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38. Food (a 1-gram piece of horse meat) could be obtained on each trial by pushing open the door bearing the positive cue. The door bearing the negative cue was locked, and self-correction was not permitted. Training sessions of 40 trials occurred at the same time each day. Trials were 1 minute apart. After the end of each training session, the animals were permitted free access to food in their home cage until evening. Water was always available in the cage.
39. The day before the 2DG experiment, the cat was transported in its home cage from New York University to Brookhaven National Laboratory by car, together with the training apparatus. At the regularly scheduled time, the animal was subjected to the usual behavioral session. A dish of food was given after this test session. No further food was provided, to ensure a high level of motivation. Water was available in the cage.
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# A Yeast Gene That Is Essential for Release from Glucose Repression Encodes a Protein Kinase

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**The *SNF1* gene plays a central role in carbon catabolite repression in the yeast *Saccharomyces cerevisiae*, namely that *SNF1* function is required for expression of glucose-repressible genes. The nucleotide sequence of the cloned *SNF1* gene was determined, and the predicted amino acid sequence shows that *SNF1* encodes a 72,040-dalton polypeptide that has significant homology to the conserved catalytic domain of mammalian protein kinases. Specific antisera were prepared and used to identify the *SNF1* protein. The protein was shown to transfer phosphate from adenosine triphosphate to serine and threonine residues in an in vitro autophosphorylation reaction. These findings indicate that *SNF1* encodes a protein kinase and suggest that protein phosphorylation plays a critical role in regulation by carbon catabolite repression in eukaryotic cells.**

CARBON CATABOLITE REPRESSION, OR GLUCOSE REPRESsion, is an important and global regulatory system in both prokaryotic and eukaryotic cells. Studies of the yeast *Saccharomyces cerevisiae* have indicated that the regulatory mechanisms effecting carbon catabolite repression in eukaryotes are different from those in bacteria, and the evidence suggests that cyclic adenosine monophosphate (AMP) is not a direct effector (*1*). Genetic studies identified *SNF1* (sucrose nonfermenting) as a gene that plays a central role in carbon catabolite repression in yeast (*2*). The *SNF1* function is required for expression of various glucose-repressible genes in response to glucose deprivation; *snf1* mutants are unable to utilize sucrose, galactose, maltose, melibiose, or nonfermentable carbon sources, and diploids homozygous for *snf1* do not sporulate. The *snf1* mutants are not healthy strains, but are not defective in induction of acid phosphatase, an enzyme that is not regulated by glucose repression. Recent experiments (*3*) have shown that *SNF1* is the same gene as *CCR1*, which was independently found to be essential for derepression of several glucose-repressible enzymes (*4*).

The role of *SNF1* in expression of the *SUC2* (invertase) gene has been examined at the molecular level. The inability of *snf1* mutants to utilize sucrose results from a failure to derepress the *SUC2* messenger RNA encoding secreted invertase (*5*). Evidence that the effects of *SNF1* on *SUC2* expression occur at the transcriptional level and are mediated by the *SUC2* upstream regulatory region was obtained by showing that *SNF1* was required for expression of a heterologous yeast promoter under the control of the *SUC2* upstream regulatory region (*6*).

We have previously cloned the *SNF1* gene and mapped it to a locus on the right arm of chromosome IV (*7*). Disruption of the

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-390                                                                                                                    CTGCAGTTTTTCTCGTAGCGGAAGGGGATG

-360  GAAATTTTTAACGTAAAAGAAAGTTGGTAAGAGTATGGCACATCAACAGGTAGCGTTATAGGGGCCAAGACATAGCTTTGGGCTTAGTACTGTAGGCTTGTTACCAGAACTTTCATTTAT

-240  CGAAATATTCTTTTTTCACATTTCCTCTTACTGCGCATTCGTGTCCAAACAGTCATTCAGGAAGTATAACGATAAACAATAGATTGAAGCCTGAAAAAAAAAAAAAAAAGCACGACAATAG

-120  AAATTGTTTCAGTGTCATTGGCTTTTGATAATATATAGAGGCTGTTTCAATAATCATAGCGAAAGAAATAGAAGTTTTTTTTTGTAACAAGTTTTGCTACACTCCCTTAATAAAGTCAAC

      Met Ser Ser Asn Asn Asn Thr Asn Thr Ala Pro Ala Asn Ala Asn Ser Ser His His His His His His His His His His His His His   30
   1  ATG AGC AGT AAC AAC AAC ACA AAC ACA GCA CCT GCC AAT GCA AAT TCT AGC CAC CAC CAC CAC CAT CAC CAC CAT CAC CAC CAC CAT CAC

      Gly His Gly Gly Ser Asn Ser Thr Leu Asn Asn Pro Lys Ser Ser Leu Ala Asp Gly Ala His Ile Gly Asn Tyr Gln Ile Val Lys Thr   60
  91  GGT CAT GGC GGA AGC AAC TCG ACG CTA AAC AAT CCC AAG TCG TCC TTA GCG GAT GGT GCA CAT ATC GGG AAC TAC CAA ATC GTC AAA ACG

