Mutational Analysis of the *Saccharomyces cerevisiae* SNF1 Protein Kinase and Evidence for Functional Interaction with the SNF4 Protein

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The *SNF1* gene of *Saccharomyces cerevisiae* encodes a protein-serine/threonine kinase that is required for derepression of gene expression in response to glucose limitation. We present evidence that the protein kinase activity is essential for *SNF1* function: substitution of Arg for Lys in the putative ATP-binding site results in a mutant phenotype. A polyhistidine tract near the N terminus was found to be dispensable. Deletion of the large region C terminal to the kinase domain only partially impaired *SNF1* function, causing expression of invertase to be somewhat reduced but still glucose repressible. The function of the *SNF4* gene, another component of the regulatory system, was required for maximal in vitro activity of the SNF1 protein kinase. Increased *SNF1* gene dosage partially alleviated the requirement for *SNF4*. C-terminal deletions of *SNF1* also reduced dependence on *SNF4*. Our findings suggest that SNF4 acts as a positive effector of the kinase but does not serve a regulatory function in signaling glucose availability.

In the yeast *Saccharomyces cerevisiae*, the expression of many genes is repressed when glucose is plentiful in the environment. The signal transduction pathway leading from the sensing of glucose to the regulation of gene expression has not been elucidated in detail for any eucaryote. Previously, we have identified components of the regulatory circuit in *Saccharomyces cerevisiae* by isolating mutations in *trans*-acting genes required for repression or derepression of the *SUC2* gene for invertase (4, 5, 22, 23). Mutations in six genes, designated *SNF1* through *SNF6* (sucrose nonfermenting), impaired growth on sucrose and raffinose, which are substrates for invertase. The functions of two of the *SNF* gene products have been determined. The *SNF3* gene encodes a high-affinity glucose transporter (10). The *SNF1* gene encodes a 72-kilodalton (kDa) protein-serine/threonine kinase; nucleotide sequence analysis revealed homology to other protein kinases, and biochemical characterization of the SNF1 protein demonstrated autophosphorylation activity in vitro (8). *SNF1* is the same gene as *CCR1* (11, 12; L. Neigeborn and M. Carlson, unpublished results) and *CAT1* (13, 14, 33, 37), which were identified in other searches for genes affecting glucose repression. Evidence is presented in this paper to support the simple but therefore unproven conclusion that the protein kinase activity of SNF1 is essential for the function of SNF1 in vivo.

The finding that *SNF1* encodes a protein kinase indicates that protein phosphorylation plays a critical role in regulation by glucose repression in yeast cells. It is important to note, however, that although the SNF1 kinase is clearly essential for release from glucose repression, the amount and gross cellular localization of SNF1 do not change in response to glucose availability (8), and there is as yet no evidence that the activity of this kinase is regulated. We have here carried out a mutational analysis of *SNF1* in an attempt to address this issue.

The identification of SNF1 as a protein kinase provides a

cornerstone for the biochemical analysis of this regulatory system. Other genes identified by genetic studies would be predicted to encode regulators, modulators, or targets of this protein kinase. One of the genes identified in our mutant search, *SNF4*, is a likely candidate to encode such a modulator or target of the SNF1 protein kinase. Genetic studies suggested a close functional relationship between the *SNF1* and *SNF4* genes. Both are required for expression of various glucose-repressible genes in response to glucose deprivation, and mutations in the two genes cause similar phenotypes; *snf1* or *snf4* mutants are unable to utilize sucrose, raffinose, galactose, maltose, or nonfermentable carbon sources, and homozygous diploids are unable to sporulate (4, 22). Both genes affect *SUC2* expression at the transcriptional level, and their effects are mediated by the *SUC2* upstream regulatory region (3, 31). Most significantly, genetic analysis showed that *snf1* and *snf4* show different patterns of suppression by *ssn6* and *ssn20* (*spt6*) mutations than do other *snf* mutations, indicating that *SNF1* and *SNF4* have related functions that are distinct from those of other *SNF* genes (22, 24). The genetic behavior of *snf1* and *snf4* mutations is consistent with several possible functions for the *SNF4* gene product. One simple model casts the SNF4 protein as a target, either direct or indirect, of the SNF1 protein kinase. Another model assigns the SNF4 protein a positive function in activating or regulating the SNF1 protein kinase. We have here used both genetic and biochemical approaches to explore the interaction of the *SNF1* and *SNF4* gene products. Molecular characterization of *SNF4* is reported in the accompanying paper (9).

In this paper, we first report a mutational analysis of the *SNF1* gene to identify functional domains of the protein. The results support the conclusion that the protein kinase activity is essential for SNF1 function and suggest that the large C-terminal region is not a regulatory domain that conveys signals regarding glucose availability. We present additional genetic evidence for a functional interaction between the SNF1 and SNF4 proteins: increased dosage of the *SNF1* gene partially compensates for a deletion in *SNF4*, restoring

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TABLE 1. *S. cerevisiae* strains

Strain ^a	Genotype
MCY888	<i>MATα snf4-Δ1 lys2-801 ura3-52 SUC2</i>
MCY1090	<i>MATα snf4-319 trp1-Δ1 SUC2</i>
MCY1093	<i>MATα his4-539 lys2-801 ura3-52 SUC2</i>
MCY1094	<i>MATα ade2-101 ura3-52 SUC2</i>
MCY1378	<i>MATα lys2-801 ura3-52 his3-Δ200 ade2-101 leu2::HIS3 SUC2</i>
MCY1595	<i>MATα snf1-Δ3 his4-539 lys2-801 ura3-52 his3-Δ200 SUC2</i>
MCY1720	<i>MATα snf4-Δ1 lys2-801 ura3-52 ade2-101 leu2::HIS3 (his3?) SUC2</i>
MCY1793	<i>MATα snf1-Δ3 snf4-Δ1 ade2-101 lys2-801 ura3-52 leu2::HIS3 SUC2</i>
MCY1828	<i>MATα snf1-Δ10 ade2-101 ura3-52 SUC2</i>
MCY1843	<i>MATα snf1-Δ10 ura3-52 his4-539 lys2-801 SUC2</i>
MCY1853	<i>MATα snf4-Δ2 ura3-52 his4-539 lys2-801 SUC2</i>
MCY1860	<i>MATα snf1-K84R ura3-52 lys2-801 SUC2</i>
YPH54 ^b	<i>MATα ade2-101 lys2-801 ura3-52 his3-Δ200 trp1-Δ1 SUC2</i>

^a All strains are isogenic or congeneric to S288C.

^b Obtained from P. Hieter.

regulated expression of the *SUC2* gene. We examine effects of C-terminal deletions of SNF1 on the requirement for SNF4 function. Finally, we report biochemical evidence that the SNF4 protein is required for maximal SNF1 protein kinase activity in vitro. These genetic and biochemical studies suggest that SNF4 functions as a positive effector of the SNF1 protein kinase but does not regulate kinase activity in response to glucose.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used are listed in Table 1. Standard genetic methods were followed (18, 34). Growth on carbon sources was scored as previously described (4) except that strains harboring plas-

mids were grown on minimal medium (SD [34]) lacking uracil and then tested for anaerobic growth on rich medium containing the specified carbon source.

