIS3 tpk3::TRP1snf1::URA3*	83/100

In both experiments, cells were inoculated in rich medium to a cell density of 0.7–1 × 10^7 cells/ml.

[a] After 24 and 52 hr (about 12 and 40 hr after medium glucose exhaustion), a 1 ml aliquot of cells was withdrawn, transferred to a 1.5-ml Eppendorf tube and incubated at 50° for 20 min, cooled briefly, diluted and plated on YPD.

[b] Isogenic to ST172-11.

[c] Isogenic to ST13-3B.

[d] As in (*a*) except that cells were treated after 20 and 24 hr (about 8 and 12 hr after medium glucose exhaustion). Cells not treated with heat served as the basis of comparison.

[e] Isogenic to ST17-1.

the *cdc35–10* mutation to suppress Δ*snf1::URA3* was temperature dependent. At the permissive temperature (24°) the *cdc35–10* Δ*snf1::URA3* double mutants did not accumulate glycogen on rich medium; after further incubation at 30°, however, they appeared to accumulate as much glycogen as the wild type (Figure 5). Similarly, on phosphate-depleted medium the double mutants hyperaccumulated glycogen only after incubation at 30° (data not shown).

Although the Δ*snf1::URA3* mutation influenced the traits associated with *ras2* and *cdc35*, it did not bypass the absolute requirement for either RAS1 and RAS2 or CDC35 (TATCHELL *et al.* 1984). In a cross between a *ras2::LEU2 snf1–172* strain and a *ras1::URA3* strain, we were unable to recover viable *ras1::URA3 ras2::LEU2 snf1–172* progeny. Similarly, the temperature-dependent lethality of *cdc35–10* was not suppressed by *snf1* (Figure 5).

As mutations which lowered the level of cAMP and/or cAPK in *snf1* mutants resulted in generally healthier, more normal cells, we decided to test the effect in a *snf1* strain of increasing the level of cAMP and/or cAPK. To this end, we transformed JC482 (wild type) and STΔ*snf1* with *RAS2^{val19}* on a single copy vector (YCp50) and selected Ura$^+$ transformants. STΔ*snf1* transformed with YCp50-*RAS2^{val19}* was, like the wild type strain containing *RAS2^{val19}*, exquisitely sensitive to heat shock and did not accumulate glycogen on rich media. The two strains had a notable difference in growth rate: JC482 containing YCp50-*RAS2^{val19}* grew only slightly slower in synthetic medium than JC482 containing YCp50 alone; conversely, STΔ*snf1* with YCp50-*RAS2^{val19}* doubled much slower than STΔ*snf1* with YCp50 alone (270 min as opposed to 160 min).

Given that the major effect of *RAS2^{val19}* is the ele-

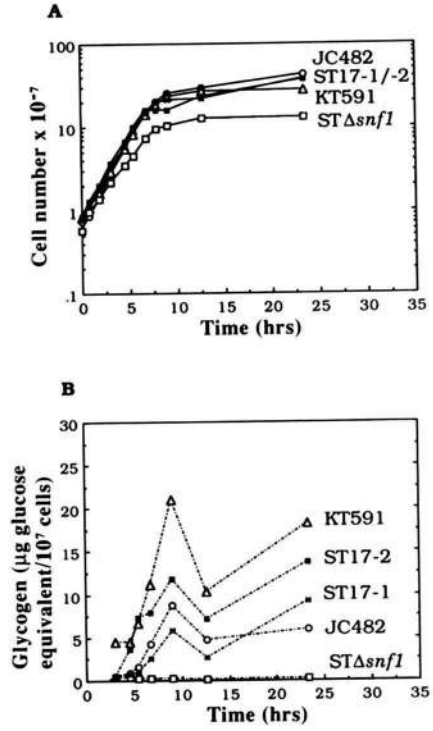

FIGURE 4.—Quantitative glycogen determinations for ST17–1 and ST17–2 during growth in YPD medium. YPD medium was inoculated with 3–6 × 10^6 cells/ml of JC482, KT591 (*ras2::LEU2*), STΔ*snf1* (Δ*snf1::URA3*), ST17–1 or ST17–2 (Δ*snf1::URA3 ras2::LEU2*). Cell counts were determined at points along the growth curve as indicated and aliquots were taken for determination of glycogen. ST172–11 (*snf1–172*, not shown) gave the same result as STΔsnf1.