      Leu Gly Glu Gly Ser Phe Gly Lys Val Lys Leu Ala Tyr His Thr Thr Thr Gly Gln Lys Val Ala Leu Lys Ile Ile Asn Lys Lys Val   90
 181  CTG GGA GAG GGG TCC TTT GGT AAA GTT AAA TTG GCA TAT CAT ACC ACT ACG GGC CAA AAA GTT GCT CTA AAA ATC ATT AAT AAG AAG GTT

      Leu Ala Lys Ser Asp Met Gln Gly Arg Ile Glu Arg Glu Ile Ser Tyr Leu Arg Leu Leu Arg His Pro His Ile Ile Lys Leu Tyr Asp  120
 271  TTG GCA AAG AGT GAT ATG CAG GGC AGA ATT GAA AGA GAA ATA TCT TAT CTG AGA CTC TTA AGA CAC CCC CAC ATC ATC AAA CTG TAT GAT

      Val Ile Lys Ser Lys Asp Glu Ile Ile Met Val Ile Glu Tyr Ala Gly Asn Glu Leu Phe Asp Tyr Ile Val Gln Arg Asp Lys Met Ser  150
 361  GTT ATC AAA TCC AAA GAT GAA ATC ATT ATG GTT ATA GAG TAC GCC GGG AAC GAA TTG TTT GAC TAT ATT GTT CAG AGA GAC AAA ATG AGC

      Glu Gln Glu Ala Arg Arg Phe Phe Gln Gln Ile Ile Ser Ala Val Glu Tyr Cys His Arg His Lys Ile Val His Arg Asp Leu Lys Pro  180
 451  GAG CAA GAG GCA AGA AGA TTT TTC CAG CAG ATC ATC AGT GCC GTC GAG TAC TGC CAT AGG CAC AAA ATT GTC CAT AGA GAT CTG AAG CCT

      Glu Asn Leu Leu Leu Asp Glu His Leu Asn Val Lys Ile Ala Asp Phe Gly Leu Ser Asn Ile Met Thr Asp Gly Asn Phe Leu Lys Thr  210
 541  GAA AAC TTA CTA CTA GAT GAG CAT CTG AAT GTA AAG ATT GCC GAT TTT GGT TTG TCA AAC ATC ATG ACT GAT GGT AAT TTC TTA AAG ACT

      Ser Cys Gly Ser Pro Asn Tyr Ala Ala Pro Glu Val Ile Ser Gly Lys Leu Tyr Ala Gly Pro Glu Val Asp Val Trp Ser Cys Gly Val  240
 631  TCT TGT GGT TCT CCC AAT TAT GCG GCT CCT GAA GTT ATC AGC GGT AAG CTG TAC GCA GGC CCA GAA GTG GAC GTG TGG TCA TGT GGG GTT

      Ile Leu Tyr Val Met Leu Cys Arg Arg Leu Pro Phe Asp Asp Glu Ser Ile Pro Val Leu Phe Lys Asn Ile Ser Asn Gly Val Tyr Thr  270
 721  ATC CTT TAT GTT ATG CTT TGT CGT CGT CTA CCG TTT GAC GAT GAA AGC ATC CCA GTG CTT TTC AAG AAT ATC AGC AAC GGT GTT TAC ACC

      Leu Pro Lys Phe Leu Ser Pro Gly Ala Ala Gly Leu Ile Lys Arg Met Leu Ile Val Asn Pro Leu Asn Arg Ile Ser Ile His Glu Ile  300
 811  TTG CCT AAA TTT TTA TCT CCT GGA GCT GCT GGG CTA ATC AAA AGA ATG TTA ATC GTT AAT CCA TTG AAC AGA ATA AGC ATT CAT GAA ATT

      Met Gln Asp Asp Trp Phe Lys Val Asp Leu Pro Glu Tyr Leu Leu Pro Pro Asp Leu Lys Pro His Pro Glu Glu Glu Asn Glu Asn Asn  330
 901  ATG CAA GAC GAT TGG TTC AAA GTT GAC CTG CCA GAA TAT CTA CTT CCA CCA GAT TTG AAA CCA CAC CCA GAA GAA GAG AAT GAA AAT AAT

      Asp Ser Lys Lys Asp Gly Ser Ser Pro Asp Asn Asp Glu Ile Asp Asp Asn Leu Val Asn Ile Leu Ser Ser Thr Met Gly Tyr Glu Lys  360
 991  GAC TCA AAA AAG GAT GGC AGC AGC CCA GAT AAC GAT GAA ATT GAT GAC AAC CTT GTC AAT ATT TTA TCA TCG ACC ATG GGT TAC GAA AAA

      Asp Glu Ile Tyr Glu Ser Leu Glu Ser Ser Glu Asp Thr Pro Ala Phe Asn Glu Ile Arg Asp Ala Tyr Met Leu Ile Lys Glu Asn Lys  390
1081  GAC GAG ATT TAT GAG TCC TTA GAA TCA TCA GAA GAC ACT CCT GCA TTC AAC GAA ATT AGG GAC GCG TAC ATG TTG ATT AAG GAG AAT AAA

