Characterization of Tobacco Protein Kinase NPK5, a Homolog of *Saccharomyces cerevisiae* SNF1 That Constitutively Activates Expression of the Glucose-Repressible *SUC2* Gene for a Secreted Invertase of *S. cerevisiae*

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We have isolated a cDNA (cNPK5) that encodes a protein kinase of 511 amino acids from suspension cultures of tobacco cells. The predicted kinase domain of NPK5 is 65% identical in terms of amino acid sequence to that of the SNF1 serine/threonine protein kinase of *Saccharomyces cerevisiae*, which plays a central role in catabolite repression in yeast cells. SNF1 positively regulates transcription of various glucose-repressible genes of the yeast, such as the *SUC2* gene for a secreted invertase, in response to glucose deprivation: *snf1* mutants cannot utilize sucrose as a carbon source. Expression of cNPK5 in yeast cells allowed the *snf1* mutant cells to utilize sucrose for growth and caused constitutive expression of the *SUC2* gene in wild-type cells even in the presence of glucose, an indication that the NPK5 protein is present in a constitutively active form in *S. cerevisiae*. On the other hand, expression of cNPK5 failed to suppress the growth defect of the *snf4* mutant cells in the presence of sucrose and to induce expression of the *SUC2* gene. These results indicate that SNF4 is required for the induction of *SUC2* expression by NPK5, as by SNF1, even if NPK5 is constitutively active in *S. cerevisiae*. The recombinant NPK5 protein is capable of autophosphorylation in vitro in a reaction that requires Mn^{2+} rather than Mg^{2+} ions but is inhibited by Ca^{2+} ions. Both dicotyledonous and monocotyledonous plants have several copies of the NPK5-related gene, which probably constitute a small gene family. NPK5-related genes were found to be expressed in the roots, leaves, and stems of tobacco plants. The high degree of structural conservation and the functional similarity of NPK5 to SNF1 lead us to speculate that NPK5 (or a related kinase) also plays a role in sugar metabolism in higher plants.

The unicellular eukaryote *Saccharomyces cerevisiae* has been a model system for studies of molecular mechanisms that control cellular responses to intracellular and extracellular signals in eukaryotes because this microorganism is suitable for both genetic and biochemical studies (14). Many yeast genes and their products, in particular those that are involved in important physiological processes, such as regulation of mitotic cell division, changes in sugar metabolism, and mating pheromone-inducible cell type specialization, have been identified and characterized. Proteins exhibiting homology to the yeast regulatory proteins have been found in other eukaryotes. The mechanism of signal transduction, elucidated with the yeast system, is considered, therefore, to be a paradigm relevant to attempts to understand analogous biological processes in multicellular eukaryotes.

Catabolite repression in *S. cerevisiae* is a typical system for the regulation of sugar metabolism and has been intensively studied. Wild-type yeast cells are able to utilize sucrose, galactose, maltose, and nonfermentable sources of carbon only in the absence of glucose in the medium: expression of the various genes that encode enzymes that catalyze the metabolism of these sugars is repressed by glucose. Recent genetic studies have identified *SNF* (sucrose nonfermenting) genes that are required for the sucrose metabolism in *S. cerevisiae* (4, 32).

Among *SNF* genes, the *SNF1* gene plays a central role in catabolite repression and encodes a protein serine/threonine

kinase (8). *snf1* mutants of *S. cerevisiae* are unable to utilize any of the sugars that are subject to catabolite repression and are unable to transcribe the glucose-repressible genes even when cells are deprived of glucose. The transcription of the gene for alcohol dehydrogenase (ADHII) of *S. cerevisiae* is similarly regulated by SNF1 (11). Since *snf1* mutants are, thus, pleiotropic and the activity of in vitro autophosphorylation of the SNF1 protein kinase is constitutive regardless of the presence of glucose (8), glucose deprivation might not directly activate the SNF1 kinase in vivo, but this enzyme does seem to be essential for the expression of glucose-repressible genes (8). The observation that mutation of a single amino acid in the putative ATP-binding site within the kinase domain of SNF1 resulted in the *snf1* mutant phenotype and inactivation of autophosphorylation activity revealed that the protein kinase activity of SNF1 is crucial for SNF1 function (9).

The SNF1 protein kinase positively regulates transcription of glucose-repressible genes (27), and the inability of *snf1* mutants to utilize sucrose is due to a failure to derepress transcription of the *SUC2* gene, which encodes a secreted invertase that hydrolyzes sucrose to glucose and fructose (5). It has been reported that SNF1 not only is required for the derepression of glucose-repressible enzymes but also affects cell growth under certain nutritioned conditions (30). The *SNF4* gene was first thought to be a gene candidate for a modulator or target of the SNF1 protein kinase (25). The results of further genetic studies suggested, however, that SNF4 acts as a positive effector of the SNF1 kinase but does not have a regulatory function in signalling the availability of glucose (9). Another gene, *SIP1*, which encodes a protein that coimmunoprecipitates with SNF1 and is phosphorylated by

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SNF1 has been isolated (33). Although several lines of genetic evidence suggest that SIP1 functions downstream of SNF1, its role in the signalling pathway is still ambiguous since an *sip1* disruption mutation has no effect on expression of invertase (33).