Preparation and manipulation of DNA. DNAs were prepared as described previously (30). Manipulation and analysis of DNA were carried out by standard methods (21).

Construction of the *snf1-K84R* mutation. The 3.2-kilobase-pair (kb) *EcoRI-BamHI* fragment from pCN9 (7; Fig. 1) was cloned in M13mp19 (25). Oligonucleotide-directed in vitro mutagenesis was carried out by using the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit and an oligonucleotide with the sequence 5'-GCTCTAAGAATCATTAAATAAG-3' (purchased from Research Genetics). This oligonucleotide changes codon 84 from AAA (lysine) to AGA (arginine). The *EcoRI-BamHI* fragments from three independent recombinant phages were subcloned into pAC120 (gift of Ezra Abrams), which was derived from YCp50 (28) by filling in the *XhoI* site with DNA polymerase I Klenow fragment and religating. A control plasmid, pCE108, was also constructed by cloning the wild-type *EcoRI-BamHI* fragment into pAC120. The *XhoI-BamHI* fragment from one of the mutant plasmids was subcloned in M13mp18 (25), and the region containing the mutation was sequenced (29), using as a primer the oligonucleotide 5'-GAGGCTGTTTCAATAATCAT-3' (Research Genetics), which starts at position -82. The sequenced *XhoI-BamHI* fragment was also cloned between the *SalI* and *BamHI* sites of pCGS40 (15) and YIp5 (2), yielding pCE110 and pCN110, respectively. To introduce the mutation into the yeast genome (32), a wild-type diploid generated by crossing MCY1093 with MCY1094 was transformed to uracil prototrophy with pCN110 DNA that had been digested with *BglII* to target integration (26) to the *SNF1* locus. Ura⁻ segregants of four independent transformants were selected by using 5-fluoro-orotic acid (1), and several Ura⁻ diploids were sporulated and dissected. Two diploids derived from independent transformants showed 2+:2- segregations for anaerobic growth on raffinose and sucrose.

Construction of the *snf1-Δ10* deletion. A multistep procedure was followed. The 4.5-kb *HindIII-BamHI* fragment

FIG. 1. Maps of the *SNF1* locus and plasmids. Symbols: —, DNA from the *SNF1* locus; □, *SNF1* coding region; ■, polyhistidine tract; [H], protein kinase domain; *, position of Lys-84 in the putative ATP-binding site. For each plasmid, the bar represents DNA from the *SNF1* locus. Plasmids are described in Materials and Methods except for pCE9 and pCN9 (7), which are derived from the vectors YEp24 and YIp5 (2), respectively. Restriction sites: B, *BamHI*; Bg, *BglII*; H, *HindIII*; Hp, *HpaI*; Hc, *HincII*; N, *NcoI*; P, *PstI*; Pv, *PvuII*; R, *EcoRI*; X, *XhoI*.

from pCN9 (Fig. 1) was subcloned into pUC18 at the corresponding sites, yielding plasmid pCC100. pCC100 was digested with *HincII*, and the vector-containing portion was gel purified and allowed to recircularize to give pCC104. The 1.4-kb *EcoRI*-*Bam*HI fragment from pCC104 was cloned into YIp5, yielding pCN105. The 1-kb *Bam*HI fragment from pCE5 (6) was then inserted into pCN105 so as to reconstruct the sequence 3' to *SNF1*. The resulting plasmid, pCN106 (Fig. 1), carries a deletion of nucleotides -3 to +1884 of *SNF1*; only the last five codons of the gene remain.

The mutation was introduced into the genome of a wild-type diploid (MCY1093 $\times$ MCY1094) by transformation with *XhoI*-digested pCN106 DNA, followed by selection on 5-fluoro-orotic acid (1) for loss of the plasmid *URA3* marker, as described above. By tetrad analysis of *Ura*⁻ diploids, we identified two diploids derived from independent transformants that showed 2+:2- segregations for the *Snf* phenotype. Southern blot analysis (35) of DNA prepared from the four spore clones from one tetrad confirmed the presence of a 1.9-kb deletion in the two *Snf*⁻ spore clones.

Construction of *SNF1*- Δ H. To delete the 12 histidine residues at positions 18 to 29, oligonucleotide-directed mutagenesis was carried out as described above with the oligonucleotide 5'-GCCAATGCAAATTCTAGCCACGGTCATGGCGGAAGC-3' (purchased from Research Genetics); 36 nucleotides are deleted between the underlined C residues. Twelve independent bacteriophages were analyzed by restriction mapping and contained the deletion. The *EcoRI*-*Bam*HI fragments from three of the phages were subcloned into pAC120. The *XhoI*-*Bam*HI fragment from one of the resulting plasmids, designated pSNF1- Δ H, was subcloned into M13mp18 and sequenced (29), using the primer used to sequence *snf1*-K84R.

Construction of C-terminal deletions. pCN9 DNA was digested with *HpaI*, treated with BAL 31 endonuclease (New England BioLabs), and treated with DNA polymerase I (Klenow fragment). This DNA was digested with *EcoRI*, and the truncated fragments were gel purified and ligated into the vector portion of pCN9 digested with *EcoRI* and *HpaI*. The recovered plasmids were digested with *Bgl*II and *Bam*HI to determine the size of each deletion. The *XhoI*-*Bam*HI fragment containing each deletion was subcloned between the *Sal*I and *Bam*HI sites of pCGS40 (15). Plasmids pCEsnf1- Δ 4, pCEsnf1- Δ 5, pCEsnf1- Δ 6, and pCEsnf1- Δ 7 carry deletions of approximately 75, 175, 325, and 475 base pairs, respectively (Fig. 1). The reading frames after the deletion endpoints were not determined. The original reading frame includes 5 codons after the *HpaI* site; the other two frames include 9 and 22 codons.

pCEsnf1- Δ 8 was constructed by a two-step procedure. The 0.4-kb *Bgl*II-*HincII* fragment of *SNF1* was cloned into pCN9 digested with *Bgl*II and *HpaI* to construct a plasmid, pCN109, with a deletion of nucleotides 925 to 1884 (codons 309 to 628) followed by the C-terminal sequence in the correct reading frame. The *EcoRI* to *Bam*HI fragment containing the deletion was then subcloned into pCGS40 to create pCEsnf1- Δ 8 (Fig. 1).