      Ser Leu Ile Lys Asp Met Lys Ala Asn Lys Ser Val Ser Asp Glu Leu Asp Thr Phe Leu Ser Gln Ser Pro Pro Thr Phe Gln Gln Gln  420
1171  TCT TTG ATC AAG GAT ATG AAG GCA AAC AAA AGC GTC AGT GAT GAA CTG GAT ACC TTT CTG TCC CAG TCA CCT CCA ACT TTT CAA CAA CAA

      Ser Lys Ser His Gln Lys Ser Gln Val Asp His Glu Thr Ala Lys Gln His Ala Arg Arg Met Ala Ser Ala Ile Thr Gln Gln Arg Thr  450
1261  AGC AAA TCC CAT CAA AAG AGT CAA GTA GAT CAT GAA ACT GCC AAG CAA CAC GCA AGA AGG ATG GCA AGT GCT ATC ACT CAA CAA AGG ACA

      Tyr His Gln Ser Pro Phe Met Asp Gln Tyr Lys Glu Glu Asp Ser Thr Val Ser Ile Leu Pro Thr Ser Leu Pro Gln Ile His Arg Ala  480
1351  TAT CAC CAA TCA CCC TTC ATG GAT CAG TAT AAA GAA GAA GAC TCT ACA GTT TCC ATT TTG CCT ACA TCT TTA CCT CAG ATC CAC AGA GCT

      Asn Met Leu Ala Gln Gly Ser Pro Ala Ala Ser Lys Ile Ser Pro Leu Val Thr Lys Lys Ser Lys Thr Arg Trp His Phe Gly Ile Arg  510
1441  AAT ATG TTA GCA CAA GGT TCG CCA GCT GCC TCT AAA ATA TCT CCT CTT GTA ACG AAA AAA TCT AAA ACG AGA TGG CAT TTT GGT ATA CGA

      Ser Arg Ser Tyr Pro Leu Asp Val Met Gly Glu Ile Tyr Ile Ala Leu Lys Asn Leu Gly Ala Glu Trp Ala Lys Pro Ser Glu Glu Asp  540
1531  TCT CGC TCA TAT CCA TTA GAC GTT ATG GGT GAA ATT TAT ATT GCC TTG AAG AAT TTG GGT GCC GAA TGG GCC AAG CCA TCT GAA GAG GAT

      Leu Trp Thr Ile Lys Leu Arg Trp Lys Tyr Asp Ile Gly Asn Lys Thr Asn Thr Asn Glu Lys Ile Pro Asp Leu Met Lys Met Val Ile  570
1621  TTA TGG ACT ATC AAA TTA AGG TGG AAA TAT GAT ATT GGA AAC AAG ACA AAC ACT AAT GAA AAA ATA CCT GAT TTA ATG AAA ATG GTA ATT

      Gln Leu Phe Gln Ile Glu Thr Asn Asn Tyr Leu Val Asp Phe Lys Phe Asp Gly Trp Glu Ser Ser Tyr Gly Asp Asp Thr Thr Val Ser  600
1711  CAA TTA TTT CAA ATT GAA ACC AAT AAT TAT TTG GTG GAT TTC AAA TTT GAC GGC TGG GAA AGT AGT TAT GGA GAT GAT ACT ACT GTT TCT

      Asn Ile Ser Glu Asp Glu Met Ser Thr Phe Ser Ala Tyr Pro Phe Leu His Leu Thr Thr Lys Leu Ile Met Glu Leu Ala Val Asn Ser  630
1801  AAT ATT TCT GAA GAT GAA ATG AGT ACT TTT TCA GCC TAC CCA TTT TTA CAT TTA ACA ACA AAA CTA ATT ATG GAA TTA GCC GTT AAC AGT

      Gln Ser Asn ***                                                                                                       633
1891  CAA AGC AAT TGATGGTGGAACGTAAAAGAATGATATGGAAGTTCCCTTTTTTTTATGTATCGTAACGTATTTGCAACATCTTTATAATATCCCTTTGTATAAAGTAAACAATCTTGA

2008  TTAAATAAACCTGAACATAATTTATTGATGTGAATGAACTTTTATTGGCATTATTTCTTCGGATGAAAAGCAAATTTCACGCTCTATGAGAAAAATTGAAAACGCTATTCTATGTGAAAA

2128  TAGAGATGAGTACTACTAGTGGCTACAATAAAAATACTGAACCTTTGACAATCGTACAACAAAAGGATCC
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Fig. 1. Nucleotide sequence of the *SNF1* gene and predicted amino acid sequence of the gene product. Restriction fragments were cloned into the vectors M13mp18 and M13mp19 (*32*), and the sequence of both strands was determined by the method of Sanger *et al.* (*33*) with the 17-nucleotide sequencing primer (Amersham). The 14 histidine residues near the amino terminus are underlined. The asterisks indicate the termination codon. Nucleotides are numbered on the left and amino acids on the right.