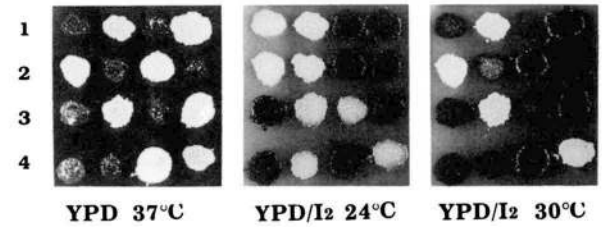

FIGURE 5.—Qualitative glycogen determinations for progeny from the cross of STΔsnf1 with LR84. STΔ*snf1* (Δ*snf1::URA3*) was mated with LR84 (*cdc35–10*), the diploid was sporulated on YPA medium at 24°, and the meiotic progeny were dissected. Spore clones (positioned left to right) from four tetrads (1–4) were patched onto three YPD plates, which were incubated at (from left to right): 37° for 24 hr, 24° for 24 hr, or 24° and then 30° for 24 hr each. The 24° and 30° plates were stained with iodine to estimate glycogen content. Lack of growth at 37° identifies the presence of *cdc35–10* (left panel). Failure to stain dark at 24° on YPD (center panel) identifies the presence of Δ*snf1::URA3*.

vation of cAMP levels, the slow growth phenotype of ST*Δsnf1* transformed with YCp50-*RAS2*^{val19} is likely to be a combination of increased levels of cAMP-dependent protein kinase in a *snf1* mutant background. To directly test this possibility we crossed a strain bearing a *HIS3*-tagged disruption of *bcy1* (EG281.2–4A) with a *Δsnf1::URA3* deletion (EG283–8A), sporulated the diploid, dissected four-spored asci on YPD (2% glucose) and incubated the plates for 5 days. Microscopic examination of the 28 dissected asci revealed that 93 out of 112 spores germinated and divided at least once, but no *Δsnf1::URA3 bcy1::HIS3* (Ura$^+$ His$^+$) spore clones grew into macroscopic colonies. Of those spore clones that could be unambiguously designated as *bcy1::HIS3 Δsnf1::URA3* double mutants (from the genotype of sister spore clones) 15 of 22 germinated and divided at least twice and most gave rise to microcolonies of 8–20 cells. We had previously observed that *bcy1* mutants grew better in liquid culture at glucose concentrations above the commonly used level of 2% (K. TATCHELL, unpublished observation). To test the possibility that the *snf1 bcy1* double mutants may be glucose remedial, we dissected the same cross onto YPD media containing 8% glucose. On this medium 12 of 14 putative *bcy1 snf1* spore clones germinated and divided at least once and eight gave rise to visible colonies. However, none of these were able to grow on YPD or synthetic media containing 2% glucose. The *bcy1 snf1* double mutants did not grow on media with 1 M sorbitol, indicating that the growth defect was not due to osmotic sensitivity. It is possible that the extreme phenotype of the *snf1 bcy1* double mutants is simply the result of the additive effect of two generally deleterious mutations. However, the doubling times of *snf1* (120–125 min) and *bcy1* (100–110 min) strains in rich medium are not dramatically different from that of the wild type JC482 (90 min). We therefore propose that the extreme phenotypes of *snf1 RAS2*^{val19} and *snf1 bcy1* indicate a synergism between the two mutations and suggest that SNF1 and the cAPK may act on the same physiological processes.

