The *Aspergillus nidulans xprF* Gene Encodes a Hexokinase-like Protein Involved in the Regulation of Extracellular Proteases

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ABSTRACT

The extracellular proteases of *Aspergillus nidulans* are produced in response to limitation of carbon, nitrogen, or sulfur, even in the absence of exogenous protein. Mutations in the *A. nidulans xprF* and *xprG* genes have been shown to result in elevated levels of extracellular protease in response to carbon limitation. The *xprF* gene was isolated and sequence analysis indicates that it encodes a 615-amino-acid protein, which represents a new type of fungal hexokinase or hexokinase-like protein. In addition to their catalytic role, hexokinases are thought to be involved in triggering carbon catabolite repression. Sequence analysis of the *xprF1* and *xprF2* alleles showed that both alleles contain nonsense mutations. No loss of glucose or fructose phosphorylating activity was detected in *xprF1* or *xprF2* mutants. There are two possible explanations for this observation: (1) the *xprF* gene may encode a minor hexokinase or (2) the *xprF* gene may encode a protein with no hexose phosphorylating activity. Genetic evidence suggests that the *xprF* and *xprG* genes are involved in the same regulatory pathway. Support for this hypothesis was provided by the identification of a new class of *xprG*$^-$ mutation that suppresses the *xprF1* mutation and results in a protease-deficient phenotype.

THE filamentous fungus *Aspergillus nidulans* can use exogenous protein as a source of carbon, nitrogen, or sulfur. Utilization of exogenous protein is dependent on the production of extracellular proteases. Two genes encoding extracellular proteases have been isolated from *A. nidulans*. The *prtA* gene encodes an alkaline/serine protease (Katz *et al.* 1994). Disruption of the *prtA* gene showed that the *prtA*-encoded serine protease is the most abundant extracellular protease detected in a wide range of pH conditions (vanKuyk *et al.* 2000). A gene (*prtB*) encoding a putative acid/aspartic protease has also been isolated from *A. nidulans* (vanKuyk *et al.* 2000). It is likely that additional genes encoding extracellular proteases are present in *A. nidulans* as six types of extracellular protease genes have been isolated from other Aspergillus species (Tatsumi *et al.* 1989, 1991; Berka *et al.* 1990; Inoue *et al.* 1991; Sirakova *et al.* 1994; van den Hombergh *et al.* 1994).

In *A. nidulans*, the production of extracellular proteases is subject to at least four regulatory mechanisms—carbon, nitrogen, and sulfur metabolite repression and pH control. A fifth regulatory mechanism, induction by exogenous protein, is present in *A. niger* (Jarai and Buxton 1994) and may also operate in *A. nidulans* under certain conditions. However, all of the *A. nidulans* extracellular proteases that have been detected are produced in the absence of exogenous protein if the fungus is exposed to carbon-, nitrogen-, or sulfur-limiting nutrient conditions (Cohen 1973).

A number of genes that may be involved, directly or indirectly, in the regulation of the extracellular proteases have been identified through genetic studies. Nitrogen metabolite repression of the *A. nidulans* extracellular proteases is mediated by the *areA* gene product, a positive-acting DNA-binding protein (Cohen 1972; Kudla *et al.* 1990). *A. nidulans* strains carrying *areA*$^-$ loss-of-function mutations are unable to use protein as a source of nitrogen (Arst and Cove 1973). Mutations in the *creB* and *creC* genes lead to higher levels of extracellular protease and to derepression of some enzymes involved in carbon catabolism (Hynes and Kelly 1977). The *creA* gene encodes a DNA-binding protein that plays a major role in carbon catabolite repression in *A. nidulans*, but it is not known whether the *creB* and *creC* genes have a direct involvement in gene regulation (Kelly 1994).

In a previous study (Katz *et al.* 1996) specifically designed to isolate *A. nidulans* mutants with altered levels of extracellular protease, three additional genes (*xprE*, *xprF*, and *xprG*) involved in the regulation of extracellular proteases were identified. The *xprE1* mutation results in a protease-deficient phenotype that, at least in the case of the *prtA* gene, is due to very low levels of protease mRNA. The isolation of *xprE1* revertants led to the characterization of the *xprF1, xprF2*, and *xprG1* mutations,

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TABLE 1

***A. nidulans* strains used in the study**

Strain	Genotype[a]	Source
G47	*pabaA22 frA1*-T1(IV; VIII) *pyroA4*	J. Clutterbuck
G715	*biA1; AcrA1; actB1 oliC1 imaA4 benC18 choA1; cbxB1*	J. Clutterbuck
MH97	*yA1 pabaA1 acuE215*	M. J. Hynes
MK82	*biA1; xprF2; riboB2*	Katz *et al.* (1996)
MK86	*suA-adE20 yA1 adE20; xprG1; niiA4 riboB2*	Katz *et al.* (1996)
MK87	*pabaA1 yA1 acuE215; xprG1*	This study
MK117	*pabaA1 yA1 acuE215; xprF1*	Katz *et al.* (1996)
MK142	*yA1 acuE215; prnΔ309 xprF1; niiA4 riboB2*	This study
MK186	*yA1 acuE215; prnΔ309 xprF1 xprG2; niiA4 riboB2*	This study
MK187	*yA1 acuE215; prnΔ309 xprF1 xprG3; niiA4 riboB2*	This study
MK192	*pabaA1 yA1 acuE215; areA102; xprG1*	This study
MK198	*pabaA1 prnΔ309 xprG2; niiA4*	This study
MK218	*yA2 xprF1 amdA7 cnxF8*	This study
MK282	*frA1*-T1(IV; VIII) *xprF1*	This study
TBH-7	*biA1; areA102; sarB7; niiA4*	M. A. Davis

[a] For the meanings of gene symbols see Clutterbuck (1993).

which suppress the protease-deficient phenotype of *xprE1* mutants.

