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Molecular Analysis of the *SNF4* Gene of *Saccharomyces cerevisiae*: Evidence for Physical Association of the SNF4 Protein with the SNF1 Protein Kinase

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The *SNF4* gene is required for expression of glucose-repressible genes in response to glucose deprivation in *Saccharomyces cerevisiae*. Previous evidence suggested that *SNF4* is functionally related to *SNF1*, another essential gene in this global regulatory system that encodes a protein kinase. Increased *SNF1* gene dosage partially compensates for a mutation in *SNF4*, and the *SNF4* function is required for maximal SNF1 protein kinase activity in vitro. We have cloned *SNF4* and identified its 1.2-kilobase RNA, which is not regulated by glucose repression. A 36-kilodalton SNF4 protein is predicted from the nucleotide sequence. Disruption of the chromosomal *SNF4* locus revealed that the requirement for *SNF4* function is less stringent at low temperature (23°C). A bifunctional *SNF4-lacZ* gene fusion that includes almost the entire *SNF4* coding sequence was constructed. The fusion protein was shown by immunofluorescence microscopy to be distributed throughout the cell, with partial localization to the nucleus. The SNF4–β-galactosidase protein coimmunoprecipitated with the SNF1 protein kinase, thus providing evidence for the physical association of the two proteins.

The expression of many genes is controlled by glucose (carbon catabolite) repression in both procaryotic and eucaryotic cells. In *Saccharomyces cerevisiae*, an important global regulatory system controls expression of these genes in response to glucose availability. Previously, we have identified components of this regulatory system by isolating mutants that are defective in regulation of the *SUC2* (invertase) gene (4, 5, 28, 29). These studies identified *SNF1* as a gene that is essential for the expression of many glucose-repressible genes in response to glucose limitation. *SNF1* is the same as *CCR1* (11, 12; L. Neigeborn and M. Carlson, unpublished results) and *CAT1* (13, 14, 39, 45), which were also found to affect glucose repression. Molecular analysis showed that *SNF1* encodes a 72-kilodalton (kDa) protein-serine/threonine kinase (8), and the accompanying paper presents genetic evidence that the protein kinase activity is crucial for SNF1 function (9). The amounts of the *SNF1* RNA and protein are not regulated by glucose repression (7, 8), and it is not yet known whether the SNF1 kinase activity is regulated in vivo. One of the key problems in understanding the biological role of protein kinases is the identification of proteins that interact with the kinase in vivo, either as targets of the kinase or as modulators or regulators of kinase activity. In yeast cells, genetic analysis can offer important clues toward resolving such problems.

Genetic studies strongly suggest a close functional relationship between *SNF1* and *SNF4*, another gene identified in our mutant search. *SNF4* is also required for expression of many glucose-repressible genes in response to glucose deprivation. In the case of the *SUC2* gene, both *SNF1* and *SNF4* have been shown to act at the transcriptional level (3, 36). Mutations in *SNF4* cause a phenotype similar to that caused by mutations in *SNF1*: defects in utilization of carbon sources that require expression of glucose-repressible genes, such as sucrose, raffinose, galactose, maltose, glycerol, and ethanol, and failure of homozygous diploids to sporulate (4, 28). Genetic analysis of the interactions of *snf1* and *snf4* mutations with extragenic suppressors of *snf* mutations, *ssn6* and *ssn20* (*spt6*), indicated that *SNF1* and *SNF4* have related functions that are distinct from those of other *SNF* genes (28, 30). In the accompanying paper (9), we present additional genetic evidence for a functional interaction between these two genes: increased dosage of *SNF1* and mutations in *SNF1* can partially compensate for the deletion of *SNF4*. We also report biochemical evidence that the *SNF4* function is required for maximal SNF1 protein kinase activity in vitro; in immunoprecipitation kinase assays, the autophosphorylation activity of the SNF1 kinase derived from a *snf4* mutant is greatly reduced relative to that of the wild type. A modest effect of SNF4 on the level of the SNF1 protein is also noted.

These genetic and biochemical studies suggest that the *SNF4* product functions as a positive effector of the SNF1 protein kinase. SNF4 might affect SNF1 function directly, perhaps via protein-protein interaction, or indirectly, via a cascade of events. To examine further the possibility that SNF4 functions as a positive effector and also to examine the mechanism of this interaction, we have undertaken a molecular analysis of the *SNF4* gene and its product. In this paper, we report the cloning and sequence analysis of the *SNF4* gene. Disruption of the chromosomal locus led to the unusual finding that *snf4* deletion mutations cause a temperature-sensitive phenotype. We constructed a bifunctional *SNF4-lacZ* fusion for use in analysis of the gene product and its localization within the cell. We demonstrate coimmunoprecipitation of the SNF4–β-galactosidase fusion protein with the SNF1 protein, thereby providing evidence for the physical association of the SNF4 protein and the SNF1 protein kinase in vivo.

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TABLE 1. *S. cerevisiae* strains

Strain[a]	Genotype
MCY501	*MATα ade2-101 SUC2*
MCY638	*MATa his4-539 lys2-801 ura3-52 SUC2*
MCY888	*MATα snf4-Δ1 lys2-801 ura3-52 SUC2*
MCY1093	*MATa his4-539 lys2-801 ura3-52 SUC2*
MCY1094	*MATα ade2-101 ura3-52 SUC2*
MCY1209	*MATa snf4-319 his4-539 ura3-52 SUC2*
MCY1217	*MATα snf4-319 his4-539 ura3-52 SUC2*
MCY1218	*MATa snf4-319 his4-539 lys2-801 ura3-52 SUC2*
MCY1595	*MATα snf1-Δ3 his4-539 lys2-801 ura3-52 his3-Δ200 SUC2*
MCY1793	*MATa snf1-Δ3 snf4-Δ1 lys2-801 ura3-52 ade2-101 leu2::HIS3 SUC2*
MCY1830	*MATa snf4-2 ade2-101 ura3-52 SUC2*
MCY1853	*MATa snf4-2 ura3-52 his4-539 lys2-801 SUC2*

[a] All strains are isogenic or congenic to S288C.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used are listed in Table 1. Standard procedures were followed for crossing, sporulation, and tetrad analysis (41) and for transformation (18, 19). Media and methods for scoring carbon source utilization have been described previously (4).