CAP2, a gene encoding a putative actin-binding protein (AMATRUDA *et al.* 1990), lies next to *BCY1* and is partially deleted in our *bcy1::HIS3* disruption. Although *cap2* deletions are viable, it is possible that the glucose remedial phenotype we observe in the *bcy1 snf1* double mutants is due to the partial deletion of *cap2*. We tested this possibility by crossing a *cap2::HIS3* deletion (CH100) with *snf1::URA3* (EG283–8A) and analyzing the meiotic products by tetrad analysis. The *cap2::HIS3 snf1::URA3* double mutants were viable and did not have the extreme phenotype observed for *bcy1 snf1* mutants.

The similar phenotypes of *snf1* and *bcy1* and the additivity of their traits suggests that the SNF1 protein kinase may act directly in the cAMP-dependent protein kinase pathway, perhaps by exerting negative control over the activity of the catalytic subunits. Alternatively, SNF1 and cAPK may act in distinct pathways, but with common substrates which they affect in an antagonistic manner. To distinguish between these two possibilities we wanted to compare the phenotype of *snf1* mutants that lack the cAPK to *snf1* mutants that contain the cAPK. If the SNF1 protein kinase acts in a distinct pathway from the cAPK, then a deletion of *snf1* in a strain lacking cAPK should have a *snf1* phenotype. However, if SNF1 acts through the cAPK pathway, then loss-of-function mutations in cAPK should be epistatic to *snf1*. Unfortunately, cAPK, encoded by *TPK1, TPK2* and *TPK3*, is essential for cell growth and division. Yeast strains must contain at least one of three *TPK* genes to grow (TODA *et al.* 1987b). We circumvented this problem in two ways. In the first, we tested the effect of a *snf1* mutation in a strain with very low levels of cAMP-dependent protein kinase activity. In the second, we tested the effect of *snf1* in a genetic background that allowed growth and division of a cAPK-deficient strain.

CAMERON *et al.* (1988) observed that yeast strains which lack the regulatory subunit of cAPK (*bcy1*) but contain only a single partially defective or attenuated catalytic subunit gene (*tpk1*^{w}) can mount a nearly normal starvation response. These strains are unable to respond through the cAMP pathway because they lack BCY1, the cAMP-binding regulatory subunit, but do not exhibit the defects of most *bcy1* strains because the cAPK levels are extremely low. These strains have no measurable cAPK activity in standard cAPK protein kinase assays on whole cell extracts (CAMERON *et al.* 1988). One such strain (JG228D) contains disruption alleles of *bcy1, tpk2* and *tpk3* and contains an attenuated allele of *tpk1* (*tpk1*^{w}). As expected, this strain has no measurable protein kinase activity (S. THOMPSON-JAEGER and J. GAUGHRAN, unpublished results) but is heat shock resistant, and accumulates glycogen. The *SNF1* coding sequence in JG228D was replaced with *URA3* (see MATERIALS AND METHODS) and transformants were tested for their response to nutritional stress. We observed many of the traits of *snf1* in these transformants. One Ura$^+$ transformant (JG228-*snf1*) for which the deletion of *snf1* was confirmed by Southern blot analysis (data not shown), was tested for glycogen accumulation, thermotolerance, and growth on ethanol and glycerol (Table 2 and Figure 6). JG228-*snf1* accumulated less glycogen than the parental strain and grew poorly on ethanol and glycerol (Figure 6). The *snf1* mutation clearly exerts a phenotype on strains with only very low levels of cAPK, and this provides evidence that SNF1 acts at least in part via a cAMP-independent pathway.

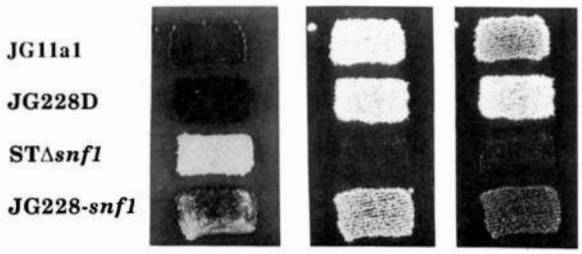

FIGURE 6.—Phenotypic characterization of JG228D-snf1. The *SNF1* coding sequence in JG228D (*bcy1::LEU2 tpk1^{w} tpk2::HIS3 tpk3::TRP1*) was deleted to create JG228-*snf1*. The strains as shown to the left of the figure were patched onto a YPD master plate and then replica-plated to various media. The plates are, from left to right, YPD which was stained with iodine vapor, 2% ethanol (YPE), and 2% glycerol (YPG). JG11a1 is the wild type.