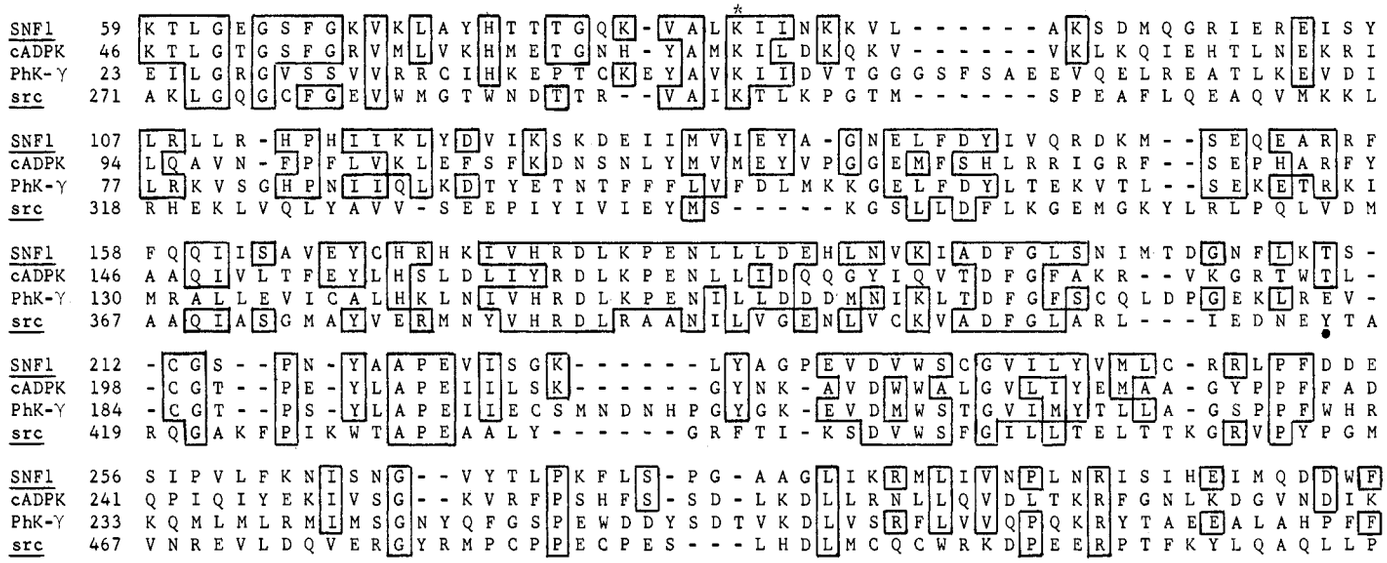


Fig. 2. Homology between *SNF1* and three mammalian protein kinases: the catalytic subunit of cyclic AMP–dependent protein kinase (cADPK) from bovine cardiac muscle (*13*); the γ subunit of phosphorylase kinase (PhK-γ) from rabbit skeletal muscle (*14*); and pp60$^{v\text{-}src}$ (*src*) of the Prague C strain of Rous sarcoma virus (predicted from the nucleotide sequence) (*15*). Amino acid sequences of the mammalian protein kinases are aligned as in (*17*); the *SNF1* protein sequence was aligned with the others to maximize the homology. Dashes indicate gaps introduced to optimize the homology. Identities with *SNF1* protein are boxed. The conserved lysine that is part of the ATP binding site (*19*) (position 71 in cADPK) is marked with an asterisk. The major phosphorylated tyrosine residue (position 416) of the *src* protein (*17*) is marked with a dot. Amino acids are numbered on the left. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

gene at its chromosomal locus conferred the same phenotype as did previously isolated missense mutations (*8*). *SNF1* was found to encode a 2.4-kb, unspliced poly(A)-containing RNA, and the amount of this RNA was not regulated by glucose repression (*8*). We report here that the *SNF1* gene encodes a protein that is homologous to mammalian protein kinases and that is phosphorylated on serine and threonine residues in an autophosphorylation reaction.

***SNF1* encodes a protein that is homologous to protein kinases.** Figure 1 shows the nucleotide sequence of the *SNF1* gene and the predicted amino acid sequence of the gene product. The open reading frame of 633 codons lies entirely within the region known to encode the 2.4-kb *SNF1* RNA, and its polarity is consistent with the direction of transcription. The 5′ end of the RNA was previously mapped (*8*) to a position 0.3 kb upstream from the initiation codon, and the 3′ end of the RNA was mapped to a position 0.2 kb downstream from the termination codon.

The sequence predicts a molecular weight of 72,040 for the polypeptide encoded by *SNF1*. A *SNF1* gene product of this size has been identified (see below), and analysis of protein fusions supported the existence of the open reading frame between positions −3 and 1884 (*9*). An unusual feature of this polypeptide is the presence of 14 histidine residues near the amino terminus, of which 13 are consecutive (positions 18 to 30 and 32). We found no significant region of hydrophobicity by plotting the hydrophobicity of segments of 21 amino acid residues using the normalized consensus scale of Eisenberg (*10*).