Cloning of the *SNF4* gene. Plasmid DNA from a YEp24 library (3) was used to transform (18) strain MCY1209 (*snf4 ura3*) to uracil independence. Transformants displaying a Snf^+ phenotype were then selected by the ability to utilize raffinose under anaerobic conditions by methods described previously (6). To recover the complementing plasmid from each transformant, total genomic DNA was isolated and used to transform *Escherichia coli* HB101 to ampicillin resistance.

To show that the cloned DNA directs integration of a plasmid to the *SNF4* locus, a 2.7-kilobase-pair (kb) fragment from one of the complementing plasmids was subcloned into the integrating vector YIp5 (2). The plasmid was used to transform MCY638 (*SNF4 ura3*), and two Ura^+ transformants were crossed to MCY1217 (*snf4 ura3*). Tetrad analysis of the resulting diploids showed that the Ura^+ and Snf^+ phenotypes cosegregated (2:2) in eight tetrads from each diploid.

Subclones. pFE27-2 is a subclone of the 4.6-kb *Hin*dIII fragment in pCGS40 (15), which contains the 2μm plasmid origin and the *URA3* gene. Plasmids pCP8, pCP17, pCP3, pCP2, and pBG5 were derived from pFE27-2. pFE27-12 contains the 1.1-kb *Cla*I fragment subcloned into pLS1, a derivative of YIp5 lacking the *Eco*RI site (35). pRA5 and pRA20 are the 0.65- and 0.45-kb *Cla*I-*Eco*RI fragments, respectively, subcloned into pSP64 (24) between the *Acc*I and *Eco*RI sites.

Preparation and analysis of DNA and RNA. DNAs were prepared as described previously (35). Manipulation and analysis of DNA were carried out by standard methods (23). Preparation and gel transfer hybridization analysis of poly(A)-containing RNAs were carried out as described previously (37).

DNA sequence analysis. Intact and BAL 31-treated restriction fragments were cloned into M13mp18 and M13mp19 (31). The sequences of both strands were determined by the method of Sanger et al. (34), using the 17-nucleotide sequencing primer (Amersham Corp.).

Construction of the *snf4-Δ1* allele. A 0.15-kb deletion was constructed within pFE27-12 by treating *Eco*RI-digested DNA with BAL 31 nuclease and ligating. The resulting deletion plasmid was inserted into the yeast genome by integrative transformation of MCY638 to uracil independence; a haploid strain was used because the previous isolation of the *snf4-319* amber allele (28) suggested that the deletion would not be lethal. Integration at the *SNF4* locus would result in duplication of *SNF4* gene sequences, and excision of the plasmid could then occur by homologous recombination between the duplicated sequences, resulting in loss of the plasmid-borne *URA3* marker (38). Ura^- segregants were selected by plating transformants on medium containing 5-fluoro-orotic acid (1). A Ura^- Snf^- strain was identified, and blot hybridization analysis of genomic DNA from this strain confirmed the presence of the deletion at the *SNF4* locus. The *snf4-Δ1* strain was crossed to a wild-type *SNF4* strain (MCY501), and tetrad analysis of the diploid showed 2:2 segregations of the Snf^- phenotype in eight tetrads.

Although this deletion has not been sequenced, further evidence suggests that the correct reading frame is not restored 3′ to the deletion. Integration of a plasmid carrying the *SNF4(321)-lacZ* fusion (described below) into the genome of a *snf4-Δ1* mutant resulted in some transformants carrying the deletion fused to *lacZ*, as judged by gel transfer hybridization (42). These transformants did not express β-galactosidase, suggesting that the deletion shifted *lacZ* out of frame.

Construction of the *snf4-Δ2* allele. A 0.25-kb deletion was constructed in pFE27-12 as described for the *snf4-Δ1* deletion. The plasmid was used to transform the diploid MCY1093 × MCY1094, and Ura^- segregants were selected by using 5-fluoro-orotic acid (1). Tetrad analysis of a Ura^- diploid showed 2:2 segregations for a temperature-sensitive Snf phenotype. Four spore clones of one tetrad were analyzed by genomic blot hybridization to confirm that the deletion mutation was segregating 2:2.

Construction of *SNF4-lacZ* fusions. pFE27-2 DNA was cleaved at restriction sites 3′ to the *SNF4* gene, treated with BAL 31 nuclease, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and digested with *Hin*dIII. Fragments in the desired size range were purified by agarose gel electrophoresis and ligated to YEp356R DNA (27) that had been digested with *Hin*dIII and *Sma*I. *E. coli* transformants were screened for plasmids with inserts of the correct size, which were then used to transform MCY888 with selection for the plasmid *URA3* marker. The *SNF4-lacZ* fusion junctions for plasmids pSL184 and pSL321 were located by sequence analysis at codons 184 and 321, respectively, of *SNF4*. Both fusion proteins were of the expected size (135 and 150 kDa, respectively), as judged by immunoblot analysis. The gene fusions were also transferred to vector YEp366R (27), which carries *LEU2* as a selectable marker.

Enzyme assays. Glucose-repressed cultures were grown to mid-log phase in synthetic complete medium containing 2% glucose, and derepressed cultures were prepared by a shift to medium containing 0.05% glucose for 3 h. Selection for plasmids was maintained by the omission of uracil from the media. Secreted invertase was assayed (16) in whole cells as described previously (6). β-Galactosidase was assayed (26) in permeabilized cells (17).