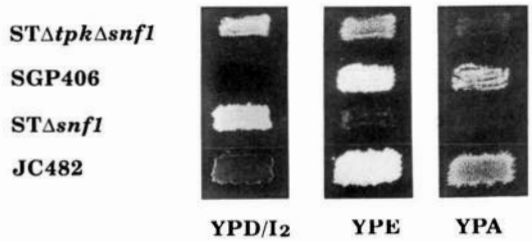

FIGURE 7.—Phenotypic characterization of STΔ*tpk*Δ*snf1*. The *SNF1* coding sequence in SGP406 (*tpk1::URA3 tpk2::HIS3 tpk3::TRP1 yak1::LEU2*) was deleted to yield STΔ*tpk*Δ*snf1*, which contains disruptions in all three genes which encode the catalytic subunit of the cAPK and a disruption of *YAK1*. The strains as shown to the left were patched onto a YPD master plate and then replica-plated to the media as shown. The plates are, from left to right, YPD which was stained with iodine, 2% ethanol (YPE) and 2% glycerol (YPG).

However, JG228-*snf1* may still contain low levels of cAPK activity from *tpk1^{w}* and it can be argued that SNF1 is acting through this residual amount of tpk1 kinase activity. Therefore, we also tested the phenotype of a *snf1* strain in a background that totally lacked cAPK.

Although cAPK is essential for growth, GARRETT and BROACH (1990) observed that strains which lack all three *TPK* genes are viable if they also contain a deletion of *YAK1*, which encodes another protein kinase. Since the *yak1* deletion does not alter the ability of either wild-type or *tpk*$^{-}$ strains to grow on nonfermentable carbon sources or accumulate glycogen, we reasoned that the deletion of *snf1* in a *yak1*$^{-}$ *tpk*$^{-}$ strain would allow us to access any traits of *snf1* in a cAPK^{-} background. We therefore disrupted *snf1* in strain SGP406 by replacing the *SNF1* coding sequence with the *ADE8* gene (see MATERIALS AND METHODS). These transformants, one of which is shown in Figure 7, clearly display the *snf1* phenotype. They fail to accumulate glycogen and do not grow well on non-fermentable carbon sources. These results show that the *snf1* phenotype is present in the absence of cAPK and provide compelling evidence that SNF1 acts at least in part through a cAMP-independent pathway. The phenotypes of the *snf1* strains in the *tpk1 tpk2 tpk3 yak1* background are not identical to the phenotypes in the wild type background. Some residual growth is observed of STΔ*tpk*Δ*snf1* on YPE, whereas STΔsnf1 fails to grow at all on YPE. We also observed that the STΔ*tpk*Δ*snf1* strain accumulated some glycogen at the periphery of the colony, suggesting that this strain can accumulate glycogen while growing, but is unable to accumulate the carbohydrate once cells have ceased to grow. In confirmation, we have quantitated glycogen in YPD liquid cultures of SGP406 and STΔ*tpk*Δ*snf1* during growth in batch culture and found that both strains accumulate high levels of glycogen in the exponential growth phase but STΔ*tpk*Δ*snf1*, unlike SGP406, is unable to maintain the glycogen levels once the cells reach stationary phase (data not shown).

DISCUSSION

SNF1 is a 72-kD serine-threonine protein kinase with a central role in glucose derepression (CIRIACY 1977; ZIMMERMANN *et al.* 1977; ENTIAN and ZIMMERMANN 1982; CELENZA and CARLSON 1984b, 1986, 1989). Much data on SNF1 focuses on its role in the control of *SUC2* (sucrose invertase) gene expression (CARLSON, OSMOND and BOTSTEIN 1981). The action of SNF1 is reportedly at the level of transcription of *SUC2* (CARLSON and BOTSTEIN 1982; SAROKIN and CARLSON 1985). Here we extend the previously reported data on SNF1 to show that this protein kinase is required not only for the derepression of glucose-repressible enzymes, but also for cell survival during several types of starvation and heat stress. In addition, SNF1 plays a role in the regulation of glycogen metabolism.