Isolation of *snf1* mutations that suppress *snf4*. pCE101 was constructed by subcloning the 2.8-kb *XhoI*-*Bam*HI fragment from pCN9 into the *Sal*I and *Bam*HI sites of a derivative of YCp50 in which the *EcoRI* and *XhoI* sites were destroyed by filling in with Klenow fragment. pCE101 DNA (10 μ g) was incubated with hydroxylamine for 20 h at 37°C (27) and then used to transform MCY888 to uracil prototrophy. Approximately 200 colonies were replica plated to score anaerobic growth on raffinose at 30°C. Three raffinose-fermenting

colonies were identified. Plasmid DNA was recovered by passage through *Escherichia coli* and reintroduced into MCY888 to retest the phenotype. To localize the mutations, three pairs of fragments were swapped between the mutant plasmid and the wild-type pCE101: the *Bgl*II fragments from the *Bgl*II site at 528 in the *SNF1* gene to the site in the vector, the *NcoI* fragments from the site at 1064 in *SNF1* to the site in the vector, and the fragments from the *PvuII* site at 1463 in *SNF1* to the *Bam*HI site at 2192. All fragments were gel purified, and the large fragment from the mutant plasmid was recombined with the small fragment from the wild-type plasmid and vice versa. Two recombinants from each of the six constructions were used to transform MCY888, and transformants were tested for anaerobic growth on sucrose and raffinose. For two of the mutant plasmids, the suppressor mutation was localized 5' to the *Bgl*II site. For the third plasmid, which had only a very weak suppressor phenotype, the mutation could not be clearly localized and was not studied further.

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).

Immunoprecipitation and kinase assay. Cultures were grown to mid-log phase in medium containing 2% glucose (glucose-repressing conditions). Strains carrying plasmids were grown in synthetic complete medium with selection for the plasmid; other strains were grown in rich medium. Crude protein lysates were prepared as described previously (8). Protein concentrations were determined with the Bio-Rad protein assay kit and were adjusted to 5 to 10 mg/ml. Samples (250 μ g) of protein were stored at -70°C. A sample was thawed on ice and diluted to 250 μ l with immunoprecipitation buffer (50 mM Tris hydrochloride [pH 7.5], 1% Triton X-100, 0.5 mg of ovalbumin per ml). The lysate was then clarified by centrifugation at 13,000 $\times$ g for 15 min at 4°C, and 100 μ l was diluted into 0.5 ml of cold immunoprecipitation buffer. Either affinity-purified anti-SNF1 antibody (4 μ l) (8) or mouse anti- β -galactosidase monoclonal antibody (0.5 μ l) (Promega Biotec) was added to the lysate; these amounts were empirically determined to provide an excess of antibody. After incubation for 2 to 4 h at 4°C, *Staphylococcus aureus* cells (Pansorbin; Calbiochem) were added, and the mixture was incubated for 1 h at 4°C. The adsorbed immune complexes were collected by centrifugation in a microfuge, washed twice with immunoprecipitation buffer, once with immunoprecipitation buffer lacking ovalbumin, and once with kinase buffer (50 mM Tris hydrochloride [pH 7.5], 1% Triton X-100, 10 mM MgCl₂) and resuspended in 50 μ l of kinase buffer. The kinase reaction was initiated by the addition of [γ -³²P]ATP (3,000 Ci/mmol; Dupont, NEN Research Products) to a final concentration of 0.15 μ M. The reaction mixture was incubated for 20 min at 4°C and terminated by the addition of 50 μ l of 2 $\times$ Laemmli buffer (19). The sample was boiled for 3 min, the *S. aureus* cells were removed by centrifugation, and a 40- μ l sample was loaded on a 6 or 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel (19). After electrophoresis, the gel was stained, washed extensively in destaining solution containing 10 mM sodium pyrophosphate, dried, and exposed to film at -70°C with an intensifying screen.

Affinity purification of antibody. The immunoglobulin G fraction of polyclonal rabbit anti-SNF1 antiserum (8) was

purified by using the Beckman rProtein A purification kit. TrpE-SNF1 fusion protein was partially purified from bacteria as described previously (8), and protein from a 25-ml culture was separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5% gel and electroblotted to nitrocellulose. The position of the 60-kDa fusion protein was determined by immunoblot analysis of small test strips, and the region of the filter containing the protein was excised and blocked in Tris-buffered saline (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl) containing 5% nonfat dry milk for 30 min at room temperature. Specific antibody was affinity purified by the method of Lillie and Brown (20), with the following changes: Tris-buffered saline was substituted for phosphate-buffered saline, antibody was eluted with 50 mM glycine [pH 2.3]–150 mM NaCl, and eluted antibody was neutralized with 1 M Tris hydrochloride (pH 7.5). Antibody was concentrated with a Centricon 30 microconcentrator (Amicon Corp.), and the buffer was changed to Tris-buffered saline containing 0.02% sodium azide.

Immunoblot analysis. Proteins were separated by electrophoresis and electroblotted to nitrocellulose as described previously (36) except that methanol was omitted. SNF1 products were detected by using polyclonal rabbit anti-SNF1 serum as described previously (8) except that 5% nonfat dry milk was added to buffers. The primary antibody was detected by using goat anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate and the ProtoBlot immunoblotting system (Promega Biotec).

Western blot filter assay for autophosphorylation. The Western blot (immunoblot) assay was carried out as described previously (8), with minor modifications. Proteins (50 μ g) were separated by SDS-PAGE in 7.5% polyacrylamide and electroblotted to nitrocellulose as described previously (36) except that methanol was omitted. The filter was incubated in 30 mM sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES; pH 7.5) containing 5% nonfat dry milk (Carnation) for 30 min at room temperature. Proteins bound to the filter were denatured by incubating the filter in 7 M guanidine hydrochloride–50 mM Tris hydrochloride (pH 8.3)–50 mM dithiothreitol–2 mM EDTA–0.25% nonfat dry milk for 1 h at room temperature. Proteins were allowed to renature in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–2 mM dithiothreitol–2 mM EDTA–0.1% Nonidet P-40–0.25% nonfat dry milk overnight at 4°C. The filter was rinsed several times with 30 mM HEPES (pH 7.5) at room temperature and transferred to a plastic bag. The kinase reaction was carried out by incubating in 0.03 μ M [γ - 32 P]ATP (3,000 Ci/mmol; Dupont, NEN)–0.1 μ M ATP–30 mM HEPES (pH 7.5)–10 mM MgCl_2 –2 mM MnCl_2 for 30 min at room temperature. After extensive washing with 30 mM HEPES (pH 7.5) at room temperature, the filter was dried in air and exposed to film.

RESULTS

Mutation of the ATP-binding site in the SNF1 protein kinase domain. We previously showed that the *SNF1* gene encodes a protein kinase, but we had no rigorous evidence that the protein kinase activity is essential for *SNF1* function. Studies of other protein kinases have shown that mutation of a conserved lysine residue in the ATP-binding site abolishes or greatly reduces protein kinase activity (17). We therefore used oligonucleotide-directed mutagenesis to change the lysine residue (Lys-84) in the predicted ATP-binding site of the SNF1 protein to an arginine residue (Fig. 2). Three plasmids carrying the substitution mutation, designated *snf1*-

FIG. 2. Amino acid sequence encoded by mutant *snf1* alleles. In panel A, the box indicates the histidine codons at positions 18 to 29 that are deleted from *SNF1-ΔH*. (B) Lys-to-Arg substitution mutation in the ATP-binding site in allele *snf1-K84R*.