To gain insight into possible functions of the *SNF1* gene product, we compared the predicted amino acid sequence to the sequences of other proteins in a large database (*11*) with the computer program SEARCH (*12*). This comparison revealed that the *SNF1* protein contains a region with striking homology to the catalytic domains of mammalian protein kinases. Figure 2 shows the homology to the catalytic subunit of cyclic AMP–dependent protein kinase from bovine cardiac muscle (*13*), the γ (catalytic) subunit of phosphorylase b kinase from rabbit skeletal muscle (*14*), and pp60$^{v\text{-}src}$, the tyrosine protein kinase encoded by the Rous sarcoma virus *src* gene (*15*). The homology between the *SNF1* protein and the others within the region shown in Fig. 2, which corresponds to the catalytic domain, is 33 percent for both cyclic AMP–dependent protein kinase and the phosphorylase kinase γ subunit and 22 percent for the *src* tyrosine protein kinase. The *SNF1* gene also shows homology to other known protein kinases including bovine lung cyclic guanosine monophosphate (GMP)–dependent protein kinase (*16*), growth factor receptors with tyrosine protein kinase activity, the other members of the oncogene family of protein kinases (*17*), and another yeast gene with homology to protein kinases, the cell division control gene *CDC28* (*18*).

Of particular significance is the finding that the amino acids that are highly conserved among other protein kinases (*17*) are conserved at homologous positions in the *SNF1* sequence. The lysine at position 71 of cyclic AMP–dependent protein kinase is conserved among all the protein kinases and has been shown to form part of the adenosine triphosphate (ATP) binding site (*19*). A lysine residue is present at the homologous position (position 84) in the *SNF1* sequence. The sequence Leu-Gly-X-Gly-X-Phe-Gly-X-Val, which is highly conserved among protein kinases at a position roughly 15 residues toward the amino terminus from this lysine, is also present in the *SNF1* protein. Other invariant sequences that are conserved in *SNF1* are Arg-Asp-Leu (residues 176 to 178 in the *SNF1* protein), Asp-Phe-Gly (residues 195 to 197) and Ala-Pro-Glu (residues 219 to 221).

The sequence comparison suggests that the *SNF1* product is likely to be a serine-threonine–specific kinase. The *SNF1* protein exhibits greater overall homology to three serine-threonine kinases (cyclic AMP–dependent and cyclic GMP–dependent protein kinases and the phosphorylase kinase γ subunit) than to any of the tyrosine kinases. Moreover, the *SNF1* sequence contains many of the residues that are highly conserved only among the serine-threonine kinases (*17*), for example, the sequence Lys-Pro-Glu-Asn (positions 179 to 182). In addition, Thr$^{210}$ in the *SNF1* protein corresponds to Thr$^{196}$ in cyclic AMP–dependent protein kinase, which is autophosphory-

lated (*13, 20*), and $Cys^{212}$ corresponds to $Cys^{198}$ in cyclic AMP–dependent protein kinase, which is affinity-labeled with peptide substrates (*21*). In contrast, the tyrosine residue at position 416 in the *src* protein, which is conserved among tyrosine kinases and is the major site of tyrosine phosphorylation (*17*), is not found in the *SNF1* protein.

**Identification of the *SNF1* protein.** To identify the authentic *SNF1* product in yeast, we prepared specific rabbit antisera. A fusion between the *Escherichia coli trpE* gene and a portion of the *SNF1* gene was constructed. The hybrid protein was expressed in bacteria and was used to generate antisera to *SNF1* determinants (*22*). The *SNF1* protein was identified by immunoblot analysis (*23*) of total yeast proteins (Fig. 3B); sera obtained from three rabbits showed similar specificity. A 72-kD protein was detected in wild-type (*SNF1*$^+$) yeast cells, and was present in greater abundance in cells carrying the *SNF1* gene on a multicopy plasmid, pMSNF1 (*24*). This protein was not present in mutants carrying deletion *snf1-Δ3* or insertion *snf1::HIS3* (*24*). Moreover, a fusion protein of the expected size (187 kD) was detected in strains harboring plasmid pSNacZ, which carries a fusion between codon 628 of *SNF1* and codon 9 of the *E. coli lacZ* gene (*24*); this *SNF1-lacZ* fusion complemented a *snf1* mutation and produced β-galactosidase activity. These findings confirmed the data showing that *SNF1* encodes a protein of the predicted size.

To examine the effects of glucose repression on the *SNF1* protein, we performed immunoblot analysis on proteins from both glucose-repressed and -derepressed wild-type cells. No differences in the amount or mobility of the *SNF1* protein were detected (Fig. 3B, lanes b and c). These findings suggest that any regulation of the activity of the *SNF1* protein is effected by interactions with other molecules or by modifications that do not alter migrational mobility.