Coimmunoprecipitation assay. The immunoprecipitation kinase assay was performed essentially as described in the accompanying paper (9) except that anti-SNF1 antiserum (2 μl) was used without affinity purification. After electrophoresis, the separated proteins were analyzed by immunoblotting (44) as described in the accompanying paper (9). The primary antibody was detected by using the ProtoBlot immu-

noblotting system (Promega Biotec). The rabbit anti-SNF1 antibody (8) was detected by using goat anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate. Mouse monoclonal anti-β-galactosidase antibody (Promega Biotec) was detected by using goat anti-mouse immunoglobulin G (heavy and light chain)-alkaline phosphatase conjugate. Before being reprobed with a different primary antibody, the blot was treated with 5% nonfat dry milk in buffer (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl, 0.05% Tween 20). The immunoblots were also exposed to film to detect ^{32}P-labeled products. Inclusion of 0.1 M NaCl, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate in the buffer used for immunoprecipitation and washing did not affect the coimmunoprecipitation of *SNF1* and *SNF4-lacZ* gene products, nor did use of affinity-purified (9) anti-SNF1 antibody.

Immunofluorescence microscopy. Cells were grown in minimal medium containing 2% glucose and lacking uracil and then were shifted to minimal medium containing 0.05% glucose (derepressing conditions) for 3 h. Cells were prepared as described previously (10) and stained with monoclonal antibody to β-galactosidase (Promega Biotec) and fluorescein isothiocyanate-conjugated F(ab')$_2$ fragment of sheep antibody to mouse immunoglobulin G (Sigma Chemical Co.). Both incubations with antibody were carried out overnight at 4°C. Cells were also stained with 4',6-diamidino-2-phenylindole. Cells were observed and photographed as described previously (10).

RESULTS

Cloning of the *SNF4* gene. Plasmids carrying the cloned *SNF4* gene were isolated from a library (3) by selecting for complementation of the *snf4* mutation in yeast cells (see Materials and Methods). Three plasmids that complement *snf4* for growth on raffinose and contain overlapping DNA segments were recovered. We subcloned a restriction fragment into an integrating vector and showed by genetic analysis that the cloned DNA directs integration of the plasmid to the chromosomal *SNF4* locus (see Materials and Methods). The *SNF4* gene was localized on the cloned DNA by testing subcloned segments for the ability to complement a *snf4* mutation. A 4.6-kb *Hin*dIII fragment common to the three *SNF4* clones was subcloned, and the resulting plasmid, pFE27-2, was found to complement *snf4* (Fig. 1 and Table 3). Derivatives of pFE27-2 were then constructed and tested for complementation. The results localized the *SNF4* gene to a 1.4-kb region (Fig. 1).

The *SNF4* RNA was identified by Northern (RNA) blot analysis of poly(A)-containing RNA (Fig. 2). A 1.2-kb RNA homologous to *SNF4* was detected by hybridization with a probe prepared from pFE27-12 (Fig. 1). This RNA was equally abundant in glucose-repressed and derepressed cells, indicating that its expression is not regulated by glucose repression. The direction of transcription (Fig. 1) was determined by using single-stranded probes prepared from pRA5 and pRA20 (Fig. 2).

Nucleotide sequence of *SNF4*. Figure 3 shows the nucleotide sequence of the 1.4-kb complementing region and the amino acid sequence of the predicted 36,398-Da *SNF4* gene product. The open reading frame of 322 amino acids could be encoded by the 1.2-kb *SNF4* RNA. No signature sequences for functional domains were identified, and a hydropathy plot (20) revealed no hydrophobic region of sufficient length to span a membrane. The amino acid sequence of the predicted protein was compared with the sequences in the Protein Identification Resource (release V19.0) of the National Biomedical Research Foundation by using the computer program FASTP (22) and also with the sequences in GenBank (release 58.0) translated in all six reading frames by using the program TFASTA (32). No significant homology was found. By examining the sequence of the yeast gene *CAT3* (40), however, we determined that *SNF4* is the same gene.

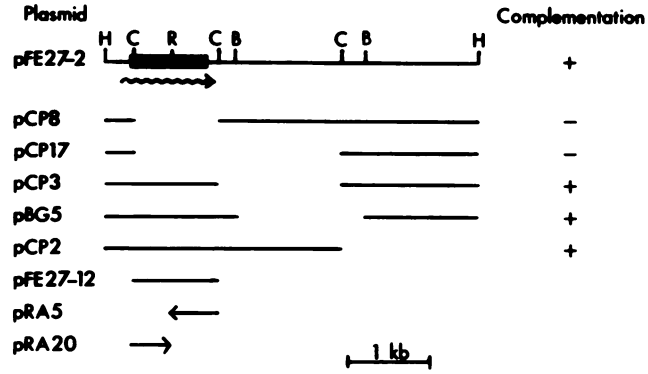

FIG. 1. Restriction maps of clones containing *SNF4* sequences. Plasmid constructions are described in Materials and Methods. Only the cloned yeast DNA is indicated. The ability of episomal plasmids to complement a *snf4* mutant (MCY1218) for growth on raffinose is indicated. Symbols: ⇝, approximate position and direction of transcription of the *SNF4* RNA; ■, 322-codon open reading frame; ⟶, ⟵, direction of transcription of the single-stranded probes prepared from plasmids pRA5 and pRA20. Restriction sites: B, *Bgl*II; C, *Cla*I; H, *Hin*dIII; R, *Eco*RI. An additional *Cla*I site at position 448 of the *SNF4* sequence (see Fig. 3) is methylated in bacteria.

Disruption of the *SNF4* chromosomal locus. The cloned DNA was next used to disrupt the *SNF4* chromosomal locus. A deletion of about 150 base pairs was constructed at the *Eco*RI site in pFE27-12. This deletion was introduced into the yeast genome by transplacement (38) to replace the wild-type *SNF4* sequence (see Materials and Methods). The newly created allele, designated *snf4-Δ1*, conferred a phenotype indistinguishable from that of a *snf4-319* amber mutant with respect to impaired growth on sucrose, raffinose, galactose, and glycerol and inability to derepress invertase (Table 2). The *snf4-Δ1* allele is recessive and fails to complement *snf4-319* for growth on raffinose. These results confirm that the cloned gene is indeed *SNF4*.