The *xprF1*, *xprF2*, and *xprG1* mutants display a number of phenotypic similarities: (1) the mutants all show increased levels of extracellular protease in response to carbon limitation and, to a much lesser extent, nitrogen limitation. (2) The ability of these mutants to utilize a number of nitrogen sources is affected. (3) The *xprF1* and *xprG1* mutations suppress the *areA217* defect in the utilization of milk as both a carbon and nitrogen source but not as a nitrogen source alone. (4) The *xprG1* mutation and, to a lesser extent, the *xprF1* and *xprF2* mutations show incomplete dominance. Here, we provide evidence for the interaction of these two genes by showing that mutations in *xprG* can suppress mutations in the *xprF* gene. We also report the isolation of the *xprF* gene and show that this gene encodes a hexokinase-like protein that appears to represent a new class in this family of proteins.

MATERIALS AND METHODS

Aspergillus strains, media, growth conditions, and genetic techniques: The *A. nidulans* strains used in this study are listed in Table 1. *A. nidulans* was cultured at 37° in the minimal medium described by Cove (1966) except that glucose was omitted from media that contained other carbon sources. For media that contained 1% skim milk as a nitrogen or carbon source, sodium deoxycholate (0.08%) was used to induce compact colony formation. The techniques used for genetic manipulation of *A. nidulans* have been described (Clutterbuck 1974).

Transformation of *A. nidulans*: The preparation and transformation of *A. nidulans* protoplasts have been described previously (Tilburn *et al.* 1983). All transformation experiments involved cotransformation with a second plasmid carrying a selectable marker—pAN222, which carries part of the *A. nidulans prn* gene cluster (Hull *et al.* 1989), or pL3, which carries the *A. nidulans riboB* gene (Oakley *et al.* 1987). Transformation recipient strains carried either the *prnΔ309* deletion mutation and/or the *riboB2* mutation. The prn$^+$ (pAN222) or ribo$^+$ (pL3) transformants were selected on medium containing proline as a nitrogen source and on medium lacking the vitamin riboflavin, respectively. Transformants were subsequently replicated to medium containing milk as a sole carbon source and medium containing hypoxanthine as a nitrogen source to test for the presence of the *xprF* gene on the nonselected DNA. The proportion of prn$^+$ or ribo$^+$ transformants that were also xpr$^+$ was 7% for cotransformations with L32F12 cosmid DNA (7 out of 96 MK142 transformants) and 7% for pMK370 plasmid DNA (7 xpr$^+$ribo$^+$ transformants out of 111 ribo$^+$ transformants of MK142 and 10 xpr$^+$ribo$^+$ transformants out of 141 ribo$^+$ transformants of MK82).

Isolation of the *xprF* gene: The *A. nidulans* chromosome-specific cosmid library, which consisted of *A. nidulans* DNA inserted in the pWE15 and Lorist2 cosmid vectors (Brody *et al.* 1991), was obtained from the Fungal Genetics Stock Center. Clones containing chromosome VII cosmids were pooled into groups of 24. Cosmid DNA from nine pools containing 203 Lorist2 clones were used together with the selectable plasmid pAN222 in cotransformation experiments that used the *xprF1* recipient strain MK142. Two *xprF*$^+$ transformants were obtained from one of the nine pools. The 11 cosmids within this pool were then tested individually and cosmid L32F12 was shown to generate *xprF*$^+$ cotransformants from the MK142 recipient strain.

To localize the *xprF* gene within L32F12, deletion subclones were generated from L32F12 by partial *Hin*dIII digestion and recircularization using T4 DNA ligase. The deletion subclones were tested for the ability to generate *xprF*$^+$ transformants from the *xprF1* recipient strain MK142 in cotransformation experiments using the pL3 selectable plasmid. Additional subclones and cosmid clones adjacent to L32F12 on the *A. nidulans* physical map were also tested.

DNA sequencing and molecular techniques: Standard techniques were used for DNA cloning, restriction enzyme digestion, Southern and Northern blot analysis, and DNA sequencing (Sambrook *et al.* 1989).

Extraction of Aspergillus DNA and RNA: Genomic DNA was extracted from *A. nidulans* by the method of Andrianopoulos

and Hynes (1988). RNA was prepared using a procedure developed by Reinert *et al.* (1981).

RT-PCR analysis: The Access reverse transcriptase (RT)-PCR System (Promega, Madison, WI) was used for all RT-PCR analyses. The reactions used 50 ng of total RNA prepared from mycelium of *A. nidulans* strain MH97 that had been grown in minimal medium containing 1% glucose as a carbon source. The reactions were performed using three pairs of primers, which are shown in Figure 4: (1) MK106 and MK110, (2) MK107 and MK110, and (3) MK113 and MK108. For each primer pair, a control PCR experiment was performed using DNA template. The RT-PCR product obtained with MK107 and MK110 was cloned using the SureClone ligation kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and the DNA sequence was determined.

Characterization of the *xprF1* and *xprF2* alleles: The entire coding region of the *xprF1* and *xprF2* alleles was amplified in PCR experiments that used two primer pairs shown in Figure 4: (1) MK106 and MK108 and (2) MK107 and MK108. The DNA sequences of the *xprF1* and *xprF2* coding regions were determined by direct sequence analysis of the PCR amplification product and by sequence analysis of PCR products, which had been cloned using the SureClone ligation kit (Amersham Pharmacia Biotech). The single base pair alterations that were detected in each allele were confirmed by sequencing both strands of the amplified DNA.

Enzyme assays: The assays used to measure protease activity in *A. nidulans* growth medium were performed as described previously (Katz *et al.* 1996). The method of Ruijter *et al.* (1996) was used to measure the phosphorylation of glucose and fructose in *A. nidulans* cell extracts prepared from mycelia grown for 16 hr in medium containing 1% glucose as a carbon source and then transferred to fresh medium containing 1% glucose for 3 hr.