**Autophosphorylation of the *SNF1* protein on serine and threonine residues.** To obtain biochemical evidence that the *SNF1* product is a protein kinase, we used the following method to demonstrate that it is phosphorylated in an autophosphorylation reaction (Fig. 3A). A Western blot (*23*) of total yeast proteins was prepared, and the proteins on the filter were exposed to the denaturant guanidine-HCl and were then allowed to renature (*25*). We then carried out an autophosphorylation reaction by incubating the filter and the proteins bound to it in buffer containing [$\gamma$-$^{32}$P]ATP and divalent cations. The *SNF1* proteins from both glucose-repressed and -derepressed wild-type (*SNF1*$^+$) cells were labeled in this reaction. No labeled *SNF1* protein was detected in *snf1* deletion or insertion mutants, and increased amounts were detected in strains carrying multiple copies of the gene on plasmid pMSNF1. The functional *SNF1-lacZ* fusion protein encoded by pSNacZ was also phosphorylated; because this fusion protein migrated with a different mobility from that of the *SNF1* protein, this finding excluded the possibility that phosphorylation was dependent on the presence of a comigrating protein. The autophosphorylation reaction required one of the divalent cations $Mg^{2+}$ or

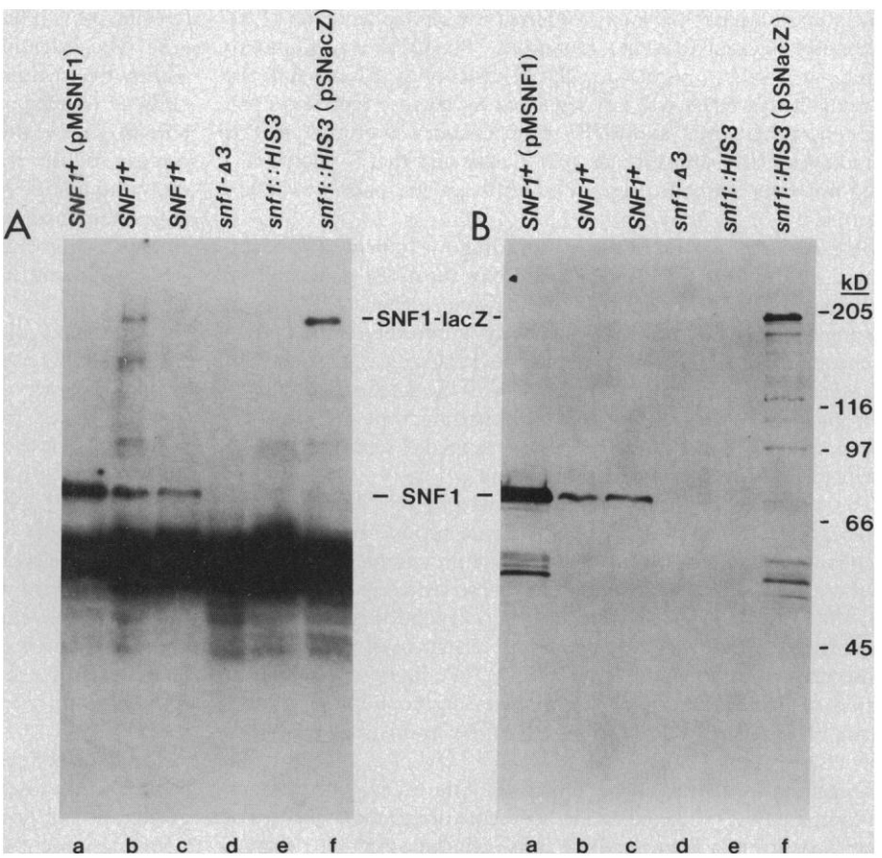


Fig. 3. Autophosphorylation assay and immunoblot analysis of the *SNF1* protein. Relevant genotypes of yeast strains: (lane a) *SNF1*$^+$(pMSNF1); (lane b) *SNF1*$^+$; (lane c) *SNF1*$^+$, glucose-derepressed; (lane d) *snf1-Δ3*; (lane e) *snf1::HIS3*; (lane f) *snf1::HIS3* (pSNacZ). Cultures were grown to mid-log phase in media containing 2 percent glucose (glucose-repressing conditions), except for the culture in lane c, which was derepressed by shifting to media containing 0.05 percent glucose for 2.5 hours (*7*). Strains carrying plasmids were grown in synthetic medium with selection for the plasmid; other strains were grown in rich medium. Cells were collected, washed, frozen in an ethanol–dry ice bath, and thawed on ice in 25 m*M* tris-phosphate (*p*H 6.7), 2 m*M* phenylmethylsulfonyl fluoride. Cells were broken in the cold by processing in a Vortex with glass beads, and lysates were clarified by centrifugation at 1000*g* for 15 minutes. Protein concentrations were determined with the Bio-Rad Protein Assay Kit. Proteins (50 μg) were separated by SDS-PAGE in 7.5 percent polyacrylamide and electroblotted to nitrocellulose as described in (*23*), except that methanol was omitted. (A) Autophosphorylation assay. The filter was blocked with 5 percent nonfat dry milk (Carnation) in 30 m*M* sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (Hepes) (*p*H 7.5). Proteins bound to the filter were denatured with 7*M* guanidine-HCl in 50 m*M* tris-HCl (*p*H 8.3), 50 m*M* dithiothreitol (DTT), 2 m*M* EDTA, 0.25 percent nonfat dry milk for 1 hour at 25°C, and were then allowed to renature in 50 m*M* tris-HCl (*p*H 7.5), 100 m*M* NaCl, 2 m*M* DTT, 2 m*M* EDTA, 0.1 percent Nonidet P-40, 0.25 percent nonfat dry milk for 16 hours at 4°C (*25*). The filter was treated with 5 percent nonfat dry milk in 30 m*M* Hepes (*p*H 7.5) for 30 minutes at 25°C and then was incubated with 0.03 μ*M* [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol, New England Nuclear) in 30 m*M* Hepes (*p*H 7.5), 10 m*M* $MgCl_2$, 2 m*M* $MnCl_2$, 0.25 percent nonfat dry milk for 30 minutes at 