Temperature-dependent phenotype of a *snf4* null mutant. In the course of testing the phenotypes of *snf4* mutants, we noted a striking effect of temperature. At 30°C, the usual

TABLE 2. Temperature dependence of the *snf4* mutant phenotype

Relevant genotype	Invertase activity[a]				Growth phenotype[b]	
	23°C		30°C			
	Repressed	Derepressed	Repressed	Derepressed	23°C	30°C
snf4-Δ1	1	19	1	1	+	−
snf4-Δ2	1	9	1	1	+	−
SNF4	1	186	1	246	+++	+++

[a] Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values for *snf4-Δ1* are averages of assays of three different mutants. Values for the wild type are averages of assays of two strains.
[b] Growth on sucrose or raffinose.

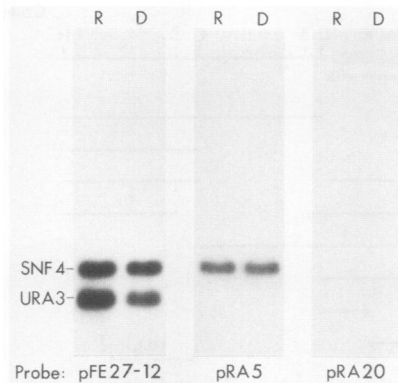

FIG. 2. Analysis of the *SNF4* RNA. Poly(A)-containing RNAs were prepared from glucose-repressed (R) and derepressed (D) cells (MCY501), separated by electrophoresis in an agarose gel containing formaldehyde (23), transferred to nitrocellulose (43), and hybridized to the indicated probes. The 1.2-kb *SNF4* RNA and the 0.9-kb *URA3* RNA were detected by hybridization with ^{32}P-labeled pFE27-12 DNA prepared by nick translation (33). Subsequent hybridization of the filters with a *SUC2* probe confirmed that the yeast cells were properly glucose repressed and derepressed; only the 1.8-kb *SUC2* mRNA was detected in the RNA from repressed cells, and both the 1.8- and 1.9-kb mRNAs were present in the derepressed sample. Single-stranded probes were prepared (25) from pRA5 and pRA20, and hybridization was performed as described previously (46). Subsequent hybridization of the filter that was probed with pRA20 confirmed the presence of intact RNA.

temperature for growth of yeast cells in the laboratory, the *snf4* mutants showed no anaerobic growth on raffinose and only weak growth on sucrose. At 23°C, however, both the *snf4-319* and *snf4-Δ1* mutants were capable of growing anaerobically on both sucrose and raffinose, although they grew more slowly than the wild type (Table 2). The same pattern was observed with respect to aerobic growth on glycerol. At 37°C, the mutants grew quite poorly on glucose and, when streaked on rich medium containing glucose, formed much smaller colonies than did the wild type. The levels of expression of secreted invertase activity at 23 and 30°C were consistent with the growth phenotypes (Table 2).

This finding that the *snf4* mutants show a temperature-sensitive phenotype raised the possibility that neither the amber mutation nor the *snf4-Δ1* deletion was truly a null mutation. We therefore constructed another, 0.25-kb deletion, designated *snf4-Δ2*, and introduced it into the genome of a diploid strain (see Materials and Methods). Tetrad analysis of the diploid revealed 2:2 segregation of a temperature-sensitive Snf phenotype indistinguishable from that of the two previous *snf4* alleles. We consider it extremely unlikely that all three alleles encode temperature-sensitive proteins. Thus, this phenotype presumably corresponds to the null phenotype. Apparently, the requirement for the SNF4 protein is less stringent when cells are grown at 23°C.

This temperature sensitivity distinguishes the *snf4* null mutant phenotype from the *snf1* phenotype, which was equally mutant at all temperatures. We tested *snf1 snf4* double mutants for anaerobic growth on sucrose and raffinose at 23°C. The phenotype of the double mutant was identical to that of the *snf1* mutant.

Increased *SNF4* gene dosage does not restore invertase derepression in a *snf1* mutant. Previous genetic evidence suggested that the *SNF4* gene and the *SNF1* protein kinase gene play functionally related roles in expression of *SUC2* (28, 30). We show in the accompanying paper (9) that increased dosage of the *SNF1* gene suppresses the defect in *SUC2* derepression caused by a mutation in *SNF4*. We therefore tested whether multiple copies of the *SNF4* gene would restore invertase expression in a *snf1* deletion mutant. The multicopy plasmid pFE27-2 did not restore invertase expression in a *snf1* mutant or affect its regulation in a wild-type strain (Table 3). This finding is consistent with biochemical evidence that SNF4 is a positive effector of the SNF1 protein kinase activity.

Coimmunoprecipitation of the SNF1 protein kinase and a SNF4–β-galactosidase fusion protein. The SNF4 protein