K84R, on a centromere-containing vector were constructed independently (see Materials and Methods) and tested for complementation of *snf1*. The mutant plasmids were used to transform strain MCY1595, carrying the *snf1-Δ3* deletion (8), to uracil prototrophy; the analogous wild-type plasmid pCE108 and the parent vector pAC120 served as controls. Four transformants carrying each plasmid were tested for growth on sucrose, raffinose, and glycerol. All three mutant plasmids failed to complement the *snf1* defect. The *snf1-K84R* allele was also transferred to a multicopy plasmid, pCE110 (see Materials and Methods); even in multiple copies, this allele failed to remedy the growth defects of a *snf1* mutant.

The *snf1-K84R* mutation was introduced into the genome of a diploid strain so as to replace the wild-type sequence as described in Materials and Methods. The mutation was recessive. Tetrad analysis of the heterozygous diploid showed 2+:2- segregation for the Snf phenotype. In a growth assay on 2% glucose, haploid segregants were generally unhealthy, although they appeared to grow slightly better than *snf1* deletion mutants. A mutant strain was assayed for secreted invertase and failed to derepress the enzyme in response to glucose limitation (data not shown).

Thus, the *snf1-K84R* allele appears to have no *SNF1* function. In experiments described below (see Fig. 3), the mutant allele was shown by immunoblot analysis to encode a protein of the same size and abundance as the wild-type SNF1 protein, as would be predicted from the conservative nature of the mutation. These findings support the notion that protein kinase activity is crucial for SNF1 function.

Deletion of the entire *SNF1* gene. The previously described *snf1-Δ3* mutation (8) is a deletion of approximately 650 base pairs in the kinase domain. To determine whether the SNF1 protein has additional functions beyond those for which the kinase domain is responsible, we constructed a new deletion that removes virtually the entire coding region of *SNF1* in plasmid pCN106 (Fig. 1). This plasmid was used to construct a new allele, *snf1-Δ10*, by replacing the wild-type genomic copy in a diploid strain with the deletion (see Materials and Methods). Tetrad analysis showed 2+:2− segregation for the Snf phenotype. Haploid segregants were indistinguishable from *snf1-Δ3* strains with respect to growth phenotypes on various carbon sources and minimal medium and also expression of secreted invertase activity (Table 2). Therefore, deletion of the entire *SNF1* gene is not lethal and confers no apparent additional phenotype beyond that caused by loss of kinase function.

Polyhistidine sequence near the N terminus of SNF1 is dispensable. An interesting feature of the SNF1 protein sequence is a block of 13 consecutive histidine residues at positions 18 to 30, followed by another histidine at position

TABLE 2. Restoration by multicopy *SNF1* plasmid pCE9 of invertase expression in a *snf4* mutant

Expt no.	Relevant genotype ^a	Plasmid	Invertase activity ^b	
			Repressed	Derepressed
1	<i>snf4</i> -Δ1	YE24	1	2
	<i>snf4</i> -Δ1	pCE9	2	41
	<i>snf1</i> -Δ3	YE24	1	2
	<i>snf1</i> -Δ3	pCE9	1	121
	Wild type	YE24	<1	175
	Wild type	pCE9	<1	202
2	<i>snf4</i> -Δ2	pCE9	<1	16
	<i>snf1</i> -Δ10	pCE9	1	168

^a Strains used were MCY888 (*snf4*-Δ1), MCY1595 (*snf1*-Δ3), MCY1093 (wild type), MCY1843 (*snf1*-Δ10), and MCY1853 (*snf4*-Δ2). YE24 (2) is the parent vector for pCE9.

^b Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are averages of assays of three transformants for experiment 1 and two transformants for experiment 2.

32. To assess the functional significance of this sequence, we used oligonucleotide-directed mutagenesis to delete 12 of the 13 histidines (Fig. 2). Three low-copy-number plasmids carrying the mutant allele, designated *SNF1*-ΔH, were constructed independently (see Materials and Methods) and tested for the ability to complement *snf1*. The three plasmids were used to transform MCY1595 (*snf1*-Δ3) and were found to complement for growth on sucrose and raffinose as well as did pCE108, the control plasmid carrying the wild-type *SNF1* gene. One of these transformants was assayed for secreted invertase and showed normal, glucose-repressible expression of the enzyme identical to that of a control transformant carrying pCE108 (data not shown). Immunoblot analysis confirmed that the *SNF1*-ΔH allele encodes a protein appropriately smaller than the wild-type SNF1 (Fig. 3). Thus, this polyhistidine tract is dispensable for SNF1 function.

Deletion of SNF1 sequences C terminal to the kinase domain. The protein kinase domain of SNF1 is located in the N-terminal part of the protein (residues 59 to 306) and is followed by 327 amino acids of C-terminal sequence. The phenotype of the *snf1*-Δ10 allele showed that deletion of the entire gene causes no apparent additional phenotype beyond that of a mutation in the kinase domain but left open the possibility that the C-terminal region has a function related to that of the kinase domain, perhaps as a regulatory domain. To assess the role of this C-terminal region of SNF1 function, a series of C-terminal deletions was constructed on a multicopy vector, and their phenotypic effects were examined. Plasmids pCEsnf1-Δ4, pCEsnf1-Δ5, pCEsnf1-Δ6, pCEsnf1-Δ7, and pCEsnf1-Δ8 carry deletions of *SNF1* coding sequence with a common endpoint at the *HpaI* site at position 1882 (codon 628) (see Materials and Methods and Fig. 1). pCEsnf1-Δ8 encodes a protein that is truncated shortly after the protein kinase domain, at codon 309.

To test these deleted plasmids for the ability to complement the null mutation *snf1*-Δ10, each plasmid was used to transform MCY1843 to uracil prototrophy. The wild-type *SNF1* plasmid pCE9 served as a control. Three transformants carrying each plasmid were tested for anaerobic growth on glucose, sucrose, raffinose, or galactose and for aerobic growth on glycerol at 23, 30, and 37°C. pCEsnf1-Δ4 and pCEsnf1-Δ5 failed to complement, and pCEsnf1-Δ6 and pCEsnf1-Δ7 complemented weakly. None of these plasmids caused a *Snf*⁻ phenotype in a wild-type (*SNF1*) strain. Immunoblot analysis showed that all of these plasmids

produced proteins of the expected sizes (Fig. 4). pCEsnf1-Δ8 complemented quite well for all phenotypes, although cells carrying pCEsnf1-Δ8 grew less well than those carrying pCE9. We cannot compare the amount of SNF1 protein produced from the two plasmids because we were unable to detect the protein encoded by pCEsnf1-Δ8 with anti-SNF1 serum from either of two rabbits; the mutant protein retains only 131 residues of the 216-amino-acid sequence used to generate antibody. Transformants carrying pCEsnf1-Δ8 were assayed for secreted invertase activity. Derepressed cells produced 25 to 50% of the wild-type level of activity, and glucose-repressed cells did not produce secreted invertase (Table 3). Thus, deletion of the entire C-terminal region of SNF1 partially impaired derepression of invertase, but glucose repression of expression occurred normally. These results are consistent with other evidence that the protein kinase domain is crucial for SNF1 function and provide evidence that the C terminus is not a regulatory domain that conveys signals regarding glucose availability.