Other *SNF* genes, *SNF2*, *SNF3*, *SNF5*, and *SNF6* (6), in which mutations cause defects in the derepression of *SUC2* and other glucose-repressible genes, have been isolated, and their nucleotide sequences and predicted amino acid sequences have been analyzed. *SNF3* was found to encode a glucose transporter (10). On the basis of the nuclear localization of the *SNF2*, *SNF5*, and *SNF6* proteins, as well as the genetic and biochemical analysis of these proteins, it has been proposed that they activate transcription of glucose-repressible genes in conjunction with gene-specific activators (32). Although the functions of the genes that are involved in glucose derepression of gene expression have been extensively studied, the entire signal pathway remains to be defined.

Recently, a cDNA clone for a protein, designated RKIN1, that is similar to SNF1 was isolated from rye, and the cDNA of the *RKIN1* gene was shown to complement a *snf1* mutation in *S. cerevisiae* (1). It remains, however, to be proven that the predicted protein functions as a protein kinase, and analysis of functions of RKIN1 in *S. cerevisiae* is still incomplete. The *RKIN1*-related gene is expressed only in developing endosperms and not in shoots. Other *SNF1*-related genes were recently isolated from barley and *Arabidopsis thaliana*, and their structure and expression were examined (15, 23). The various results suggest that plants of the Gramineae, a family of monocotyledonous plants that includes rye and barley, and *A. thaliana* have SNF1-related protein kinases, although the general distribution of an SNF1 homolog throughout the plant kingdom and its function in plants remain to be established.

In the present study, we isolated from tobacco a cDNA (designated cNPK5) which encodes a protein kinase homologous to SNF1 in the course of experiments in which we were trying to clone a plant homolog of protein kinase C (3). In contrast to *RKIN1* of rye, the *NPK5*-related genes of tobacco were expressed in roots, leaves, and stems of tobacco and in suspension-cultured cells of tobacco. Expression of cNPK5 complemented the *snf1* mutation and induced constitutive expression of the *SUC2* gene for invertase both in the *snf1* mutant cells and in wild-type yeast cells that were cultured in the presence of glucose. These results indicate that homologs of the yeast SNF1 protein kinase are present in a wide variety of higher plants. In addition, our observations in the experiments with the NPK5 protein kinase may shed some light on the mechanism of the yeast signal transduction system that involves the SNF1 kinase.

MATERIALS AND METHODS

Plant materials. *Nicotiana tabacum* SR1 was grown in a greenhouse with a daytime (16 h) temperature of 28°C and a nighttime (8 h) temperature of 20°C. Suspension cultures of the BY-2 cell line of tobacco (21) were maintained in modified Linsmaier and Skoog medium (26) with 0.2 mg of 2,4-dichlorophenoxyacetic acid per liter, with weekly dilutions.

Screening for cNPK5 and sequencing of DNA. A plasmid clone containing the DNA fragment (CC5) that can encode a portion of a tobacco homolog of the SNF1 protein kinase was isolated from a library of PCR-amplified DNA fragments, which were amplified by PCR with primers, and a cDNA library, prepared from poly(A)⁺ RNA from BY-2 cells (3), was screened with the CC5 DNA fragment as a probe. Positive cDNA inserts were analyzed with restriction endonucleases

and one of the cDNA inserts was recloned into pBluescript (SK⁻) (Stratagene, La Jolla, Calif.) to produce the plasmid pcNPK5. Both strands of the full-length cDNA (cNPK5) were sequenced by the dideoxy chain termination method with Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) and synthetic oligonucleotide primers.

Construction of plasmids. The *Bam*HI fragment containing cNPK5 was cloned into the *Bam*HI site of pUC18 to produce pXK1, in which the cNPK5 sequence was oriented in such a way that the 5' end of the sense sequence was located next to the *Xba*I site in the multiple cloning site and the 3' end was next to the *Kpn*I site. pXK1R, in which the cNPK5 sequence was oriented in the inverted direction, was also produced. To produce pXK1(K48R) in which Lys at residue 48 was replaced by Arg in the putative ATP-binding site of NPK5 (Fig. 1), the A residue at nucleotide position 293 was replaced by a G residue by oligonucleotide-directed mutagenesis (17).

To create a construct for production of the recombinant NPK5 protein in *Escherichia coli* cells, a *Bam*HI site was generated immediately upstream of the putative initiator codon of the *NPK5* reading frame (the corresponding sequences is as follows: GGGATCCCCATG--). The *Bam*HI fragment containing the whole reading frame was then inserted into the *Bam*HI site of pGEX-3X (Pharmacia, Uppsala, Sweden) (28) to link the coding sequence for glutathione *S*-transferase (GST) to that of cNPK5 in frame. The resulting construct was designated pGEX-NPK5 and carried the *GST-NPK5* fusion. To produce pGEX-NPK5(K48R) which had a base substitution mutation in the coding sequence of cNPK5 that corresponded to the putative ATP-binding site of the kinase domain, the *Hind*III-*Xho*I fragment encoding the ATP-binding site in pGEX-NPK5 was replaced by the corresponding DNA fragment that encoded a mutated ATP-binding site in pXK1(K48R).

To generate the pYNPK5, pYNPK5R, and pYNPK5(K48R) plasmids for expression of cNPK5 in *S. cerevisiae*, each of the *Xba*I-*Kpn*I fragments isolated from pXK1, pXK1R, and pXK1(K48R) was cloned between the *Xba*I and *Kpn*I sites of the yeast expression vector pGT5 (24).