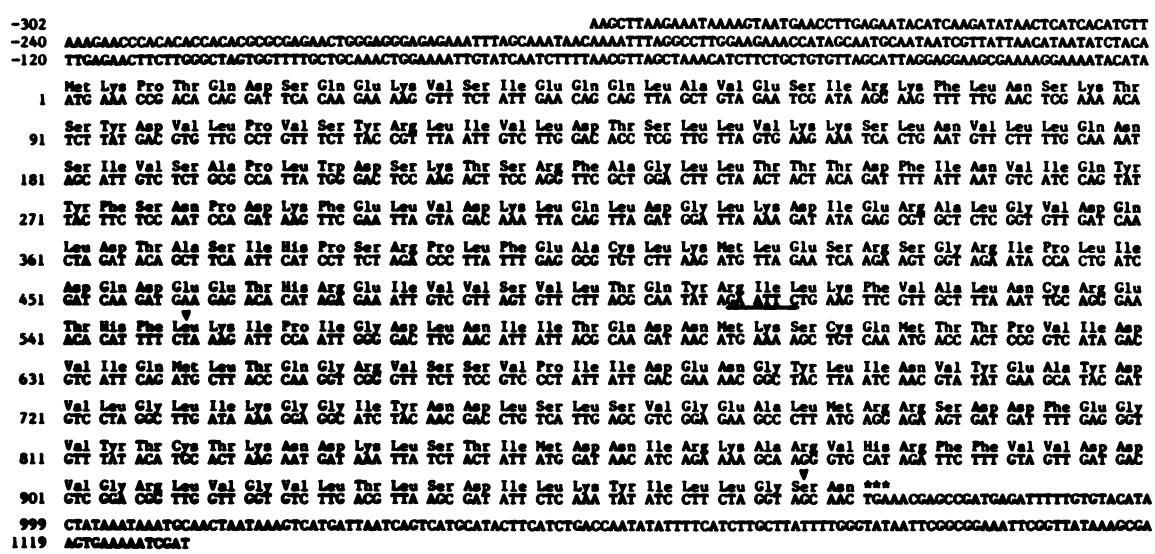

FIG. 3. Nucleotide sequence of the *SNF4* gene and deduced amino acid sequence. Nucleotides are numbered at the left, and amino acids are numbered at the right. Symbols: *, first in-frame termination codon; ▼, fusion sites of the *SNF4-lacZ* fusions at amino acids 184 and 321. The *Eco*RI site used for construction of deletions *snf4-Δ1* and *snf4-Δ2* is underlined. The sizes of these *SNF4-lacZ* fusion proteins and of two bacterial proteins produced from fusions between the N-terminal coding region of the *E. coli trpE* gene to codons 21 and 169 of *SNF4* confirmed the reading frame at those positions (data not shown).

TABLE 3. Effects of a multicopy *SNF4* plasmid on invertase expression

Relevant genotype[a]	Secreted invertase activity[b]	
	Repressed	Derepressed
snf4-Δ1 (pCGS40)	1	3
snf4-Δ1 (pFE27-2)	1	208
snf1-Δ3 (pCGS40)	1	1
snf1-Δ3 (pFE27-2)	<1	<1
Wild type (pCGS40)	<1	153
Wild type (pFE27-2)	<1	170

[a] Strains used were MCY888 (*snf4-Δ1*), MCY1595 (*snf1-Δ3*), and MCY1093 (*SNF1 SNF4*). pCGS40 (15) is the parent vector for pFE27-2.

[b] Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are averages of assays of three transformants.

could affect the activity of the SNF1 protein kinase by a direct mechanism, such as protein-protein interaction, or by an indirect process, perhaps involving a cascade of events. To examine the possibility that the SNF1 and SNF4 proteins are physically associated, we first tagged the *SNF4* gene product by constructing a fusion between codon 321 of the *SNF4* gene and the *E. coli lacZ* gene (see Materials and Methods). The plasmid-borne gene fusion, designated *SNF4(321)-lacZ*, complemented a *snf4* mutation for raffinose utilization. The fusion also produced β-galactosidase activity in yeast cells that was not regulated by glucose repression (30 to 75 U of activity normalized to cell density as described by Miller [26] in both glucose-repressed and derepressed cells). We also constructed a second fusion at codon 184 of the *SNF4* gene, *SNF4(184)-lacZ*, which failed to complement *snf4* but did express β-galactosidase activity. Both fusions produced proteins of the expected sizes (150 and 135 kDa). Consistent with the complementation data, SNF1 protein kinase activity was detected in immunoprecipitates derived from *snf4* mutants carrying the *SNF4(321)-lacZ* fusion but not the *SNF4(184)-lacZ* fusion (data not shown).

We could now test for coprecipitation of these proteins in immune complexes. Figure 4 shows the results of two experiments. In the first experiment (Fig. 4A and B), anti-SNF1 serum was used for immunoprecipitation; in the second experiment (Fig. 4C and D), anti-β-galactosidase antibody was used. In each experiment, the immunoprecipitated proteins were analyzed by immunoblotting, and the two antibody preparations were used sequentially to probe the blot.

In the first experiment, antiserum specific to the SNF1 protein (8) was used to precipitate the SNF1 protein from an extract of a strain carrying the *SNF4(321)-lacZ* fusion, and then the immunoprecipitate was examined for the presence of the *SNF4(321)-lacZ* fusion protein by immunoblot analysis, using antibody to β-galactosidase. The fusion protein coimmunoprecipitated with the SNF1 protein (Fig. 4A, lane a). Recovery of the fusion protein depended on precipitation of the SNF1 protein because no fusion protein was recovered in immune complexes prepared from a *snf1* deletion mutant (Fig. 4A, lane b). In the second experiment to test for coimmunoprecipitation, the fusion protein was first precipitated with anti-β-galactosidase antibody, and the immunoprecipitate was then subjected to immunoblot analysis, using anti-SNF1 antiserum. The SNF1 protein coimmunoprecipitated with the *SNF4(321)-lacZ* fusion product (Fig. 4C, lane e). Precipitation of the SNF1 protein was dependent on the presence of the *SNF4*–β-galactosidase fusion protein in the extract (Fig. 4C, lane h). Rough quantitation of the immunoblots suggested that a substantial fraction of the immunoprecipitable SNF1 protein coimmunoprecipitated with the fusion protein and vice versa.