The response of the *snf1–172* mutant (ST172–11) to the four types of starvation was not identical. Glycogen accumulation was near normal when ST172–11 was starved of sulfate or nitrogen at 24° and 30°, but was abnormally low under phosphate or glucose starvation. During glucose depletion this is not surprising, as the *snf1–172* strain does not derepress gluconeogenic enzymes (S. THOMPSON-JAEGER, J. FRANÇOIS and K. TATCHELL, unpublished observations) and thus cannot make the glucose needed for synthesis of glycogen. In contrast, the reason for decreased carbohydrate accumulation in *snf1–172* cells starved of phosphate is obscure, but may be related to the regulation of intracellular phosphate. An absence of available phosphate from intracellular stores could disrupt a number of cellular functions. Here it must be noted that *snf1* strains are not defective in acid phosphatase (CELENZA and CARLSON 1986); however, the level of intracellular polyphosphate has to our knowledge not been quantified for any *snf1* strain.

The *snf1–172* strain rapidly lost viability when transferred from rich medium, in which it had been exponentially growing, to minimal medium lacking glucose. However, this same strain did not lose viability when grown to saturation in rich medium. Although in both cases glucose is depleted, the two regimes are known to result in different responses. For example, rapid withdrawal of glucose can result in cells arresting at different points in the cell cycle, whereas gradual glucose depletion allows cells to arrest uniformly in G_1 (PRINGLE and HARTWELL, 1981). *snf1* mutants appear less able to adapt to the abrupt loss of glucose. Cells grown to saturation in rich medium become oxygen- as well as glucose-limited (LILLIE and PRINGLE 1980). As *snf1–172* cells approach stationary phase in rich medium it may be the decrease in oxygen availability that protects them from death. Here, when testing cells for loss of viability in glucose-depleted minimal medium, the cells were diluted such that oxygen may not have been limiting.

There is a remarkable phenotypic similarity between *bcy1/sra1* strains (which have high, unregulated activity of the cAPK) and our *snf1* strains. All share a reduced tolerance to heat and starvation, do not sporulate, and fail to accumulate glycogen, as well as becoming abnormally large when grown in rich medium. Through genetic analysis we have explored the relationship between the adenylate cyclase pathway and the SNF1 protein kinase. Here, we found that *snf1* mutants carrying a disruption of the *RAS2* gene (RAS2 is a positive regulator of adenylate cyclase; BROEK *et al.* 1985; TODA *et al.* 1985) resembled the wild type rather than either single-mutation parent in their ability to accumulate glycogen and sporulate, as if the two mutations had counteracted one another. We believe that the partial suppression of *snf1* by *ras2::LEU2* is related to the role of *RAS2* in the activation of adenylate cyclase (CYR1/CDC35), because the *cdc35–10* mutation also suppressed the defective glycogen accumulation phenotype of *snf1* strains which were incubated at the nonpermissive temperature. Similarly, in a strain with only attenuated cAPK activity, the loss of the *SNF1* coding sequence caused a phenotype intermediate between either single mutation with regard to glycogen accumulation and growth on gluconeogenic substrates.

If mutations that decrease cAMP levels in the cell attenuate the phenotype of *snf1*, one might expect mutations which activate adenylate cyclase or the cAPK to alter the traits of *snf1* strains as well. This appears to be the case as both *bcy1 snf1* and $RAS2^{val19}$ *snf1* mutants were extremely unhealthy, and the former grew only on 5% or higher concentrations of glucose, if at all. That the $RAS2^{val19}$ *snf1* and *bcy1 snf1* mutants grow so much more poorly than either single mutant emphasizes the possibility of SNF1 involvement in areas other than derepression of glucose-repressible enzymes. In this regard, DENIS (1984) and CIRIACY (1977) have inferred that SNF1 is active during growth on glucose.