Purification of fusion proteins. Fifty-milliliter cultures of *E. coli* NM522 or HB101 cells that carried pGEX-NPK5 or pGEX-NPK5(K48R) were grown at 25°C to an optical density at 600 nm of 0.6. Isopropylthio-β-D-galactoside (IPTG) was then added to a final concentration of 1 mM, and the cultures were further incubated at 25°C for 16 h. Cells were pelleted and suspended in 40 ml of L buffer (50 mM Tris-HCl [pH 7.5], 25% sucrose, 0.5% Tween 20, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. After centrifugation, 5 ml of a suspension of glutathione-Sepharose 4B (Pharmacia) was added to the supernatant and proteins were allowed to adsorb to the resin with gentle mixing for 16 h at 4°C. The glutathione-Sepharose was washed twice with 50 ml of WE buffer (20 mM Tris-HCl [pH 7.5], 2 mM MgCl₂, 1 mM dithiothreitol) and was then poured into a column. The adsorbed proteins were eluted with 20 ml of G buffer (5 mM glutathione [reduced form], 50 mM Tris-HCl [pH 9.6]). Fractions that contained the GST-NPK5 protein were combined and dialyzed extensively against WE buffer for 16 h at 4°C.

Autophosphorylation assay. One microgram of partially purified protein was incubated for 40 min at room temperature with 0.1 mM [γ-³²P]ATP (6,000 Ci/mmol) in 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 2 mM MgCl₂, in a final volume of 10 to 20 ml. One volume of 2× sodium dodecyl sulfate (SDS) gel loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was then added, and the mixture was heated at 65°C

for 10 min. The mixture was subjected to electrophoresis on an SDS-polyacrylamide gradient gel (10 to 20%). The gel was then stained with Coomassie brilliant blue, dried, and exposed overnight to X-ray film at -80°C .

Yeast complementation assays. *S. cerevisiae* MCY1093 (*MATa his4-539 lys2-801 ura3-52 SUC2*) (9), MCY1846 (*MATa snf1- Δ 10 lys2-801 ura3-52 SUC2*) (9), and MCY1853 (*MATa snf4- Δ 2 his4-539 lys2-801 ura3-52 SUC2*) (9) were kindly provided by M. Carlson (Columbia University, New York, N.Y.). All strains were isogenic or congenic to S288C (9). MCY1093, MCY1846, and MCY1853 were each transformed with pGT5, pYNPK5, pYNPK5R, or pYNPK5(K48R). To examine the effects of various carbon sources on the growth of the transformed cells, cells were plated on the medium supplemented with 2% glucose or 2% sucrose and cultured at 30°C under anaerobic conditions in a GasPak disposable anaerobic system (BBL Microbiology Systems, Cockeysville, Md.) to reduce background growth.

Assay for invertase activity. Glucose-repressed and derepressed yeast cells were prepared basically as described elsewhere (7). Glucose-repressed cells were prepared by growing cells in SD medium (0.7% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, Mich.], 2% glucose) at 30°C , with aeration. Cells growing exponentially were collected by centrifugation and chilled. To obtain derepressed cells, the harvested cells were washed twice with distilled water, suspended in medium that contained 0.05% glucose, and further incubated for 2 h at 30°C . Cells were harvested by centrifugation and chilled. Secreted invertase was then assayed essentially as described elsewhere (13).

Southern and Northern analyses of plants. Southern blots of plant genomic DNAs were probed with the putative kinase domain sequence from pXK1. Poly(A)⁺ RNAs were prepared from tobacco leaves, stems, roots, and suspension-cultured cells, and Northern (RNA) blot analysis was carried out with the ³²P-labeled CC5 DNA fragment as a probe, as described elsewhere (3).

Nucleotide sequence accession number. The nucleotide sequence of the cDNA insert in the representative clone has been assigned GenBank accession number D26602.

RESULTS

Nucleotide and deduced amino acid sequences of the tobacco homolog of SNF1. To isolate fragments of DNA that can encode plant homologs of the cyclic AMP-dependent protein kinase or the protein kinase C, we prepared cDNAs from mRNA that had been isolated from suspension cultures of the tobacco cell line BY-2 and then amplified DNA fragments by PCR with synthetic oligonucleotide primers, sequences of which had been deduced from consensus amino acid sequences for the protein kinases above (3). Analysis of nucleotide sequences of PCR fragments of DNA, which were cloned into plasmids, showed that one of the PCR fragments, the CC5 fragment, appeared to encode a part of the amino acid sequence homologous to that of the SNF1 protein kinase of a budding yeast. Six cDNA clones that hybridized to the CC5 DNA fragment were isolated and subjected to analysis with restriction endonucleases. The results showed that five of the clones had essentially identical physical structures. However, one clone was different from others in terms of the gel electrophoretic patterns of restriction digests even though the intensity of the hybridization signal of this clone was similar to those of the major clones (data not shown), an indication that tobacco has at least two copies of the sequence that is homologous to the CC5 DNA fragment. In the present study,

the cDNA of one of the major clones, which was approximately 2 kbp in length, was subjected to further analysis.