To show that the coimmunoprecipitation of these proteins was dependent on the *SNF4*-encoded portion of the fusion protein rather than the β-galactosidase moiety, we tested for coimmunoprecipitation of the SNF1 protein and the *SNF4(184)-lacZ* fusion protein, which did not exhibit *SNF4* function. In the first test, the *SNF4(184)-lacZ* fusion protein

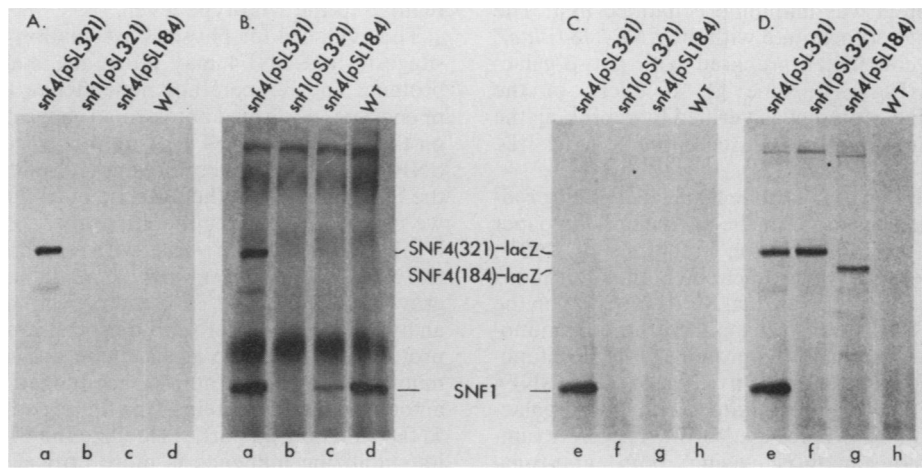

FIG. 4. Coimmunoprecipitation of the SNF1 protein and SNF4–β-galactosidase fusion proteins. (A) Proteins were immunoprecipitated with anti-SNF1 antiserum (8), separated by electrophoresis in 6% sodium dodecyl sulfate–polyacrylamide (21), and then examined by immunoblot analysis (44), using anti-β-galactosidase antibody as described in Materials and Methods. (B) The blot shown in panel A was reprobed with anti-SNF1 serum to detect the immunoprecipitated SNF1 protein. The extra bands correspond to rabbit serum proteins in the immunoprecipitate that reacted with the anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate used to detect the anti-SNF1 antibody. (C) Proteins were immunoprecipitated with anti-β-galactosidase antibody and subjected to immunoblot analysis, using anti-SNF1 antiserum. (D) The blot shown in panel C was reprobed with anti-β-galactosidase antibody to detect immunoprecipitated SNF4–β-galactosidase fusion proteins. The positions of the *SNF1*, *SNF4(321)-lacZ*, and *SNF4(184)-lacZ* gene products are indicated. Lanes contained proteins immunoprecipitated from extracts prepared from the following strains: a and e, MCY888 (pSL321); b and f, MCY1595 (pSL321); c and g, MCY888 (pSL184); d and h, MCY1093. Relevant genotypes are indicated above the lanes. Plasmids pSL321 and pSL184 carry the *SNF4(321)-lacZ* and *SNF4(184)-lacZ* fusions, respectively.

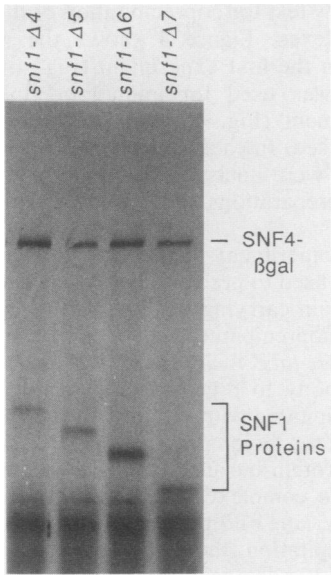

FIG. 5. Coimmunoprecipitation of C-terminally deleted SNF1 products with SNF4–β-galactosidase. Proteins were immunoprecipitated with anti-β-galactosidase antibody, separated by electrophoresis in 7.5% sodium dodecyl sulfate–polyacrylamide, and then examined by immunoblot analysis, using both anti-β-galactosidase antibody and anti-SNF1 serum as described in Materials and Methods. The products of the *snf1* deletion alleles (9) and the *SNF4(321)-lacZ* fusion are indicated. Proteins were immunoprecipitated from extracts prepared from strain MCY1793 (*snf1-Δ3 snf4-Δ1*) carrying the *SNF4(321)-lacZ* fusion on vector YEp366R and also one of the plasmids pCEsnf1-Δ4, pCEsnf1-Δ5, pCEsnf1-Δ6, or pCEsnf1-Δ7. Strains were grown with selection for maintenance of both plasmids.

did not coprecipitate with the immunoprecipitated SNF1 protein (Fig. 4A, lane c). Recovery of the SNF1 protein was verified by subsequent probing of the same immunoblot with anti-SNF1 antiserum (Fig. 4B, lane c). In the second test, the defective fusion protein was immunoprecipitated first. The SNF1 protein did not coprecipitate with the *SNF4(184)-lacZ* protein in immune complexes prepared with anti-β-galactosidase antibody (Fig. 4C, lane g); recovery of the *SNF4(184)-lacZ* fusion protein was verified by reprobing the immunoblot with anti-β-galactosidase antibody (Fig. 4D, lane g).

Coimmunoprecipitation of C-terminally deleted SNF1 products with SNF4–β-galactosidase. In the accompanying paper (9), we describe C-terminal deletion mutations of *SNF1*. Some evidence is presented that truncation of the C terminus reduces the dependence of the residual SNF1 function on the presence of SNF4. We therefore examined the coimmunoprecipitation of the truncated SNF1 proteins with the bifunctional SNF4–β-galactosidase protein (Fig. 5). The SNF4 fusion protein was immunoprecipitated with anti-β-galactosidase antibody, and the recovered proteins were examined by immunoblot analysis, using both anti-β-galactosidase antibody and anti-SNF1 serum. Each of the four truncated SNF1 proteins was detected. The proportion of the protein that was coimmunoprecipitated could not be estimated. This evidence suggests that these C-terminal deletions do not eliminate the association of the mutant SNF1 proteins with SNF4.