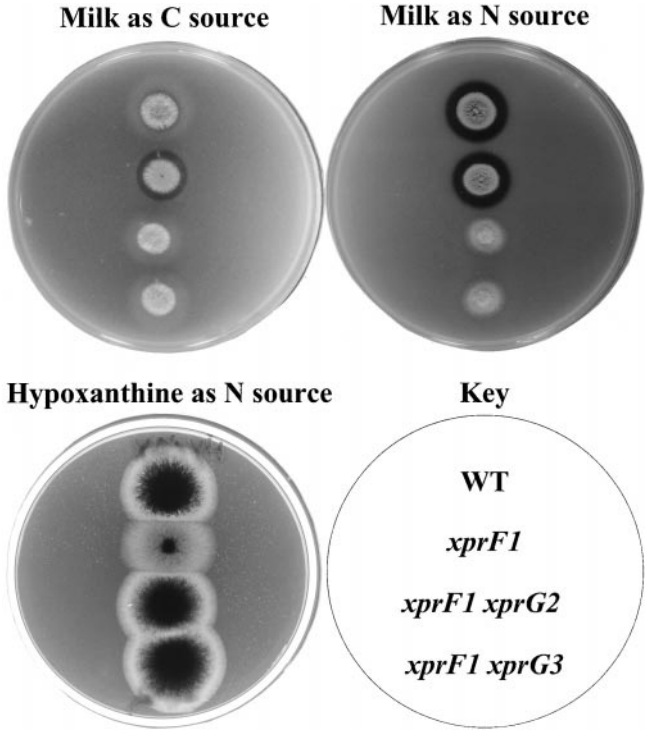


Figure 1.—Phenotype of *xprF1* revertants. Extracellular protease production of MH97 (wild type), MK142 (*xprF1*), and two revertant strains, MK186 (*xprF1 xprG2*) and MK187 (*xprF1 xprG3*), on medium containing 1% skim milk as a sole source of carbon (C) or nitrogen (N) are shown in the top row. The growth of the strains on medium containing 10 mm hypoxanthine as a sole nitrogen source is shown in the lower plate. The full genotypes of the strains are given in Table 1. Aspergillus media and growth conditions are described in materials and methods.

RESULTS

Isolation of *xprF1* revertants: Strains carrying the *xprF1* mutation are easily distinguished by two methods. The *xprF1* mutation has a negative effect on the utilization of a number of nitrogen sources, particularly hypoxanthine, and causes a high level of extracellular protease activity on medium containing milk as a carbon source (Figure 1). The latter phenotype results in the production of colonies surrounded by a clear halo, due to extracellular protease activity. The *xprF2* and *xprG1* mutations have similar phenotypic effects. Wild-type strains produce high levels of extracellular protease activity on medium containing milk as a nitrogen source but are not surrounded by a clear halo after 48 hr of growth on medium containing milk as a carbon source (Figure 1).

Two *xprF1* revertants (MK186 and MK187), which showed strong growth on medium containing hypoxanthine, were isolated. These revertants both had pale conidia and also showed a loss of extracellular protease activity on medium containing milk as a carbon or nitrogen source (Figure 1). Genetic analysis showed that both revertants carried extragenic suppressors that were unlinked to the *xprF1* mutation. The phenotype of strains carrying only the suppressor mutation was indistinguishable from the phenotype of strains carrying both the *xprF1* and suppressor mutations. The suppressor mutations from MK186 and MK187 were mapped to chromosome VII by haploidization analysis. The *xprF* and *xprG* genes are both located on chromosome VII but are unlinked. To determine whether the suppressor mutations were alleles of the *xprG* gene, a strain (MK198) carrying the MK186 suppressor mutation was crossed to an *xprG1* strain (MK86). Only segregants that did not have pale conidia were scored for growth on hypoxanthine medium and extracellular production on medium containing milk as a carbon source. All of the 819 segregants that were scored proved to have an *xprG1* phenotype. The lack of wild-type segregants from this cross suggests that the suppressor mutation in MK186 is an allele of the *xprG* gene and has thus been designated *xprG2*.

Two lines of evidence indicate that the *xprF1* suppressor in MK187 is also an allele of the *xprG* gene (*xprG3*): (1) in a cross between strain MK198, which carries *xprG2*, and MK187, no wild-type segregants were detected among the 434 segregants scored and (2) a diploid constructed from MK198 and MK187 displayed a phenotype that was identical to both haploid strains

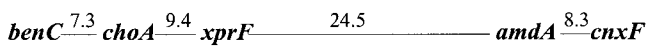


FIGURE 2.—Partial linkage map of chromosome VII showing the location of the *xprF* gene. Gene order and linkage distances in centimorgans (cM) were derived from the analysis of 192 segregants from a cross between MK218 and G715. The distance between *benC* and *choA* has been reported as 6 cM and the distance between *amdA* and *cnxF* as 13 cM according to CLUTTERBUCK (1993).

unlike diploids constructed between haploid strains carrying each suppressor mutation and wild-type haploids. The latter *xprG2/xprG*$^+$ and *xprG3/xprG*$^+$ diploids had levels of extracellular protease that were similar to wild-type strains on medium containing milk as a nitrogen source, indicating that the *xprG2* and *xprG3* mutations are recessive.