Analysis of the nucleotide sequence of the cDNA insert in the representative clone showed that it consisted of 2,041 bp and contained a single, uninterrupted open reading frame which began at nucleotide position 28 and terminated at position 1683. Northern blot analysis with this insert as a probe gave a hybridization signal with a position that corresponded to approximately 2.1 kbp (see Fig. 4B), indicating that the insert analyzed here was almost full length. If the first ATG codon, located at nucleotide position 151, is used for the initiation of translation, this open reading frame is predicted to encode a polypeptide of 511 amino acids (calculated molecular mass, 68 kDa) (Fig. 1). Since the second ATG at nucleotide position 329 is in the coding region of the putative catalytic domain of a protein kinase, as pointed out below, the first ATG codon is likely to be used for the initiation of translation.

Analysis of the amino acid sequence of the predicted protein revealed that the N-terminal half (amino acid positions 1 to 274) contains the consensus sequence of the catalytic domain of a protein serine/threonine kinase, which consists of 12 subdomains (Fig. 1). The most striking feature of the predicted protein is that it includes a region that is highly homologous to a region of the SNF1 protein serine/threonine kinase of *S. cerevisiae* (8): the amino acid sequence of the N-terminal half (amino acid positions 17 to 285) is 65% identical to that of the corresponding region of the SNF1 kinase, whereas the C-terminal half has no significant homology outside a very limited region (35% identity from amino acid position 386 to 420) (Fig. 1). In particular, the region extending from subdomain VI to subdomain IX (amino acid positions 122 to 229) is 88% identical to the corresponding region of the SNF1 kinase. A marked difference is, however, that the 13 contiguous histidine residues near the N terminus of the SNF1 protein are missing in the predicted protein of tobacco. In fact, this histidine tract has been shown not to be essential for the activity of the SNF1 kinase and the utilization of sucrose (9). We designated the gene that encodes the putative protein kinase of tobacco *NPK5*.

As shown in Fig. 1, the catalytic domain and the kinase-unrelated C-terminal region of *NPK5* are 76 and 56% identical, respectively, to those of RKIN1 from rye (1), indicating that the genes are closely related even though expression of these genes is regulated by different mechanisms, as described in Discussion.

In addition to the homology of the predicted amino acid sequences, the nucleotide sequence corresponding to the putative kinase domain of *NPK5* (amino acid positions 1 to 274) exhibits 70% homology to that of the *SNF1* gene, but that corresponding to the C-terminal half exhibits no significant homology.

NPK5 protein can act as a protein kinase. To obtain biochemical evidence that the *NPK5* protein can act as a protein kinase, we constructed a chimeric gene in which the cNPK5 sequence was linked to the gene for GST from *Schistosoma japonica*. We also constructed a fusion gene that encoded the GST-*NPK5*(K48R) protein in which the Lys residue at position 48 in the putative ATP-binding site was replaced by an Arg residue (Fig. 1). The GST-*NPK5* and GST-*NPK5*(K48R) fusion proteins were produced in *E. coli* cells and partially purified by affinity chromatography on glutathione-Sepharose (28). When proteins in both samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), three bands of protein corresponding to molecular

T	MDGSTVQ-----GG--SSVESF-----RNYKLGKT	24
Y	MSSNNNTNTAPANANSSHHHHHHHHHHHGGSSNSTLNNPKSSLADGAHIGNYQIVKT	60
R	MDG-----GGEHS--EA-L-----KNYYLGKI	19
T	LGICSGFGVKIAEHTLTGHKVAVKILNRKIKNMEMEERVRREIKILRLFM---HPHIIK	81
Y	LGEGSPGKVLAYHTTTGQKVALKILNKVLAQSDMQGRIEREISYLRLLR---HPHIIK	117
R	LGVGTEAKVITAEHKHTRHKVAIKVLNRQMRAPEMEERAKREIKILRLFIDLIHPHIIK	79
	I II III IV	
T	LYEVVETESDIYVMVEYVKSGELFDYIIVEKGRLOEDEARKFFQOITISGVEYCHRMVVR	141
Y	LYDVIKSKDEIIMVIEYAGN-ELFDYIVQDKMSEQEARFFQOITISAVEYCHRHKIVHR	176
R	VIEVIVTEKDFVVMVEYCONGDLDYIILEKRRLOEDEARRTFQOITISAVEYCHRNKVHR	139
	V VIa	
T	DLKPENLLDSKNVVKIADFGLSNIMRDGHFLKTS CGSPNYAAPEVISGKLYAGPEVDVM	201
Y	DLKPENLLDEHLNVKIAADFGLSNIMTDGNFLKTS CGSPNYAAPEVISGKLYAGPEVDVM	236
R	DLKPENLLDSKNVVKIADFGLSNIMVDGHFLKTS CGSLNYAAPEVISGKLYAGPEIDVM	199
	VIb VII VIII IX	
T	SCGVILYALLCGTLPFDDENIPNLFKKIKGGMISLPSHLSAGARDLIPRMLIVDPMKRMT	261
Y	SCGVILYMLCRRLPFDDESIPVLFKNISNGVYTIKFLSPGAAGLIKRMILVNPLNRIS	296
R	SCGVILYALLCGAVPFDDDNIPNLFKKIKGCTYILPIYLSDLVRDLISRMILIVDPMKRIT	259
	X XI	
T	IPEIRMPHFWQAHLPYRLAVPPDTHQQAQKIDE-----DILQEVV--	302
Y	IHEIMQDDWTKVDLPEYL--FPDLKPHPEEENNNDSKDKGSSPDNDEIDNLVNILSS	354
R	IGEIRKHSWQNRILPYRLAVPPDTHQQAQKIDE-----DILRDVY--	300
T	KRGFDNRSLVASLGNRVQ---NEGTVAYYLLLENQ---FRA--S-SGYMGAE----	345
Y	TMGYEKDEIYESLESSEDTAPAFNEIRDAYMLIKENKSLIKDMKANKSVSDELDTFLSQSP	414
R	KLGVDKDHVCESLGNRLQ---NEETVAYYLLLDNR---FRA--TSGYLGAH----	343
T	--FQ-----ETMEYGYHQINSSEVL-----LF-----CWQHLP	371
Y	PTFQQQSKSHQKSQVDHETAKQHARR---ASAITQQRTHQSPFMDQYKEEDSTVSILP	471
R	--YQ-----QPM-----ESA-----SF-----STRSYLP	360
T	QIMDF-----QOVGAR--QFF-VERK---WALGLQ-SRAHPREIMTEVLKALQGLN	415
Y	TSLEPQIHANMLAQGSPAASKISLVTKSKTRWHFGIR-SRSYPLDVMEIYIALKNLG	530
R	GSNDS-----QSGSLRP--YY-RVERK---WALGLQSRAPPRAIMIEVLKALKEIN	406
T	VRNKHIG---PINMKCONVGVFGHHEGMS--NNSIHIOFFGDSTV-IE-NGGVTI-PN	467
Y	AENAKPSEEDLWTIKLRNKYDIGNKTNT---NEKIPDLMKMVIQLFQIETN---MY-LV	582
R	VCWKING---DCYNKGRNCFGFRVSDMLLDANHSFVD-----DCA--IKDNGDANSRLP	457
T	AV-KFEVQLYKTRREKYLLDLORVOG-----PQFLFLDLCAAFLAQLRVL	511
Y	DF-KFDGWESSYGDDTTVSNISEDEMSTFSAYP-FLHITTKLIME--LAVNSQSN	633
R	AVIKFEIQLYKTKDDKYLLDMQRTG-----PQLLFLFCAAFLTNLRVL	502