25°C. After several washings with 30 m*M* Hepes (*p*H 7.5) and 0.25 percent nonfat dry milk at 4°C, the filter was dried in air and exposed to film for 4 hours at −70°C with a screen. (B) Immunoblot analysis. Proteins carrying *SNF1* determinants were detected on the filter shown in (A). Antiserum raised against the *trpE-SNF1* fusion protein (diluted 1:200) was used with the Bio-Rad Immun-Blot Assay Kit with goat antibody to rabbit immunoglobulin G conjugated to horseradish peroxidase, except that 0.25 percent nonfat dry milk was substituted for gelatin. The minor bands in lane a are degradation products. Numbers at right mark positions of protein standards.

$Mn^{2+}$. In addition to the *SNF1* protein, yeast cells contain other proteins that were phosphorylated in this reaction, most of which migrated faster than the *SNF1* protein; faint bands above the *SNF1* band were often, but not reproducibly, detected (Fig. 3A, lane b).

The autophosphorylation activity of the *SNF1* proteins isolated from glucose-repressed and -derepressed cells was reproducibly the same within a factor of 2. Thus, no covalent modification of the protein that was stable during the isolation procedure and that significantly modified the activity of the enzyme in this assay occurred under repressing or derepressing conditions.

As a means of identifying the amino acid residues that were phosphorylated in this reaction, the labeled *SNF1* protein from a strain carrying pMSNF1 was eluted from the filter (*26*) and subjected to phosphoamino acid analysis (*27*). Phosphoserine and phosphothreonine were detected, but phosphotyrosine was not (Fig. 4). Analysis of the phosphorylated *SNF1-lacZ* fusion protein yieided similar results.

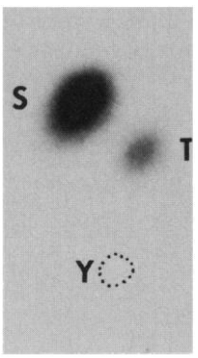


Fig. 4. Phosphoamino acid analysis. A 14-cm wide Western blot of proteins from a strain carrying pMSNF1 was prepared as in Fig. 3. The region of the filter containing the *SNF1* protein was identified by immunoblot analysis of test strips and was excised. An autophosphorylation reaction was carried out as in Fig. 3. The phosphorylated protein was eluted from the filter by incubating in 40 percent acetonitrile, 0.1*M* ammonium acetate (*p*H 8.9) for 3 hours at 37°C (*26*). The protein was lyophilized and subjected to partial amino acid hydrolysis, and phosphoamino acids were separated by two-dimensional thin-layer electrophoresis (*27*). Exposure was for 5 days. S, phosphoserine; T, phosphothreonine; and Y, phosphotyrosine.

**Localization of the *SNF1* protein.** Indirect immunofluorescence (*28*) was used to localize the *SNF1* protein within the yeast cell. In wild-type cells, the *SNF1* protein was distributed throughout the cell in a somewhat punctate pattern (Fig. 5). This pattern was identical in both glucose-repressed and -derepressed cells; thus, there was no indication that the location of the *SNF1* protein changed in response to glucose availability. To confirm that the staining pattern reflected the distribution of the *SNF1* protein, two control strains were examined: a strain carrying the *SNF1* gene on the multicopy plasmid pCE9 (*8*) and a *snf1-Δ3* deletion mutant (Fig. 5). Cells carrying pCE9 resembled the wild type, except that the intensity of the fluorescence was much greater. Deletion mutants showed no fluorescence above the background level detected in control experiments in which the antibody to *SNF1* protein was omitted.