Interactions with *cre* genes: To examine the interactions between the *xprF1* mutation and mutations in the *creA*, *creB*, and *creC* genes, double mutants were constructed. Because the *creA204*, *creB15*, and *creC27* mutations relieve carbon catabolite repression of genes involved in ethanol metabolism, strains carrying these mutations are more sensitive than cre$^+$ strains to the presence of allyl alcohol in growth medium containing a repressing carbon source such as sucrose. The *xprF1* mutant does not show increased sensitivity to allyl alcohol. Strains carrying the *creB15* and *creC27* mutation have elevated levels of extracellular protease activity and reduced growth on hypoxanthine medium but to a lesser degree than the *xprF1* mutant. The *creA204* mutation does not increase extracellular protease production, nor affect utilization of hypoxanthine. The *creB15 xprF1* and *creC27 xprF1* double mutants showed a more extreme phenotype than *xprF1*, *creB15*, or *creC27* single mutants. The double mutants produced higher levels of extracellular protease on medium containing milk as a carbon source and grew more poorly on medium containing hypoxanthine as a nitrogen source. The *creA204 xprF1* mutants showed no evidence of an additive effect for the two mutations. These results suggest that mutations in the *creB* and *creC* genes affect the same regulatory pathway as mutations in the *xprF* gene.

Mapping of the *xprF* and *xprG* genes: Previous studies had shown that the *xprF* and *xprG* genes were both located on *A. nidulans* chromosome VII but were unlinked to each other (KATZ *et al.* 1996). Genetic mapping was used to determine the position of the *xprF* gene on chromosome VII (Figure 2). The *xprG* gene was mapped to within 20 map units of the *phenB* gene.

To determine if either of these two genes were alleles of genes known to have a role in carbon or nitrogen regulation, we looked to see if any such genes are found on chromosome VII. The *sarA* and *sarB* genes are thought to have a role in nitrogen regulation (POLKINGHORNE and HYNES 1975). Though both genes are located on chromosome VII, their positions on the chromosome are unknown. The *sarA3* and *sarB7* mutations suppress the strong growth of the *areA102* gain-of-function mutant on nitrogen sources such as lysine and histidine. Neither the *xprF* or *xprG* gene appeared to be linked to *sarA*, but the *xprG* gene proved to be very tightly linked to *sarB*. Of 1149 segregants from a cross between strains MK192 (*xprG1*) and TBH-7 (*sarB7*), three proved to be recombinants, including one that displayed the high extracellular protease levels of an *xprG1* mutant and reduced ability to utilize lysine as a nitrogen source characteristic of a *sarB7* mutant. The recovery of a segregant that showed a *xprG1sarB7* phenotype makes it unlikely that *sarB7* and *xprG1* are alleles of the same gene but this possibility cannot be ruled out.

Isolation of the *xprF* gene: The *xprF* gene was isolated from the chromosome-specific *A. nidulans* cosmid library (BRODY *et al.* 1991) by cotransformation of an *xprF1* strain (MK142) with DNA from pooled chromosome VII-specific cosmids and a plasmid containing a selectable *A. nidulans* marker. A single cosmid (L32F12) that complemented the *xprF1* mutation was obtained. The *xprF* gene was localized within the cosmid in similar cotransformation experiments using partial deletion derivatives of L32F12, plasmid subclones of L32F12, and adjacent cosmid clones from the physical map of *A. nidulans* that had subsequently become available (PRADE *et al.* 1997). The smallest segment of L32F12 DNA, which was capable of generating *xprF*$^+$ transformants from an *xprF1* recipient strain, was the 2.3-kb fragment inserted in pMK370 (Figure 3). Transformants that contained a single copy of pMK370 displayed normal levels of extracellular protease on media containing milk as a carbon or nitrogen source. Transformants that contained more than one copy of pMK370 had reduced levels of extracellular protease relative to a wild-type strain and distinctly paler conidial coloring though not as marked as in the *xprG2* and *xprG3* mutants. The pMK370 plasmid was also able to generate *xprF*$^+$ transformants from an *xprF2* strain (MK82), thereby confirming that the *xprF1* and *xprF2* mutations were alleles of the same gene.

DNA sequence analysis of the *xprF* gene: Sequence analysis revealed that pMK370 contained an open reading frame of 615 amino acids interrupted by a single intron, the boundaries of which were confirmed by RT-PCR (Figure 4). As the open reading frame ends 54 bp from the end of the pMK370 insert, it is possible that pMK370 does not contain the *xprF* polyadenylation signal. No significant similarity between the 2.3-kb DNA sequence of the fragment containing the *xprF* gene and the sequences in GenBank was detected. However, the deduced amino acid sequence showed similarity to a large number of hexokinases and glucokinases (aldohexose-specific hexokinases). Surprisingly, the putative XprF sequence showed no greater similarity to fungal hexokinases than to human or plant hexokinases (16–