FIG. 1. Comparison of the deduced amino acid sequence of NPK5 of tobacco (T) with those of SNF1 of *S. cerevisiae* (Y) and of RKIN1 of rye (R). Amino acid positions from the initiator codon are indicated on the right. Sequences are aligned, and dashes have been introduced to give maximum matching. Residues identical to those in at least two sequences are shaded. Roman numerals below the sequences indicate subdomains of protein kinases (16). An asterisk indicates the Lys residue in the putative ATP-binding site of NPK5, which was replaced by an Arg residue in mutant NPK5(K48R).

masses of 84, 80, and 59 kDa were visualized by staining with Coomassie brilliant blue (Fig. 2A). The size of the 84-kDa protein was in good agreement with the predicted molecular mass of GST-NPK5 or GST-NPK5(K48R). After incubation of each of these GST fusion proteins with [γ - 32 P]ATP, the proteins were fractionated by SDS-PAGE. Figure 2B shows that two proteins (84 and 80 kDa) were phosphorylated in the sample that contained the GST-NPK5 fusion protein (lane 1), whereas an 84-kDa protein was missing from the analysis of the sample that contained the GST-NPK5(K48R) fusion (lane 2). This missing band would have been expected from a protein with the predicted molecular mass of the GST-NPK5 protein or the GST-NPK5(K48R) protein. The 80-kDa protein was observed also in the sample which was prepared from the *E. coli* cells that carried only vector plasmid, but not in that from the *E. coli* cells without the vector (data not shown), suggesting

that it is a plasmid-encoded protein, either a protein kinase or a substrate protein which can be phosphorylated by a protein kinase of *E. coli*. Therefore, it is likely that the protein of 84 kDa was a phosphorylated form of GST-NPK5, suggesting that NPK5 is capable of autophosphorylation. We could not rule out the possibility, however, that the GST-NPK5 phosphorylated a protein from *E. coli* cells that had a molecular mass of 84 kDa.

Dependence on divalent cations of the activity of the GST-NPK5 protein was examined. As shown in Fig. 2B (lanes 3 to 7), either Mg^{2+} or Mn^{2+} ions were required for the kinase activity and Mn^{3+} ions supported higher enzymatic activity than Mg^{2+} ions (lanes 3 and 4). No phosphorylation activity of GST-NPK5 was detected in the presence of Ca^{2+} ions (lanes 5), even in combination with Mg^{2+} ions (lane 7), whereas Ca^{2+} ions supported activity of the 80-kDa protein kinase of *E. coli*.

FIG. 2. Autophosphorylation of the partially purified GST-NPK5 fusion protein. (A) Proteins stained with Coomassie brilliant blue. One microgram of partially purified protein was fractionated by SDS-PAGE, and the gel was stained. Lane 1, GST-NPK5; lane 2, GST-NPK5(K48R). (B) Assay of autophosphorylation in vitro of the sample of GST-NPK5 (lanes 1 and 3 to 7) and the sample of GST-NPK5(K48R) (lane 2). Autophosphorylation reactions and SDS-PAGE were carried out as described in Materials and Methods, with the exception that reaction mixtures contained divalent cations (2 mM each) as follows: lanes 1, 2, and 3, Mg^{2+} ; lane 4, Mn^{2+} ; lane 5, Ca^{2+} ; lane 6, Mg^{2+} and Mn^{2+} ; lane 7, Mg^{2+} and Ca^{2+} . In the experiments corresponding to lanes 3 to 7, fusion proteins adsorbed to glutathione-Sepharose were used.