To examine further the subcellular localization of the *SNF1* protein, lysates of wild-type cells were prepared as described in the legend to Fig. 3. The lysates were clarified by centrifugation at 10,000*g* for 15 minutes and were then fractionated by centrifugation at 100,000*g* for 90 minutes. The distribution of *SNF1* protein between the sedimented pellet and the supernatant was assayed by immunoblot analysis. Most of the *SNF1* protein (50 to 70 percent) reproducibly was recovered in the 100,000*g* particulate fraction from both glucose-repressed and -derepressed cultures. To determine whether the *SNF1* protein could be solubilized by detergents or salt, the sedimented pellet was washed and suspended in buffer. The suspended material was incubated in 10 m*M* EDTA and detergent or salt, and centrifuged again at 100,000*g*. None of the *SNF1* protein was released from the insoluble material by 2 percent Triton X-100, but about half was released by 2 percent sodium desoxycholate or 0.5*M* NaCl. The *SNF1* protein was solubilized by 2 percent sodium dodecyl sulfate (SDS) or 2 percent sodium lauroylsarcosine. These results suggest that a major fraction of the *SNF1* protein is either membrane-associated or insoluble in the absence of strong detergents or high salt.

In summary, these data indicate that the *SNF1* gene encodes a serine-threonine specific protein kinase. Because *SNF1* function is required for derepression of glucose-repressible genes, we suggest that protein phosphorylation serves as a signal in the regulatory circuitry for carbon catabolite repression in yeast. The activity of the *SNF1* protein may be modulated in response to glucose availability; the amount of protein and its location do not appear to be regulated. These findings provide further evidence that in eukaryotic cells carbon catabolite repression is effected by different mechanisms from those in *E. coli*, as was suggested by previous evidence that cyclic AMP is not a direct effector (*1*).

An important direction of research in the elucidation of complex pathways involving protein kinases is the identification of proteins that serve as substrates and regulators of the kinases. In yeast, genetic analysis of such pathways is useful in identifying candidates. The following genetic evidence suggests the *SSN6* gene product as a possible target of the *SNF1* activity. The defect in *SUC2* expression in *snf1* mutants can be suppressed by mutations at the *SSN6* (*CYC8*) locus (*29*). Both *ssn6* mutants and *snf1 ssn6* double mutants show high-level, constitutive (glucose-insensitive) synthesis of secreted invertase. On the basis of these results, we have suggested (*29*) that *SSN6* is a negative regulator of *SUC2* and that *SNF1* acts to prevent the repressive effect of *SSN6*. We can imagine that the *SNF1* protein kinase inactivates the *SSN6* gene product by phosphorylation. However, *SSN6* cannot be the only target of *SNF1* because *ssn6* mutations do not suppress all the pleiotropic defects of *snf1*.

The *SNF4* gene product is another candidate for a protein that may interact directly with the *SNF1* protein. Like *SNF1*, *SNF4* is required for derepression of glucose-repressible genes, including *SUC2* (*30*). Genetic evidence suggests that *SNF1* and *SNF4* play functionally related regulatory roles; the genetic interactions of *snf1* and *snf4* mutations with mutations at other loci (*ssn1*, *ssn6*, and *ssn20*) are similar and distinguish *snf1* and *snf4* from mutations in other genes required for derepression of *SUC2* (*30*, *31*). One

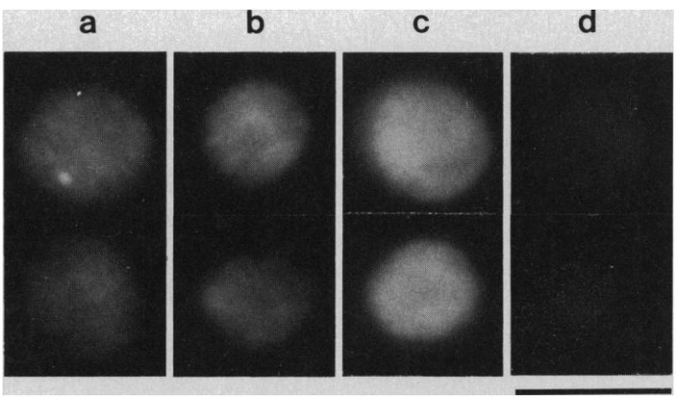


Fig. 5. Localization of the *SNF1* protein by indirect immunofluorescence. Cultures were prepared as described in Fig. 3. Cells were fixed with formaldehyde, treated with Glusulase (DuPont), immobilized on polylysine-coated slides, and stained with affinity-purified antibody to *SNF1* protein and affinity-purified FITC-conjugated goat antibody to rabbit immunoglobulin G (Sigma) as described in (*28*). Antibody to *SNF1* was purified by chromatography on DEAE Affi-Gel Blue (Bio-Rad); we then subjected it to affinity purification using, as the absorbant, gel-purified *trpE-SNF1* fusion protein coupled to CNBr-activated Sepharose 4B as described in (*34*). Cells were examined with a Zeiss Photomicroscope III equipped for epi-illumination fluorescence with a standard FITC filter set. Cells were photographed with Kodak Tri-X pan film and a 100× objective. Exposure times were 2 minutes. (a) Glucose-repressed wild type (*SNF1*$^+$); (b) glucose-derepressed wild type; (c) glucose-repressed wild type carrying pCE9; (d) glucose-repressed *snf1-Δ3* deletion mutant. Bar, 5 μm.

possibility is that the *SNF4* product serves as a primary target of *SNF1* and mediates its effects on other regulators such as *SSN6*. Alternatively, *SNF4* may regulate *SNF1* activity.

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