Increased *SNF1* gene dosage compensates for a mutation in *SNF4*. Previous genetic evidence suggested that the *SNF1* and *SNF4* genes play functionally related roles in expression of *SUC2* (22, 24). We therefore tested whether increased dosage of the *SNF1* gene could suppress the defect in *SUC2* derepression caused by a mutation in *SNF4*. A strain harboring the *snf4*-319 amber mutation was transformed with pCE9, a multicopy plasmid carrying the *SNF1* gene (7). The transformants showed improved growth on sucrose. The experiment was then repeated with strains MCY888 and MCY1853, which carry *snf4* deletion mutations that are described in the accompanying paper (9). The presence of multiple copies of *SNF1* restored substantial invertase derepression in these strains (Table 2). Moreover, this expression was glucose repressible, implying that in cells with increased *SNF1* gene dosage, appropriate regulatory signals can be conveyed in the absence of *SNF4* function. Multiple copies of a bifunctional *SNF1*-*lacZ* fusion (8) were also effective in suppression (data not shown).

This finding that the presence of multiple copies of the *SNF1* gene partially alleviates the requirement for *SNF4* function provides further evidence that the two genes serve related functions in the derepression of gene expression in response to glucose limitation. A simple model consistent with the genetic data is that the *SNF4* gene product has a positive effect on the expression, stability, or activity of the SNF1 protein kinase.

Function of C-terminal-deleted SNF1 is independent of *SNF4*. To determine whether the C-terminal region of SNF1 is involved in mediating the effect of *SNF4* on *SNF1*, we used the multicopy plasmids carrying the *SNF1* C-terminal deletions and the wild-type *SNF1* plasmid pCE9 to transform strains MCY1853 (*snf4*-Δ2) and MCY1793 (*snf1*-Δ3 *snf4*-Δ1). For each plasmid, three transformants of each strain were scored for anaerobic growth on sucrose and raffinose and compared with the transformants of MCY1843 (*snf1*-Δ10). pCEsnf1-Δ8, which carries the deletion that truncates SNF1 immediately after the protein kinase domain, suppressed the *snf4* defect better than did the wild-type plasmid pCE9, although pCEsnf1-Δ8 complements *snf1* less well than pCE9. The *snf4* and *snf1 snf4* mutants carrying pCEsnf1-Δ8 grew better than those carrying pCE9. The *snf1*, *snf1 snf4*, and *snf4* transformants carrying pCEsnf1-Δ8 showed very little difference in growth phenotype. Assays of secreted invertase activity confirmed that these transformants produced close to the same invertase activity, taking into account the expected variation in plasmid copy number,

TABLE 3. Demonstration that the function of truncated SNF1 encoded by *snf1-Δ8* is independent of *SNF4* genotype

Strain	Relevant genotype	Plasmid	Transformant	Invertase activity ^a	
				Repressed	Derepressed
MCY1843	<i>snf1-Δ10</i>	pCEsnf1-Δ8	1	1	44
			2	<1	88
MCY1793	<i>snf1-Δ3</i> <i>snf4-Δ1</i>	pCEsnf1-Δ8	1	<1	58
			2	<1	29
MCY1853	<i>snf4-Δ2</i>	pCEsnf1-Δ8	1	<1	25
			2	1	66

^a Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells.

despite the difference in *SNF4* genotype (Table 3). These findings suggest that the residual function of this truncated SNF1 protein is independent of *SNF4* function. This conclusion is subject to the reservation that we cannot assess the level of SNF1 protein produced from pCEsnf1-Δ8 in these strains. The four deletion plasmids that complement *snf1* weakly or not at all also conferred the same growth phenotypes in all hosts regardless of the *SNF4* genotype.

Point mutation in *SNF1* that compensates for deletion of *SNF4*. We next attempted to isolate dominant point mutations in *SNF1* that suppress the requirement for *SNF4*. A centromere-containing plasmid carrying the *SNF1* gene was mutagenized in vitro with hydroxylamine and used to transform MCY888 (*snf4-Δ1*) as described in Materials and Methods. Transformed colonies (~200) were screened by replica plating for the ability to utilize raffinose, and plasmids were recovered from three colonies. Upon retesting, two of the plasmids suppressed a *snf4* mutation with respect to both growth phenotype and derepression of invertase (Table 4). The plasmids also complemented a *snf1* mutation, as assayed by transformation of MCY1595 (the average derepressed invertase activity for six transformants was 81). These two plasmids contained suppressor mutations that were localized 5' to the *Bgl*II site at position 528 by a fragment-swapping procedure (see Materials and Methods). Sequence analysis of the region between nucleotides -347 and +1243 revealed that the two plasmids contained the same single-base substitution: G to A at position 157. Since the plasmids were recovered from two transformants in the same experiment, their independence is not certain. The mutation resulted in the alteration of Gly-53 to Arg, and the allele is therefore designated *SNF1-G53R*. This alteration is near the N terminus of the protein kinase domain. Thus, a point mutation in *SNF1* can alleviate the requirement for *SNF4*.

Protein kinase activity of *snf1* mutants. To extend our genetic analysis of *SNF1* function with biochemical analysis of the mutant SNF1 products, we assayed the protein kinase activity in wild-type and *snf1* mutant strains. The SNF1 protein was immunoprecipitated from wild-type and *snf1* mutant extracts by using affinity-purified polyclonal anti-SNF1 serum (8), and the immunoprecipitates were incubated with [γ -³²P]ATP (see Materials and Methods). The proteins were then separated by SDS-PAGE, and labeled products were detected by autoradiography (Fig. 3A). The *SNF1*-encoded proteins were detected by immunoblot analysis of half of the assayed sample (Fig. 3B).

In the wild-type immunoprecipitate, the SNF1 protein was labeled, as were several other proteins, including a prominent 66-kDa species and an approximately 180-kDa protein

that was less consistently labeled (Fig. 3A, lane a). The same results were obtained when extracts from glucose-repressed or derepressed cells were used (data not shown). When an extract from the *snf1-Δ10* deletion mutant was assayed, no labeled SNF1 product was detected, and phosphorylation of other proteins was virtually abolished. Immunoblot analysis of the sample confirmed the absence of the SNF1 protein (Fig. 3, lanes b). Identical results were obtained in assays of a *snf1-Δ3* mutant (data not shown).

Immune complexes prepared from the *snf1-K84R* strain showed no labeling of the mutant SNF1 protein and reduced labeling of the other two prominent species (Fig. 3, lanes c). The amount of the *snf1-K84R*-encoded protein present in the immunoprecipitate was the same as for the wild-type SNF1. This result shows that the SNF1 protein kinase activity is required for phosphorylation of the SNF1 protein in this assay.