This result showed that Ca^{2+} ions inhibited the enzymatic activity of NPK5.

Functional analyses of NPK5 in yeast cells. We examined whether or not the predicted NPK5 protein was able to complement an *snf1* mutation in *S. cerevisiae* and to induce expression of the *SUC2* gene. To express the cNPK5 sequence in yeast cells, cNPK5 was linked to the *GAL1/GAL10* promoter of *S. cerevisiae*, which can be activated by galactose, and the resultant construct was introduced into *snf1* mutant cells (MCY1846). Although we thus attempted to use this yeast promoter for expression of cNPK5, we noticed that as described below, the cNPK5 sequence itself contained a sequence which can function as a constitutive transcriptional promoter. Figure 3 shows that the *snf1* mutant cells that carried the cNPK5 construct grew on medium supplemented with sucrose although the growth of these cells was slower than that of wild-type cells. The mutant cells having only the vector sequence or cNPK5(K48R) did not grow under the same condi-

tions (Fig. 3). Thus, only the wild-type cNPK5 allowed the *snf1* mutant cells of *S. cerevisiae* to utilize sucrose for growth. Taken together with the results in Fig. 2, this result shows that the kinase activity of the NPK5 protein is critical for suppression of the *snf1* mutation.

The observation that the *snf1* mutant cells that harbored the cNPK5 construct grew in the presence of only sucrose (without galactose as an inducer of the *GAL1/GAL10* promoter) was not initially expected. This phenomenon may have been due to the presence of a promoter sequence in the 5' noncoding region of the cNPK5 sequence, which would be active in yeast cells. This explanation is supported by the fact that other *snf1* mutant cells, which carried the cNPK5R construct in which cNPK5 was linked to the *GAL1/GAL10* promoter in the antisense orientation, were also able to grow in the presence of only sucrose (Fig. 3). When we analyzed transcripts in yeast cells which carried both the sense and the antisense constructs, only sense transcripts were in fact detected (data not shown). A similar observation was made when a cDNA from human cells was expressed in *S. cerevisiae* (19).

Subsequently, we examined whether expression of cNPK5 can bypass the requirement of SNF4, the mutation of which is shown to cause the same spectrum of pleiotropic phenotypes as do mutations in *SNF1*. Since the SNF4 protein can be physically associated with the SNF1 kinase, it is thought to be a positive effector of SNF1 (9). As shown in Fig. 3, the *snf4* mutant cells that carried cNPK5 were unable to grow in the presence of sucrose while a wild-type *S. cerevisiae* grew under the same conditions. Thus, NPK5 failed to suppress the *snf4* mutation.

We next examined whether expression of the cNPK5 sequence causes induction of the secreted invertase activity in the *snf1* mutant, since expression of the *SUC2* gene for the secreted invertase has been shown to be induced by glucose deprivation and to require function of SNF1 (6). As shown in Table 1, invertase activity was detected in the *snf1* mutant cells that carried pYNPK5 and had been incubated either under repressed conditions (2% glucose) or under derepressed conditions (0.05% glucose), although invertase activity was detected in the wild-type *S. cerevisiae* only in the absence of glucose. In addition, expression of cNPK5 in the wild-type cells resulted in the induction of invertase activity even when

FIG. 3. Complementation of the yeast *snf1* mutant by tobacco cNPK5. Cells of *S. cerevisiae* MCY1093 (wild type), MCY1846 (*snf1*), and MCY1853 (*snf4*) harboring only the vector plasmid pGT5, pYNPK5, pYNPK5R, or pYNPK5(K48R) were streaked on agar plates supplemented with 2% glucose (Glc) or 2% sucrose (Suc). Cells of MCY1093 and MCY1846 were grown at 30°C for 5 days, and cells of MCY1853 were grown at 30°C for 8 days under the same conditions.

TABLE 1. Induction by cNPK5 of the activity of a secreted invertase in yeast cells

Yeast strain and plasmid	Invertase activity ^a	
	Repressed	Derepressed
MCY1093 (wild type)		
pGT5	<1	156
pYNPK5	56	164
pYNPK5(K48R)	<1	139
MCY1846 (<i>snf1</i>)		
pGT5	1	3
pYNPK5	70	77
pYNPK5(K48R)	1	3
MCY1853 (<i>snf4</i>)		
pGT5	1	1
pYNPK5	3	6
pYNPK5(K48R)	2	2

^a Expressed as micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are average results of assays of three separate transformants. Details of the repressed (2% glucose) and derepressed (0.05% glucose) conditions are given in Materials and Methods.

glucose was present in the medium. Thus, the predicted NPK5 protein can constitutively induce expression of the *SUC2* gene for invertase. We also examined whether NPK5 could suppress the *snf4* mutation to induce expression of the *SUC2* gene. The result (Table 1) showed that expression of cNPK5 failed to induce *SUC2* expression in the *snf4* background.