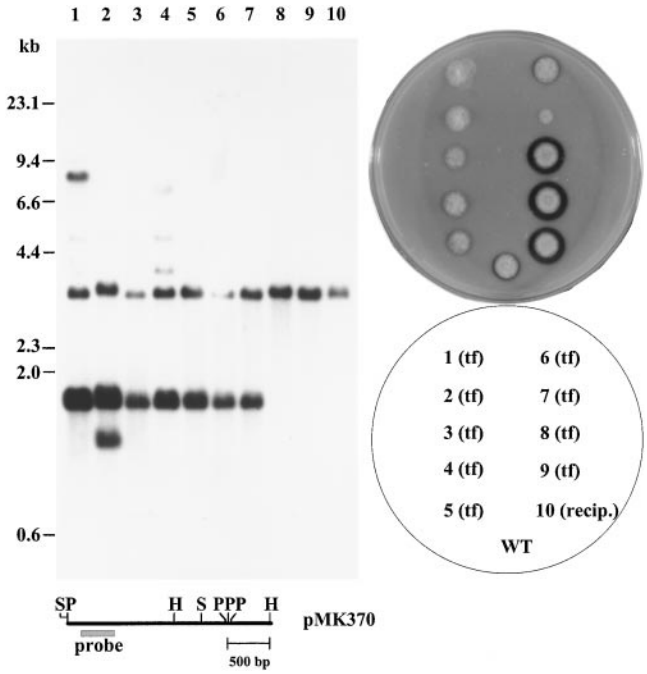


FIGURE 3.—Southern blot analysis and extracellular protease production of MK142 transformants. Transformants were generated by cotransformation of an *xprF1 riboB2* strain (MK142) with pMK370 (containing the *xprF* gene) and pL3 (containing the *riboB* gene). Lanes 1–7 contain *Sma*I-digested genomic DNA from seven ribo$^+$ xpr$^+$ transformants of MK142, lanes 8 and 9 contain genomic DNA from two ribo$^+$ transformants that did not display an xpr$^+$ phenotype, and lane 10 contains DNA from the recipient strain, MK142. The blot was probed with the 0.3-kb *Hin*dII fragment indicated under the restriction map of pMK370 (S, *Sma*I; P, *Pst*I; H, *Hin*dIII). Transformants that have acquired a copy of pMK370 are expected to contain an additional 1.6-kb *Sma*I fragment. Extracellular protease production of the transformants, the *xprF1* recipient strain MK142, and a wild-type strain (MH97), on medium containing 1% skim milk as a carbon source, is shown on the right.

19% amino acid identity with fungal hexokinases, mammalian glucokinases, *Arabidopsis thaliana* hexokinases, *Entamoeba histolytica* hexokinases, and *Schistosoma mansoni* hexokinases).

The isolation of three Aspergillus hexokinase genes has been reported, one from *A. nidulans* (*frA*, RUIJTER *et al.* 1996) and two from *A. niger* (*glkA* and *hxkA*, PANNEMAN *et al.* 1996, 1998). Only part of the sequence from the *A. nidulans frA* gene product is available (RUIJTER *et al.* 1996). Comparison of the partial FrA sequence to the deduced amino acid sequence of the *xprF* gene shows many differences and the *frA* gene does not map to *A. nidulans* chromosome VII. The XprF sequence shows 18% amino acid identity to the *A. niger* hexokinase, HxkA, and 17% to the *A. niger* glucokinase, GlkA. In contrast, HxkA showed over 50% amino acid identity to hexokinases from *Saccharomyces cerevisiae, Kluyveromyces lactis, Schwanniomyces occidentalis,* and *Yarrowia lipolytica* (PANNEMAN *et al.* 1998) and GlkA showed 39% amino acid identity with the *S. cerevisiae* glucokinase and *Schizosaccharomyces pombe* hexokinase 2 (PANNEMAN *et al.* 1996).

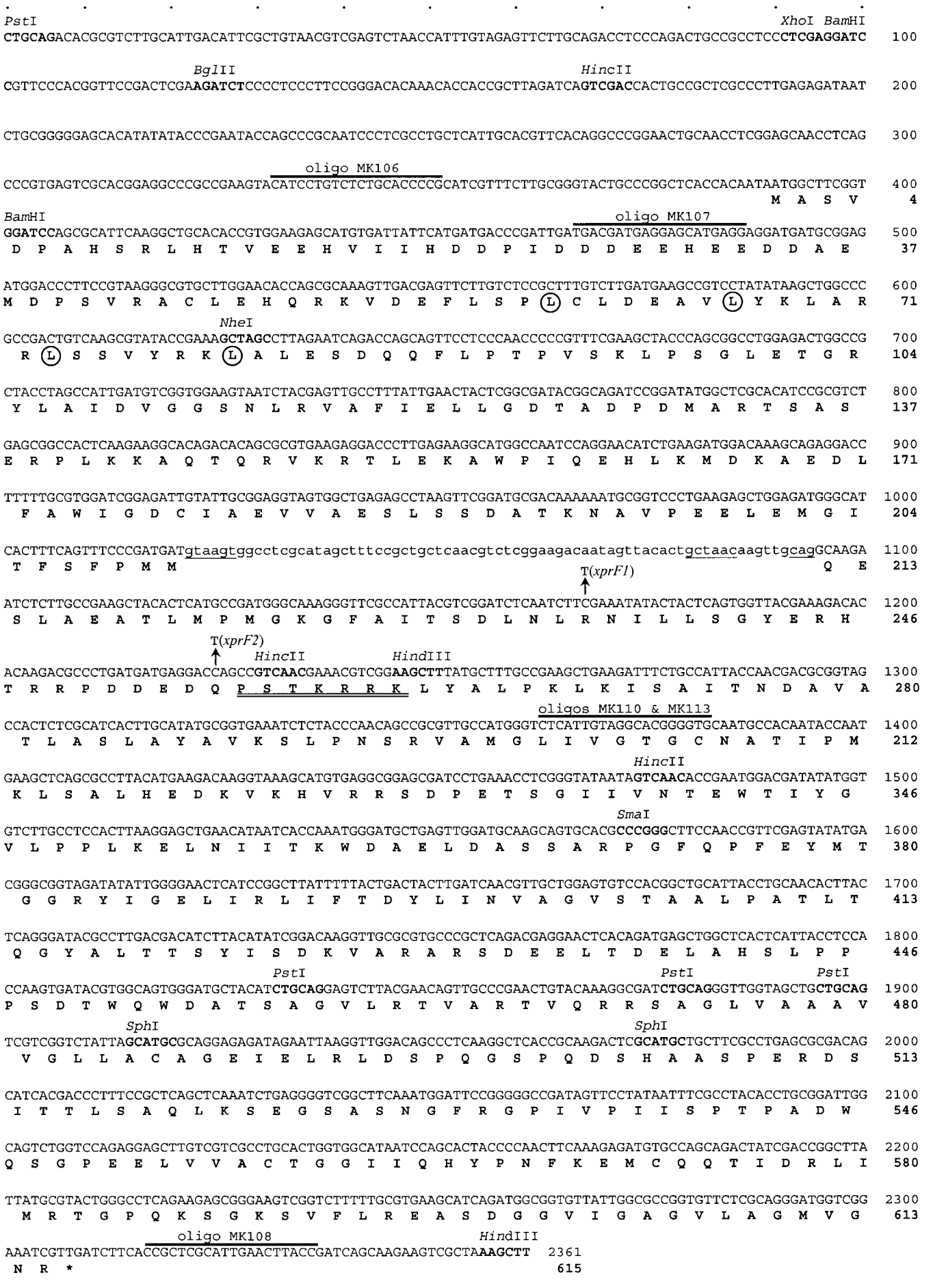
Phylogenetic analysis confirmed that the XprF sequence was not closely related to other fungal hexokinases (Figure 5). Although the XprF sequence does not cluster with other fungal hexokinases, it is more closely related to hexokinases as a group than to other sugar kinases. In these analyses, the fungal hexokinases of all types formed a separate group, as do mammalian and plant hexokinases, suggesting that the development of multiple hexokinases has occurred independently within each lineage (BORK *et al.* 1993).