Labeling of the 66- and 180-kDa proteins was only reduced, not abolished, in the *snf1-K84R* immune complexes. To test the possibility that the mutant *snf1-K84R* protein exhibits low-level activity in vitro, we assayed a strain carrying the *snf1-K84R* allele on a multicopy plasmid. No labeling of the mutant SNF1 protein was detected despite the increased amount of the protein shown by immunoblot analysis, and no increased labeling of other species was observed (Fig. 3, lanes e). This result provides further evidence that the phosphorylation of the SNF1 product in this assay is dependent on the SNF1 kinase activity. Also, this result does not support the notion that the mutant protein phosphorylates the 66- and 180-kDa proteins. Another possibility is that a second protein kinase activity coimmunoprecipitates with the *snf1-K84R* mutant protein and is responsible for the residual phosphorylation of the 66- and 180-kDa species.

The *SNF1-ΔH* deletion caused no apparent phenotypic defect. The in vitro activity of the *SNF1-ΔH* product was examined by assaying extracts from MCY1595 (*snf1-Δ10*) cells carrying *SNF1-ΔH* or the wild-type *SNF1* gene on centromere-containing plasmids. Immune complexes from the two extracts yielded the same array of labeled proteins except that the protein encoded by *SNF1-ΔH* was appropriately smaller than the wild-type protein (Fig. 3, lanes f and g).

The C-terminal deletion mutants were also assayed for kinase activity (Fig. 4). Three of the four deletions that showed reduced or no ability to complement *snf1* correspondingly encoded products that showed reduced or no labeling in the in vitro assay. In the *snf1-Δ5* immunoprecip-

TABLE 4. Derepression of invertase in *snf4* mutants carrying a suppressor mutation in *SNF1*

Plasmid ^a	Gene	Transformant	Invertase activity ^b	
			Repressed	Derepressed
A	<i>SNF1-G53R</i>	1	1	43
		2	1	53
B	<i>SNF1-G53R</i>	1	1	32
		2	1	24
pCE101	<i>SNF1</i>		1	4
pFE27-2	<i>SNF4</i>		1	248

^a Strain MCY888 (*snf4-Δ2*) was transformed with the two plasmids carrying suppressor mutations (A and B), the parent plasmid pCE101, and pFE27-2, which carries the *SNF4* gene (9).

^b Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells.

FIG. 3. Defective in vitro activity of the SNF1 protein kinase isolated from a *snf4* mutant. Protein kinase activity was assayed in immunoprecipitates prepared with affinity-purified anti-SNF1 serum as described in Materials and Methods. Half of the sample (A) was analyzed by SDS-PAGE in 7.5% polyacrylamide, and 32 P-labeled proteins were visualized by autoradiography (for 6 h for lanes a to e) at -70°C with an intensifying screen. Positions of the SNF1 protein and size standards (in kilodaltons) are indicated on the left. Longer exposure did not reveal any labeling of the SNF1 protein immunoprecipitated from the *snf4* mutant. The other half of the sample (B) was subjected to immunoblot analysis, using anti-SNF1 serum. Lanes contained protein from the following strains: a, MCY1093 (wild type); b, MCY1843 (*snf1-Δ10*); c, MCY1860 (*snf1-K84R*); d, MCY1853 (*snf4-Δ2*); e, MCY1828 (*snf1-Δ10*) carrying the multicopy plasmid pCE110 (*snf1-K84R*); f and g, MCY1595 (*snf1-Δ3*) carrying centromere-containing plasmids pCE108 and pSNF1-ΔH, which carry the wild-type and SNF1-ΔH alleles, respectively. Relevant genotypes are indicated above the lanes. WT, Wild type. The antibody used for immunoprecipitation of the samples in lanes f and g was not affinity purified.

itate, several degradation products were labeled better than the full-sized protein. In the *snf1-Δ7* immunoprecipitate, the total phosphorylation of the truncated SNF1 product was roughly comparable to that of the wild-type protein; however, the amount of protein detected by immunoblot analysis was much greater for the *snf1-Δ7* product than for the wild-type SNF1. We were not able to detect the truncated protein made from pCEsnf1-Δ8 with our antisera. It is noteworthy that the 66-kDa species was not labeled in immune complexes from any of the deletion mutants.

SNF4 function is required for maximal SNF1 protein kinase activity in vitro. The genetic evidence that increased *SNF1* gene dosage compensates for a defect in *SNF4* function suggested the possibility that the activity of the SNF1 protein kinase depends on *SNF4* function. To test this idea, we examined the in vitro activity of the SNF1 protein kinase immunoprecipitated from a *snf4* mutant. No phosphorylation of the SNF1 protein was detected, and neither the 66-kDa nor the 180-kDa product was labeled (Fig. 3, lanes d). Immunoblot analysis of the precipitated proteins confirmed that the SNF1 protein was recovered from the *snf4* mutant extract, although the amount of SNF1 protein present in a *snf4* mutant was a fewfold lower than that found in the wild type. These findings suggest that the SNF1 protein kinase exhibits greatly reduced activity in a *snf4* mutant. Low-level protein kinase activity was detected in immunoprecipitates from a *snf4* mutant carrying multiple copies of the *SNF1* gene (Fig. 5, lanes b), as would be predicted from the phenotypic effect of the increased *SNF1* gene dosage. In this experiment, the amounts of SNF1 protein immunoprecipitated from the wild-type and *snf4* mutant extracts were the same.

FIG. 4. Protein kinase activity of C-terminally deleted SNF1 products. SNF1 proteins were immunoprecipitated, and protein kinase assays were carried out (see Materials and Methods). Proteins were separated by SDS-PAGE in 7.5% polyacrylamide and subjected to immunoblot analysis, using anti-SNF1 serum (B). 32 P-labeled proteins were detected by autoradiography of the immunoblot (A). Strains assayed were MCY1843 (*snf1-Δ10*) (lanes a to e) and MCY1853 (*snf4-Δ2*) (lanes f to j) carrying pCE9 (lanes a and f), pCEsnf1-Δ4 (lanes b and g), pCEsnf1-Δ5 (lanes c and h), pCEsnf1-Δ6 (lanes d and i), and pCEsnf1-Δ7 (lanes e and j). Relevant alleles in the host strain and the SNF1 allele on the plasmid are indicated above the lanes. WT, Wild type. Strains were grown in complete minimal medium (SC [34]) lacking uracil.