Effect of SNF4 on the level of SNF1 protein. In the accompanying paper (9), we noted that the amount of SNF1 protein recovered by immunoprecipitation from a *snf4* mutant was reduced a fewfold relative to the wild-type level. This effect,

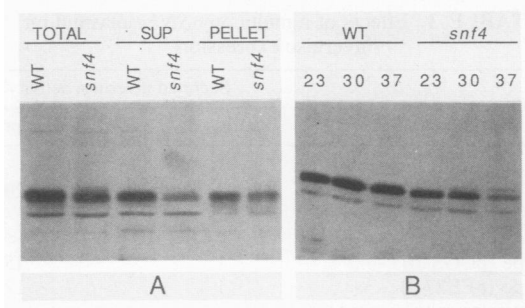

FIG. 6. Immunoblot analysis of SNF1 protein in *snf4* mutant and wild-type cells. Strains were grown to mid-log phase in rich medium containing glucose at the indicated temperature. Each sample represents the protein derived from 40 μg of total protein. Samples were analyzed by electrophoresis on a 7.5% sodium dodecyl sulfate–polyacrylamide gel (21) and immunoblotting (44). SNF1 protein was detected with anti-SNF1 serum. (A) Wild-type (MCY1093) and *snf4-Δ2* mutant (MCY1853) cultures grown at 30°C. Total protein (TOTAL) was prepared and fractionated by centrifugation at 13,000 × *g* as described for the immunoprecipitation kinase assay. The supernatant (SUP) was loaded on the gel directly, and the pellet (PELLET) was washed once with immunoprecipitation buffer, collected again by centrifugation at 13,000 × *g*, and suspended in sample buffer. (B) Wild-type (MCY1093) and *snf4-Δ2* mutant (MCY1830) strains grown at 23, 30, or 37°C, as indicated. Total protein was analyzed by immunoblotting as for panel A.

although modest, was reproducible. To confirm that SNF4 affects the amount of the SNF1 protein present in the cell rather than simply its immunoprecipitation, we compared the SNF1 protein present in wild-type and *snf4* mutant extracts by immunoblot analysis of total protein (Fig. 6A). The level of SNF1 protein was a fewfold reduced in the *snf4* mutant. In the same experiment, we also crudely fractionated the extracts by centrifugation at 13,000 × *g*, as is done to clear the lysate before immunoprecipitation. Immunoblot analysis showed that the amount of SNF1 protein present in the supernatant from the mutant extract was again reduced relative to the wild-type level.

The evidence for physical association of the two proteins suggests that SNF4 may affect the stability of the SNF1 protein. The temperature dependence of the *snf4* mutant phenotype, described above, also suggests an effect of SNF4 on the stability of SNF1. A more stringent requirement for SNF4 at higher temperatures could reflect thermolability of the SNF1 protein in the absence of SNF4. To test this idea, we examined whether the effect of a *snf4* mutation on the level of SNF1 protein varies with temperature. The amounts of SNF1 protein present in *snf4* mutant and wild-type strains grown at 23, 30, and 37°C were compared by immunoblot analysis (Fig. 6B). In the wild-type strain, the level of SNF1 protein was the same at all temperatures. In contrast, *snf4* mutant cells grown at 37°C contained a greatly reduced amount of SNF1 protein. This finding supports the idea that SNF4 functions in part to stabilize the SNF1 kinase and that its stabilizing influence is more critical at higher temperatures. However, stabilization of SNF1 does not appear to be the major function of SNF4. The amount of SNF1 protein in *snf4* cells grown at 23 and 30°C was not markedly different despite the marked difference in phenotype at these two temperatures. Moreover, *snf4* strains exhibited a distinctly mutant phenotype even at 23°C, a temperature at which the level of the SNF1 protein kinase was only a fewfold reduced relative to the wild-type level. Thus, although SNF4 appears to have a role in stabilizing the SNF1 protein, the level of SNF1 protein does not correlate well with growth pheno-

type, suggesting that stabilization is not the major function of SNF4. These results are consistent with the finding that SNF4 profoundly affects the in vitro kinase activity of SNF1 (9).

Localization of the SNF4 protein by immunofluorescence. Indirect immunofluorescence microscopy was used to localize the *SNF4(321)-lacZ* fusion product within the yeast cell. Cells carrying the gene fusion on a multicopy plasmid were stained with monoclonal antibody to β-galactosidase. In many cells, the fusion protein was partially localized to the nucleus, although a substantial portion remained in the cytoplasm (Fig. 7). The staining pattern appeared the same in cells prepared under conditions of glucose repression or derepression. We were unable to detect specific staining of cells carrying the fusion on a centromere-containing plasmid.

Previously, we showed by immunofluorescence microscopy that the SNF1 protein is distributed throughout the cell, with no apparent nuclear localization (8). Additional studies of both the SNF1 protein and a bifunctional SNF1–β-galactosidase protein confirmed this result. Thus, the SNF1 and SNF4–β-galactosidase proteins appear to some extent differently distributed in the cell. Interpretation of these results is subject to the reservation that we cannot be certain that the staining patterns accurately reflect the localization of the native proteins. It is possible that some fraction of the SNF1 or SNF4 protein is not physically associated with the other or that the stoichiometry of their association is different in the nucleus. Our coimmunoprecipitation experiments are not sufficiently quantitative to rule out the possibility that a fraction of one protein failed to coimmunoprecipitate with the other. Another possibility is that a fraction of one protein is not accessible for immunoprecipitation, and we note that a portion of the SNF1 protein was removed by clearing the lysate before immunoprecipitation (Fig. 6B).

DISCUSSION

Previous genetic evidence suggested a functional relationship between the *SNF4* gene and the *SNF1* protein kinase gene, both of which are required for expression of glucose-repressible genes in response to glucose deprivation. In the accompanying paper (9), we report that increased dosage of the *SNF1* gene compensates for a mutation in *SNF4*, restoring glucose-repressible invertase expression. We also report biochemical evidence that the *SNF4* gene product is required for maximal activity of the SNF1 protein kinase in vitro. These findings suggested that SNF4 acts as a positive effector of the SNF1 kinase. In this study, we have carried out a molecular analysis of the *SNF4* gene and its product. We report evidence that the SNF4 protein and the SNF1 protein kinase are physically associated. These findings provide a molecular basis for understanding both the effects of SNF4 on the function of the SNF1 kinase and also the genetic properties of *SNF1* and *SNF4*.