Structure of XprF: Most of the conserved amino acid residues, which are found in the ATP-binding sites and sugar-binding sites of all other eukaryotic hexokinases, are present in the predicted *xprF* gene product (Figure 6). The putative hexose-binding site of XprF (GITFSFP) differs from the hexose-binding sites in most organisms, which contain the sequence GFTFSF/YP. The only other exceptions are the two hexokinases from *E. histolytica* (GLTFSFA). In addition, all other reported hexokinase sequences contain the sequence WTKGF seven amino acids from the C-terminal side of the sugar-binding site, whereas XprF contains the sequence MGKGF (Figure 6).

The XprF sequence is longer than other fungal hexokinases that have been characterized, the longest of which is the *Y. lipolytica* hexokinase at 534 amino acids (PETIT and GANCEDO 1999). The N terminus of the XprF protein contains 37 amino acid residues not found in other hexokinases. Fifteen of these 37 amino acids are acidic. Similarity to other hexokinases commences at a second methionine residue. RT-PCR experiments showed that *xprF* mRNA includes the sequences encoding this acidic region. Four leucine residues, at 7-amino-acid intervals, are found after this region (Figure 4). A potential nuclear localization sequence (KALDERON *et al.* 1984) is found at amino acids 256–262 (Figure 4). The XprF protein contains a stretch of some 60 amino acids in the C-terminal part of the protein that are not found in other hexokinases and interrupts part of the ATP-binding site (Figure 6).

Molecular characterization of the *xprF1* and *xprF2* mutations: The nature of the *xprF1* and *xprF2* mutations was determined by amplification and sequence analysis of the *xprF* coding region from each mutant. A single base pair alteration was detected at nucleotide 1168 in the *xprF1* allele and at nucleotide 1225 in the *xprF2* allele. In both cases the base pair substitutions cause nonsense mutations (Figure 4).

Hexokinase activity in *xprF*$^-$ mutants: Since sequence analysis of the *xprF* gene suggested that it encodes a putative hexokinase, the *xprF1* and *xprF2* mutants were assayed for the ability to phosphorylate glucose and fructose (Table 2). The assays were performed in the presence and absence of trehalose 6-phosphate, which