FIG. 5. Protein kinase activity in *snf4* mutants carrying *SNF1* in multicopy and a *SNF1-lacZ* fusion. Immunoprecipitation and protein kinase assays (A and C) and immunoblot analysis (B and D) were carried out as for Fig. 3 except as noted. Positions of the SNF1 protein, SNF1-β-galactosidase fusion protein, and size standards (in kilodaltons) are marked. (A and B) Lanes: a, MCY1093 (wild type) carrying the multicopy *SNF1* plasmid pCE9; b, MCY888 (*snf4-Δ1*) carrying pCE9. Anti-SNF1 serum was used for immunoprecipitation. (C and D) Lanes: c, MCY1093 (wild type [wt]); d and e, YPH54 (wild type) carrying the *SNF1-lacZ* fusion plasmid pSNacZ; f and g, MCY1090 (*snf4-319*) carrying pSNacZ. Relevant genotypes are indicated above the lanes. Immunoprecipitation was carried out by using affinity-purified anti-SNF1 antibody (lanes c, d, and f) or monoclonal anti-β-galactosidase antibody (lanes e and g). Samples were analyzed by electrophoresis in 6% SDS-polyacrylamide.

We carried out a further control to exclude the possibility that these results depended in any way on the antibody used for immunoprecipitation. We previously constructed a gene fusion between codon 628 at the end of *SNF1* and *lacZ*; this *SNF1-lacZ* fusion encodes a hybrid protein exhibiting both SNF1 function and β-galactosidase activity in yeast cells (8). The fusion protein is recognized by both our rabbit polyclonal anti-SNF1 antiserum (8) and commercial mouse monoclonal anti-β-galactosidase antibody. Extracts were prepared from wild-type and *snf4* mutant strains carrying the gene fusion on the multicopy plasmid pSNacZ. The *SNF1-lacZ* fusion product was immunoprecipitated by using each of the two antibodies, and kinase activity was assayed (Fig. 5C and D, lanes d to g). In each case, the *SNF1-lacZ* fusion protein recovered from the wild-type extract was labeled, as was the 66-kDa protein. When the fusion protein was precipitated from a *snf4* mutant extract by using either antibody, phosphorylation of the *SNF1-lacZ* fusion product was greatly reduced. Moreover, no phosphorylation of the 66-kDa protein was detected. Immunoblot analysis showed that equal amounts of the fusion protein were immunoprecipitated from the wild-type and *snf4* mutant extracts.

The genetic analysis above suggested that the truncated SNF1 product encoded by *snf1-Δ8* functions relatively independently of SNF4 compared with the wild-type SNF1 protein. Unfortunately, we cannot immunoprecipitate the *snf1-Δ8* product with the available antisera to assess its activity in vitro. However, the growth phenotypes suggested

that two other C-terminal deletion mutants that complement *snf1* weakly also function independently of SNF4. To examine this possibility further, *snf4* mutants carrying the *SNF1* C-terminal deletion plasmids were assayed for protein kinase activity. In contrast to the results obtained with the wild-type SNF1 protein, the truncated proteins encoded by *snf1-Δ6* and *snf1-Δ7* exhibited the same level of phosphorylation in the *snf4* mutant extract as in a wild-type (*SNF4*) extract (Fig. 4). Immunoblot analysis showed that in each case, the same amount of protein was immunoprecipitated from the two extracts.

Characterization of SNF1 protein kinase activity in a filter assay for autophosphorylation. We next compared the activity of the SNF1 protein kinase derived from wild-type and *snf4* mutant cells in a different in vitro assay. We previously described an autophosphorylation assay carried out on a Western blot filter after renaturation of the bound proteins (8). Briefly, proteins were separated by SDS-PAGE, transferred to nitrocellulose, treated with the denaturant guanidine hydrochloride, and allowed to renature in buffer. The proteins bound to the filter were then incubated with [γ - 32 P]ATP, and the labeled products were detected by autoradiography. In this assay, autophosphorylation of both the native SNF1 protein and the *SNF1-lacZ* gene fusion product was detected. Neither protein was associated with the SNF4 protein on the filter because they were separated by electrophoresis; *SNF4* encodes a protein that is much smaller than SNF1 (9). It is, however, possible that before electrophore-

FIG. 6. Filter assay for autophosphorylation activity. (A) Proteins were separated by SDS-PAGE in 7.5% polyacrylamide, transferred to nitrocellulose, denatured with guanidine hydrochloride, allowed to renature in buffer, and assayed for autophosphorylation activity as described in Materials and Methods. The filter was washed, dried, and exposed to film for 8 h at -70°C with an intensifying screen. (B) The filter shown in panel A was subjected to immunoblot analysis, using anti-SNF1 antiserum. Coomassie blue staining of the gel also showed that the amount of the *SNF1-lacZ* fusion product was the same in both lanes. Positions of the *SNF1-lacZ* fusion product and the SNF1 protein are indicated. Lanes contained proteins prepared from the following strains: a, MCY1378 (pMSNF1); b, MCY1720 (pMSNF1); c, YPH54 (pSNacZ); d, MCY1090 (pSNacZ). Relevant genotypes are indicated above the lanes. WT, wild type.

sis the SNF1 product was covalently modified in a reaction requiring the SNF4 protein. To determine whether the autophosphorylation activity in this assay was affected by the *SNF4* genotype of the strain from which the SNF1 kinase activity was isolated, we assayed activity of the SNF1 and SNF1- β -galactosidase proteins derived from the wild type and a *snf4* mutant. The strains carried either the *SNF1* gene on the high-copy-number plasmid pMSNF1 (8) or the gene fusion on pSNacZ. The activities of the renatured SNF1 and SNF1- β -galactosidase proteins were the same within a factor of 2 regardless of the *SNF4* genotype (Fig. 6). Immunoblot analysis confirmed that the same amount of protein was bound to the filter for the wild-type and mutant samples (Fig. 6). Thus, the autophosphorylation activity detected by this filter assay is *SNF4* independent, and the SNF4 protein does not affect the autophosphorylation activity of the SNF1 protein kinase by means of a covalent modification that is stable in this assay. The *SNF4*-independent activity detected in this filter assay may correspond to the low-level *SNF4*-independent activity detected in immune complex assays of *snf4* mutants carrying multiple copies of *SNF1*.

DISCUSSION

Previous studies established that the *SNF1* gene is essential for release of gene expression from glucose repression and that *SNF1* encodes a protein kinase. Here we present evidence that the protein kinase activity of SNF1 is crucial for SNF1 function: a Lys-to-Arg substitution in the putative ATP-binding site within the kinase domain causes a mutant phenotype. We also show by deletion of the entire gene that *SNF1* is not essential for viability. Deletion of the sequence C terminal to the kinase domain partially impaired SNF1

function but caused no regulatory aberration in glucose repression. Thus, the C-terminal region does not constitute a regulatory domain that responds to glucose availability and is relatively dispensable.

Genetic evidence suggested that *SNF1* is functionally related to *SNF4*: mutations cause similar phenotypes and show similar interactions with extragenic suppressors (22, 24). We show here that increased dosage of the cloned *SNF1* gene partially compensates for a deletion of *SNF4*. This result supports the genetic evidence for a functional relationship between these genes. Also, the finding that increased *SNF1* gene dosage restores glucose-repressible *SUC2* expression indicates that in this situation regulatory signals regarding glucose availability can be conveyed in the absence of the *SNF4* gene product. This finding argues against a regulatory role for *SNF4*.