Genomic analysis of NPK5-related genes and their expression in tobacco plants. Southern blot analysis with genomic DNAs from *N. tabacum* and its two parental species (*Nicotiana sylvestris* and *Nicotiana tomentosiformis*) was carried out with the cNPK5 sequence as a probe. Figure 4A shows that *N. tabacum* has several copies of NPK5-related genes, which are probably derived from both the parental species. *Arabidopsis*

thaliana, *Oryza sativa*, and *Zea mays* also contain NPK5 homologs in their genomes. Thus, genes that are highly homologous to the NPK5 gene and, therefore, to the yeast *SNF1* gene are present in both dicotyledonous and monocotyledonous plants.

Poly(A)⁺ RNAs were prepared from BY-2 cells at the logarithmic phase and from leaves, stems, and roots of mature tobacco plants and subjected to Northern blot analysis with the cNPK5 sequence as the probe. As shown in Fig. 4B, an NPK5-related transcript of 2.1 kbp was detected in all the organs examined, as well as in the BY-2 suspension-cultured cells. The transcript was present at other stages of the growth of BY-2 cells also (data not shown).

DISCUSSION

This report describes four lines of evidence that indicate the presence of a gene in tobacco that encodes a protein kinase analogous to the SNF1 protein of *S. cerevisiae*. (i) The predicted NPK5 protein contains a domain that exhibits 65% identity in terms of amino acid sequence to the catalytic domain of the SNF1 protein kinase (Fig. 1). (ii) Analysis of the recombinant GST-NPK5 protein showed that the NPK5 protein had the activity of a protein kinase (Fig. 2). (iii) The defect in growth of the *snf1* mutant cells in sucrose-containing medium was complemented by expression of cNPK5 (Fig. 3). This observation is consistent with earlier studies with the rye homolog (1). (iv) Expression of cNPK5 both in *snf1* mutant and wild-type cells of *S. cerevisiae* resulted in the constitutive induction of the expression of the *SUC2* gene for invertase, regardless of the presence of glucose (Table 1). The induction of *SUC2* expression by NPK5, as by SNF1, required functions of SNF4.

The finding that expression of cNPK5 in wild-type yeast cells caused constitutive induction of the expression of *SUC2* may provide a clue to the understanding of a signalling process mediated by SNF1 in *S. cerevisiae*. Since NPK5 has an amino acid sequence that is highly homologous to that of SNF1, it is likely that the predicted NPK5 protein can constitutively activate the SNF1-mediated signal pathway of *S. cerevisiae*, which is normally initiated by glucose deprivation. Although genetic and biochemical studies of SNF1 have established that its protein kinase activity is strictly required for derepression of glucose-repressible genes (9), there is, as yet, no evidence that the activity of SNF1 is modulated in response to the availability of glucose: the autophosphorylation activities of SNF1 isolated from glucose-repressed and glucose-derepressed cells are the same (8). In addition, the amount of protein and its cellular localization are identical in both types of yeast cell (8). Therefore, the possibility that the protein kinase is required for phosphorylation of critical regulatory proteins that are involved in glucose derepression, while its own activity is not regulated, cannot be excluded (see Discussion in reference 9). The present results relating to the expression of cNPK5 in yeast cells suggest that the NPK5 kinase is constitutively active in yeast cells, so that cNPK5 resembles a dominant active mutant of SNF1, while such an SNF1 mutant has yet to be isolated. Therefore, only activation of the SNF1 protein kinase in yeast cells seems to be sufficient to derepress expression of the *SUC2* gene. Our observation is in agreement with the idea that activation of the SNF1 kinase may be induced by glucose deprivation. Alternatively, expression of the NPK5 kinase might activate another unknown pathway, which in turn might induce expression of the *SUC2* gene in the presence of glucose. The latter possibility may, however, be unlikely because the amino acid sequence identity between NPK5 and SNF1 is

FIG. 4. (A) Southern blot analysis of genomic DNAs from *N. sylvestris* (lane 1), *N. tomentosiformis* (lane 2), *N. tabacum* (lane 3), *A. thaliana* (lane 4), *O. sativa* (lane 5), and *Z. mays* (lane 6). Samples of genomic DNA from each plant were digested with *EcoRI*. The digested DNAs were fractionated on a 0.7% agarose gel, transferred to a nylon membrane, and probed with ³²P-labeled putative kinase domain sequence from pXK1. Numbers on both sides of the gel indicate the sizes of DNA markers. (B) Expression of NPK5-related genes. Poly(A)⁺ RNA prepared from cultured cells (lane 1), leaves (lane 2), stems (lane 3), and roots (lane 4) of tobacco was analyzed as described in Materials and Methods. Numbers on the left indicate the sizes of marker RNA molecules.

rather high and the induction of *SUC2* expression by NPK5 requires the *SNF4* gene (Table 1).

Our results of the experiments with cNPK5 in the *snf4* mutant of *S. cerevisiae* may also shed some light on the SNF1-regulated induction of gene expression. Expression of cNPK5 failed to suppress the growth defect of the *snf4* mutant cells in the presence of sucrose and to induce *SUC2* expression (Fig. 3 and Table 1). These results indicate that the induction of *SUC2* expression by NPK5 requires functions of the SNF4 protein in *S. cerevisiae*. Since the SNF1 kinase is shown to be physically associated with the SNF4 protein (9), NPK5 may be also somehow associated with it in yeast cells, which must be essential for the induction of *SUC2* expression even if the NPK5 protein kinase produced in yeast cells is constitutively active, as we discussed above. If the SNF1 kinase can be activated in response to glucose deprivation, the association between SNF1 and SNF4 may occur after or independently from the activation of the SNF1 kinase.