That strains with low or attenuated cAPK activity and no SNF1 resembled the wild type with regard to several traits could support a model in which SNF1 acts either directly through the cAPK (for example, as a negative regulator of catalytic activity), or, alternatively, in a separate nutrient-sensing signal transduction pathway. Several genetic results do not support the model in which SNF1 mediates the activity of the cAPK. First, the absence of a functional SNF1 protein kinase did not rescue *ras1 ras2* mutants which are inviable unless they also have unregulated cAPK catalytic activity (TODA *et al.* 1985). Second, the *snf1* null mutation did not suppress the temperature sensitive phenotype of the *cdc35–10* allele. Third, when *SNF1* was deleted in a strain lacking any cAPK catalytic subunits (and deleted of *YAK1* as well) the strain no longer grew on gluconeogenic substrates and failed to maintain glycogen stores once growth had ceased. All these indicate that SNF1 may not act through the cAPK. That the strain devoid of both SNF1 and the cAPK accumulated but then could not maintain glycogen in stationary phase may be a result of overactive glycogen phosphorylase, as witnessed in other *snf1* strains (S. THOMPSON-JAEGER and K. TATCHELL, unpublished data).

Interestingly, although the absence of any cAPK in strain SGP406 allows for the hyperaccumulation of glycogen, these same cells are not resistant to heat shock or starvation (S. THOMPSON-JAEGER and K. TATCHELL, unpublished observations) and thus hyperaccumulation of storage carbohydrates does not preclude heat shock and starvation sensitivity.

Taken together, our results are consistent with a model in which the SNF1 kinase and cAPK are members of antagonistic, parallel pathways. SCHULTZ and CARLSON (1987) have also observed antagonism between the SNF1 and cAPK pathways. Ascospores containing *ssn6*, an extragenic suppressor of *snf1*, and *cyr1–2*, a temperature-sensitive allele in adenylate cyclase, were largely inviable and most of the few viable spore clones were unhealthy. Analysis of the few viable *ssn6 cyr1–2* double mutants showed that there was partial suppression of the invertase deficiency of *cyr1–2*, but no clear epistasis was observed.

One target of yeast cAPK is the yeast transcriptional activator ADR1 (CHERRY *et al.* 1989). Phosphorylation of ADR1 at serine residue 230 partially inactivates ADR1, and prevents ADR1-dependent expression of glucose-repressible alcohol dehydrogenase (ADHII) (CIRIACY 1975). SNF1 protein kinase is additionally required for the full derepression of *ADH2*, although its mode of action has not been elucidated

(DENIS 1987). Based on our observation that SNF1 may act antagonistically to cAPK, it is tempting to speculate that SNF1 may play a role in ADR1 activation through dephosphorylation. However, BEMIS and DENIS (1988) have shown by deletion studies on ADR1 that SNF1 cannot act solely through the ADR1 phosphorylation site at residue 230; perhaps other phosphorylation sites are important, or the regulation by SNF1 may be unrelated to ADR1 dephosphorylation. CHERRY *et al.* (1989) suggest that SNF1 may be required for the phosphorylation of proteins which act in concert with ADR1 to express *ADH2*.

We have observed in assays on whole cell extracts of *snf1* strains that glycogen synthase and glycogen phosphorylase remain to a large degree in the phosphorylated forms when grown in rich medium (S. THOMPSON-JAEGER and J. FRANÇOIS, unpublished observations), even as glucose becomes depleted and the dephosphorylation of both enzymes normally occurs. Here again, one could consider a role for SNF1 in the dephosphorylation of possible target proteins of cAPK.

Whatever the function(s) of the cAPK and SNF1 protein kinase are, their possible joint role in the starvation response and glucose repression argues that genetic, physiological and biochemical studies of components of one pathway should take into account the other pathway as well.

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