Analysis of the *SNF1* C-terminal deletion mutants suggested that deletion of this region alleviates the dependence on *SNF4* function. The C terminus may be involved in mediating the effects of SNF4. The isolation of a suppressor mutation in codon 53 of *SNF1* that confers *SNF4*-independent function implies that this region near the N terminus of the kinase domain has a significant role in SNF1 function, perhaps in its interactions with other proteins.

We obtained biochemical evidence regarding the nature of the functional interaction of the SNF1 and SNF4 proteins by assaying the *in vitro* activity of the SNF1 protein kinase immunoprecipitated from the wild type or a *snf4* mutant. To demonstrate the specificity of this assay, we first showed that autophosphorylation is dependent on the SNF1 kinase activity by using the *snf1-K84R* mutant as a control. We would expect the *snf1-K84R* product to behave like the wild-type SNF1 protein with respect to immunoprecipitation because of the conservative nature of the amino acid substitution, and we have subsequently confirmed that the mutant product shows coimmunoprecipitation with SNF4 (unpublished results). Using this assay, we found that autophosphorylation activities of SNF1 and a SNF1- β -galactosidase fusion protein were greatly reduced in a *snf4* mutant extract, indicating that the SNF4 protein is required for maximal SNF1 protein kinase activity *in vitro*. Both biochemical and genetic evidence suggest, however, that the SNF1 protein kinase has a low residual activity in the absence of SNF4. Low-level kinase activity was detected in immunoprecipitates from *snf4* mutants carrying multiple copies of *SNF1* or the *SNF1-lacZ* fusion; also, autophosphorylation was detected in the Western blot filter assay. The finding that increased gene dosage of *SNF1* or the *SNF1-lacZ* fusion partially restores invertase derepression in a *snf4* mutant provides genetic evidence for residual SNF1 protein kinase activity *in vivo* in the absence of SNF4 function. Taken together, these results suggest that the SNF4 protein affects positively the SNF1 protein kinase activity but is not absolutely required for SNF1 function. Evidence presented in the accompanying paper (9) that the SNF4 protein is partially dispensable at 23°C also supports this view.

The evidence that the *SNF4* gene product affects the level of the SNF1 protein deserves mention. The amount of immunoprecipitable SNF1 protein was reproducibly a few-fold lower in *snf4* mutants than in the wild type. The accompanying paper presents evidence that the SNF4 protein affects the stability of SNF1 (9). The fewfold difference in SNF1 levels in the *snf4* mutant and the wild type seems unlikely to account for the profound difference in their phenotypes. Moreover, increased *SNF1* gene dosage significantly elevated the protein level but did not completely

restore the wild-type phenotype. Thus, we think it most likely that SNF4 affects the SNF1 kinase activity in vivo as well as in vitro.

The kinase assays of SNF1 immune complexes also provide evidence against an alternate model for SNF4 function: that SNF4 serves as a target for the SNF1 protein kinase. We report in the accompanying paper that *SNF4* encodes a protein with a predicted molecular size of 36 kDa and that the size of a bifunctional SNF4- β -galactosidase fusion protein was consistent with this prediction (9). We also present evidence that this fusion protein coimmunoprecipitates with SNF1, and we would therefore expect the native SNF4 protein likewise to coimmunoprecipitate. No protein of appropriate size was phosphorylated in these assays, consistent with the view that SNF4 is a positive effector, rather than a substrate, of the SNF1 protein kinase.

SNF4 could act as a positive effector of the SNF1 kinase either directly, by interaction with the kinase, or indirectly, via a cascade of events. Evidence that the SNF4 and SNF1 proteins are physically associated (9) strongly suggests that SNF4 affects SNF1 function by a direct mechanism. SNF4 could act directly to stimulate the kinase activity of SNF1. Alternatively, SNF4 could counteract or inhibit a negative effector of the kinase. This latter possibility is attractive in view of genetic evidence that truncation of the C terminus of SNF1 appears to relieve the dependence on SNF4; the C terminus could mediate the action of the putative inhibitor, or perhaps the C terminus itself has an inhibitory function. We also cannot exclude the possibility that SNF4 promotes the effectiveness of the SNF1 kinase by preventing the action of a phosphatase that functions antagonistically to SNF1.

Although the mechanism of SNF4 action remains unclear, we do not favor the idea that SNF4 affects the kinase activity via modification of the SNF1 protein. The autophosphorylation activity of SNF1 and the SNF1- β -galactosidase fusion protein in the Western blot filter assay, in which neither protein is physically associated with the SNF4 protein, was the same regardless of whether the protein was derived from a *snf4* mutant or the wild type. This experiment excludes effects of covalent modifications that are stable in the assay procedure.

Two unidentified proteins, approximately 66 and 180 kDa in size, were prominent substrates for phosphorylation in immune complexes containing SNF1. The detection of these labeled species depended on the *SNF1* and *SNF4* genotypes; the experiments do not distinguish between effects on their immunoprecipitation or phosphorylation, and neither species corresponded to a prominent protein immunoprecipitated from ³⁵S-labeled extracts (data not shown). We do not know whether either protein is physiologically relevant to SNF1 function, but this question clearly merits further study.

What are the roles of the SNF1 protein kinase and the SNF4 protein in the regulation of gene expression by glucose repression? Genetic evidence establishes that *SNF1* and *SNF4* are essential for derepression of many glucose-repressible genes. The simple model is that either the expression or the activity of the SNF1 protein kinase is modulated in response to glucose availability. There is no evidence for regulation of the expression or localization of the SNF1 protein in response to glucose availability (7, 8). Genetic evidence argues against a regulatory role for the SNF4 protein because increased dosage of *SNF1* restores glucose-repressible invertase expression in a *snf4* deletion mutant. We therefore favor a model in which the SNF4 protein acts

as a positive effector of the SNF1 protein kinase but does not modulate its activity in response to glucose availability. Is the enzymatic activity of the SNF1 protein kinase regulated by a mechanism that does not involve SNF4? Thus far, we have been unable to detect any difference in the in vitro activity of the SNF1 protein kinase derived from glucose-repressed or derepressed cells, and we cannot exclude the possibility that the protein kinase is required to phosphorylate critical regulatory proteins but that its activity is not regulated. The resolution of this question must await identification of the in vivo substrates of this protein kinase. In this genetic system, genetic approaches can be applied to this problem: candidates for genes encoding targets of the protein kinase can be identified by selecting suppressors of *snf1* mutations.

ACKNOWLEDGMENTS

We thank Jackie Lee for isolation of the suppressor mutations in *SNF1*, Michelle Treitel for sequence analysis, and Laura Vallier for help with invertase assays. We thank Rob Rees-Jones, David Julius, and Gerard Housey for helpful discussions.

This work was supported by Public Health Service grant GM34095 from the National Institutes of Health and by an American Cancer Society faculty research award to M.C.

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