With regard to an *snf4* mutant, Estruch et al. (12) reported that the *SNF1-G53R* mutation that was isolated as a suppressor of the requirement for *SNF4* replaces the Gly residue at position 53 with an Arg residue. The SNF1-G53R protein has greatly elevated kinase activity in immune complex assays and has the ability to express the *SUC2* gene in the background of an *snf4* mutation while the *SUC2* expression still responds to glucose deprivation. On the basis of several lines of genetic evidence, they postulated that the elevated activity of this mutant protein and the suppression of the *snf4* mutation are due to an altered interaction with an unknown molecule in the immune complex (12). Our results relating to expression of cNPK5 in the *snf4* mutant suggest that the NPK5 protein cannot interact with the molecules in the immune complex without the SNF4 protein, although NPK5 has already an Arg residue at the position corresponding to position 53 of SNF1 (Fig. 1).

It is worth noting here the effects of divalent cations on the autophosphorylation activity of NPK5. Mn^{2+} ions were more effective in supporting the activity of the NPK5 protein kinase than were Mg^{2+} ions. Recently, two other protein kinases from tobacco which were isolated in our laboratory were also shown to be activated by Mn^{2+} more efficiently than by Mg^{2+} ions (unpublished data). A preference for Mn^{2+} ions for the activity of a protein kinase from *A. thaliana* has also been reported (18). Such a preference may be characteristic of protein kinases from plants. Inhibitory effects of Ca^{2+} ions may also be of interest, since these ions did not show such inhibitory effects on activities of the other protein kinases as described above. To elucidate functions of Ca^{2+} ions in inhibition of the activity of NPK5, further biochemical analysis of NPK5 should be, however, carried out.

Southern blot analysis in the present study (Fig. 4A) and results from other groups (1, 15, 23) suggested that NPK5-related genes are ubiquitous in higher plants. In addition, the observation that several hybridization signals with different intensities were detected in the analysis of each plant examined indicated the presence of small multigene families of NPK5-related genes. Northern blot analysis indicated that the NPK5-related gene is expressed in roots, stems, and leaves of a tobacco plant (Fig. 4B) and in cultured cells at all stages of growth. These results imply that the function of NPK5, although it has yet to be demonstrated, is not controlled at the level of gene expression and that its gene product may be required for basic physiological processes in plant cells. The small size of the multigene family of NPK5-related genes suggests that each member of the gene family might be

expressed in a different organ of the tobacco plant and might have a different physiological role.

The pattern of expression of the NPK5-related genes contrasts with that of expression of the *RKIN1* gene, a rye homolog of yeast *SNF1* which is expressed predominantly in developing endosperms, but not in shoots (1). Therefore, the expression of NPK5 and that of *RKIN1* seem to be under different types of control. However, their products presumably have similar roles in different aspects of physiological pathways of plants because the amino acid sequence identities of the respective kinase domains and the respective kinase-unrelated regions are rather high (76% identity for kinase domains and 56% for kinase-unrelated regions).

From the structural and functional similarities between the NPK5 and SNF1 kinases, we can speculate that they are responsible for similar aspects of carbon metabolism that involve a secreted invertase. In yeast cells, the requirement for SNF1 in derepression of glucose-repressible genes has been established, although our understanding of the mechanism of regulation by SNF1 is still incomplete, as described above. In plants, a number of extracellular cell wall invertases, which are thought to be responsible for utilization of sucrose (a predominant transported sugar), have also been purified and partially characterized (22, 32). A cDNA clone that encodes a cell wall invertase with significant sequence homology to the *SUC2* invertase has recently been isolated from cultured carrot cells (29) and mung bean hypocotyls (2). Transcription of the gene for invertase in suspension-cultured carrot cells is not prevented by replacing sucrose with glucose, unlike the case of *SUC2* of *S. cerevisiae* (29). The activity of the cell wall invertase of suspension-cultured tobacco cells is also not affected by glucose deprivation (31). The level of expression in carrot roots is greatly enhanced by extracellular stimuli, such as mechanical wounding and bacterial pathogens (29). Although the roles of the invertase in the wound response and in the response to pathogens are unknown, it has been suggested that, in stress situations, plant cells have a high demand for hexoses that are required to fulfill the energy and carbon requirements needed for adequate responses (29). The plant SNF1 homologs might be involved in expression of the gene for invertase in response to such stimuli.

In addition to expression of *SUC2*, the yeast SNF1 protein regulates expression of genes that are responsible for the metabolism of various nonfermentable carbohydrates, including galactose, maltose, melibiose, and alcohol, as described in the introduction (11, 32). Plants contain a wide variety of sugars as cell wall components and are known to consume some of these components as energy sources. Functions of SNF1 homologs might be required in the metabolism of these carbohydrates in higher plants. It has been shown that expression of the gene for α -amylase is depressed by a carbohydrate, such as glucose, in suspension-cultured cells of rice (34) and in isolated embryos of rice (20). NPK5-related genes may regulate the expression of such glucose-repressible genes.

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