We cloned the *SNF4* gene by complementation of a *snf4* mutation. Sequence analysis indicated that *SNF4* encodes a 322-amino-acid protein with no unusual features. Sequence comparison revealed that *SNF4* is the same gene as *CAT3* (40), which was originally identified as a gene affecting expression of several glucose-repressible enzymes (14). The identity was not suspected earlier because the original *cat3* mutants showed no defect in derepression of invertase (14) or growth defects on sucrose, raffinose, and galactose (39). Disruption of *CAT3* was recently reported to cause such defects (40).

Disruption of *SNF4* led to the unexpected finding that the *snf4* null phenotype is temperature dependent. Two *snf4* deletion mutants and an amber mutant exhibited a temperature-sensitive phenotype: whereas at 30 and 37°C these strains showed a clear Snf$^-$ phenotype, at 23°C they showed only a partially mutant phenotype. These three alleles are unlikely all to encode temperature-sensitive SNF4 proteins, and we therefore favor the idea that the requirement for SNF4 is more stringent at higher temperatures. The finding that deletion of *SNF4* causes only a partial Snf$^-$ phenotype at 23°C provides further genetic evidence that SNF4 affects, but is not absolutely required for, SNF1 function.

Genetic evidence argued against a regulatory role for SNF4 in glucose repression because increased *SNF1* gene dosage restored glucose-repressible invertase expression in a *snf4* null mutant (9). The findings presented here are consistent with that view. We show that neither expression of the *SNF4* RNA nor production of a bifunctional SNF4–β-galactosidase fusion protein is regulated by glucose repression and that the distribution of the SNF4–β-galactosidase fusion protein in the cell does not change.

The most important finding with regard to the mechanism of SNF4 action is that a bifunctional SNF4–β-galactosidase fusion protein coimmunoprecipitates with the SNF1 protein kinase. This result indicates that the two proteins are physically associated. Presumably the same is true of the native SNF4 protein. Although these coimmunoprecipitation experiments were not rigorously quantitative, it appeared that a substantial fraction of each of the two proteins was associated with the other. The association represents relatively tight binding, as the complex survived immunoprecipitation under conditions of moderate salt and detergent concentrations.

We note the possibility that at least some fraction of the SNF1 or SNF4 protein may not be physically associated with the other. Immunofluorescent staining revealed somewhat different distributions for the SNF1 (8) and SNF4–β-galactosidase proteins in the cell, with the latter protein being partially localized to the nucleus. The evidence of Schuller and Entian is also consistent with partial nuclear localization: they isolated yeast nuclei and found that 70% of the β-galactosidase activity of a *CAT3-lacZ* fusion product was present in the nuclear fraction (40).

What is the function of the SNF4 protein? The simple model consistent with both genetic and biochemical evidence is that the SNF4 protein positively affects the function of the SNF1 protein kinase by virtue of its presence in a complex with the protein kinase. One possibility is that the SNF4 protein stimulates the SNF1 protein kinase via direct protein-protein interaction. Another possibility is that SNF4 inhibits or counteracts a negative effector of SNF1. The latter idea is consistent with evidence suggesting that deletion of the C terminus of *SNF1* results is a truncated protein with residual function that is independent of SNF4 (9). According to this model, the C-terminal domain would mediate interaction with the putative negative effector or would itself act as a negative effector. SNF4 would counteract the negative effector but need not interact directly with the C-terminal region. More complicated mechanisms of SNF4 function can also be imagined; for example, we cannot exclude the possibility that SNF4 acts to protect phosphorylated targets of the SNF1 kinase, also present in the complex, from attack by a phosphatase.

SNF4 has a modest fewfold effect on the level of the SNF1 protein. Studies in the accompanying paper suggested that SNF4 was likely to affect the stability of the SNF1 protein

FIG. 7. Localization of a bifunctional SNF4–β-galactosidase fusion protein by immunofluorescence. Two transformants of strain MCY888 carrying the *SNF4(321)-lacZ* fusion on pSL321 were grown under conditions of glucose repression (a to d) and derepression (e to h). Cells were stained with mouse monoclonal antibody to β-galactosidase and fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of sheep antibody to mouse immunoglobulin G, as described in Materials and Methods (a, c, e, and g). No staining was detected in cells lacking the fusion protein. No staining was detected when the anti-β-galactosidase antibody was omitted. Cells were also stained with 4′,6-diamidino-2-phenylindole to localize nuclei and mitochondria (b, d, f, and h). Bar, 5 μm.

rather than its expression: *snf4* and wild-type strains contained the same amount of a *SNF1-lacZ* fusion protein, which should be subject to the same transcriptional and translational controls as the SNF1 protein, and multicopy *SNF1* plasmids also produced about the same amount of SNF1 regardless of the *SNF4* genotype (9). The greatly reduced level of SNF1 protein observed in a *snf4* mutant at high temperature also suggests that SNF1 is less stable in the absence of SNF4. In view of the evidence for physical association of the two proteins, the idea that SNF4 affects the stability of SNF1 seems both plausible and attractive.

It is important to emphasize, however, that the modest effect of SNF4 on the level of the SNF1 protein kinase does not account for the *snf4* mutant phenotype. Elevation of SNF1 levels in a *snf4* mutant does not completely restore a wild-type phenotype (9), and *snf4* mutants exhibit a difference in phenotype at 23 and 30°C that is not reflected in the levels of SNF1 protein. We observed a substantial effect of SNF4 on the in vitro kinase activity of SNF1 (9), and we believe that the major role of SNF4 is to affect the function of the SNF1 protein kinase.

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