PstI
XhoI BamHI
CTGCAGACACGCGTCTTGCATTGACATTCGCTGTAACGTCGAGTCTAACCATTTGTAGAGTTCTTGCAGACCTCCCAGACTGCCGCCTCCCTCGAGGATC 100
BglII
HincII
CGTTCCCACGGTTCCGACTCGAAGATCTCCCCTCCCTTCCGGGACACAAACACCACCGCTTAGATCAGTCGACCACTGCCGCTCGCCCTTGAGAGATAAT 200
CTGCGGGGGAGCACATATATACCCGAATACCAGCCCGCAATCCCTCGCCTGCTCATTGCACGTTCACAGGCCCGGAACTGCAACCTCGGAGCAACCTCAG 300
oligo MK106
CCCGTGAGTCGCACGGAGGCCCGCCGAAGTACATCCTGTCTCTGCACCCCGCATCGTTTCTTGCGGGTACTGCCCGGCTCACCACAATAATGGCTTCGGT 400
M A S V 4
BamHI
oligo MK107
GGATCCAGCGCATTCAAGGCTGCACACCGTGGAAGAGCATGTGATTATTCATGATGACCCGATTGATGACGATGAGGAGCATGAGGAGGATGATGCGGAG 500
D P A H S R L H T V E E H V I I H D D P I D D D E E H E E D D A E 37
ATGGACCCTTCCGTAAGGGCGTGCTTGGAACACCAGCGCAAAGTTGACGAGTTCTTGTCTCCGCTTTGTCTTGATGAAGCCGTCCTATATAAGCTGGCCC 600
M D P S V R A C L E H Q R K V D E F L S P L C L D E A V L Y K L A R 71
NheI
GCCGACTGTCAAGCGTATACCGAAAGCTAGCCTTAGAATCAGACCAGCAGTTCCTCCCAACCCCCGTTTCGAAGCTACCCAGCGGCCTGGAGACTGGCCG 700
R L S S V Y R K L A L E S D Q Q F L P T P V S K L P S G L E T G R 104
CTACCTAGCCATTGATGTCGGTGGAAGTAATCTACGAGTTGCCTTTATTGAACTACTCGGCGATACGGCAGATCCGGATATGGCTCGCACATCCGCGTCT 800
Y L A I D V G G S N L R V A F I E L L G D T A D P D M A R T S A S 137
GAGCGGCCACTCAAGAAGGCACAGACACAGCGCGTGAAGAGGACCCTTGAGAAGGCATGGCCAATCCAGGAACATCTGAAGATGGACAAAGCAGAGGACC 900
E R P L K K A Q T Q R V K R T L E K A W P I Q E H L K M D K A E D L 171
TTTTTGCGTGGATCGGAGATTGTATTGCGGAGGTAGTGGCTGAGAGCCTAAGTTCGGATGCGACAAAAAATGCGGTCCCTGAAGAGCTGGAGATGGGCAT 1000
F A W I G D C I A E V V A E S L S S D A T K N A V P E E L E M G I 204
CACTTTCAGTTTCCCGATGATgtaagtggcctcgcatagctttccgctgctcaacgtctcggaagacaatagttacactgctaacaagttgcagGCAAGA 1100
T F S F P M M Q E 213
T(xprF1)
ATCTCTTGCCGAAGCTACACTCATGCCGATGGGCAAAGGGTTCGCCATTACGTCGGATCTCAATCTTCGAAATATACTACTCAGTGGTTACGAAAGACAC 1200
S L A E A T L M P M G K G F A I T S D L N L R N I L L S G Y E R H 246
T(xprF2)
HincII
HindIII
ACAAGACGCCCTGATGATGAGGACCAGCCGTCAACGAAACGTCGGAAGCTTTATGCTTTGCCGAAGCTGAAGATTTCTGCCATTACCAACGACGCGGTAG 1300
T R R P D D E D Q P S T K R R K L Y A L P K L K I S A I T N D A V A 280
oligos MK110 & MK113
CCACTCTCGCATCACTTGCATATGCGGTGAAATCTCTACCCAACAGCCGCGTTGCCATGGGTCTCATTGTAGGCACGGGGTGCAATGCCACAATACCAAT 1400
T L A S L A Y A V K S L P N S R V A M G L I V G T G C N A T I P M 212
HincII
GAAGCTCAGCGCCTTACATGAAGACAAGGTAAAGCATGTGAGGCGGAGCGATCCTGAAACCTCGGGTATAATAGTCAACACCGAATGGACGATATATGGT 1500
K L S A L H E D K V K H V R R S D P E T S G I I V N T E W T I Y G 346
SmaI
GTCTTGCCTCCACTTAAGGAGCTGAACATAATCACCAAATGGGATGCTGAGTTGGATGCAAGCAGTGCACGCCCGGGCTTCCAACCGTTCGAGTATATGA 1600
V L P P L K E L N I I T K W D A E L D A S S A R P G F Q P F E Y M T 380
CGGGCGGTAGATATATTGGGGAACTCATCCGGCTTATTTTTACTGACTACTTGATCAACGTTGCTGGAGTGTCCACGGCTGCATTACCTGCAACACTTAC 1700
G G R Y I G E L I R L I F T D Y L I N V A G V S T A A L P A T L T 413
TCAGGGATACGCCTTGACGACATCTTACATATCGGACAAGGTTGCGCGTGCCCGCTCAGACGAGGAACTCACAGATGAGCTGGCTCACTCATTACCTCCA 1800
Q G Y A L T T S Y I S D K V A R A R S D E E L T D E L A H S L P P 446
PstI
PstI
PstI
CCAAGTGATACGTGGCAGTGGGATGCTACATCTGCAGGAGTCTTACGAACAGTTGCCCGAACTGTACAAAGGCGATCTGCAGGGTTGGTAGCTGCTGCAG 1900
P S D T W Q W D A T S A G V L R T V A R T V Q R R S A G L V A A A V 480
SphI
SphI
TCGTCGGTCTATTAGCATGCGCAGGAGAGATAGAATTAAGGTTGGACAGCCCTCAAGGCTCACCGCAAGACTCGCATGCTGCTTCGCCTGAGCGCGACAG 2000
V G L L A C A G E I E L R L D S P Q G S P Q D S H A A S P E R D S 513
CATCACGACCCTTTCCGCTCAGCTCAAATCTGAGGGGTCGGCTTCAAATGGATTCCGGGGGCCGATAGTTCCTATAATTTCGCCTACACCTGCGGATTGG 2100
I T T L S A Q L K S E G S A S N G F R G P I V P I I S P T P A D W 546
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Q S G P E E L V V A C T G G I I Q H Y P N F K E M C Q Q T I D R L I 580
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M R T G P Q K S G K S V F L R E A S D G G V I G A G V L A G M V G 613
oligo MK108
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N R * 615

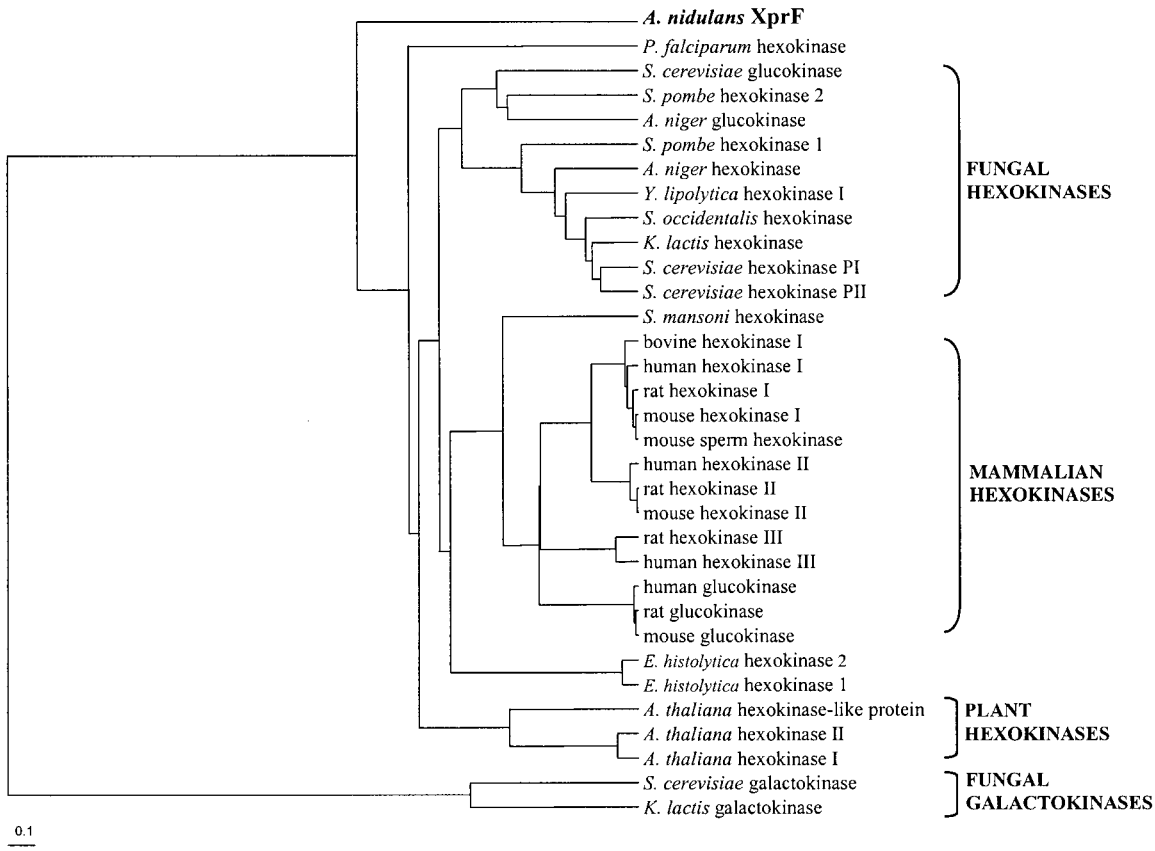


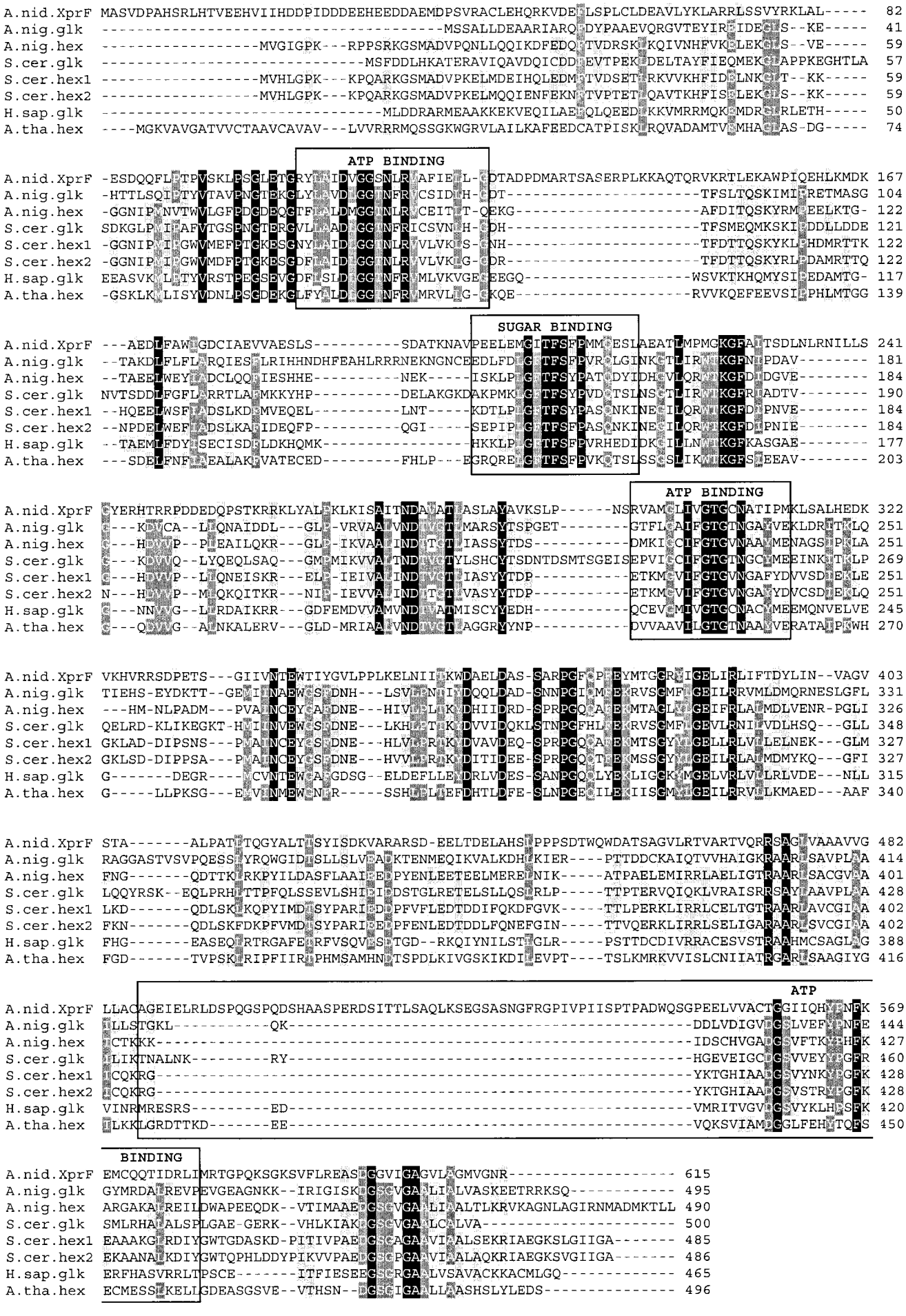
FIGURE 5.—Phylogenetic tree of the *A. nidulans* XprF sequence and hexokinases from other species. The sources for the amino acid sequences are as follows: *A. thaliana* hexokinase I and II (JANG *et al.* 1997) and hexokinase-like protein (GenPept accession no. CAB38932); *A. niger* glucokinase (PANNEMAN *et al.* 1996) and hexokinase (PANNEMAN *et al.* 1998); bovine hexokinase I (GRIFFIN *et al.* 1991); *E. histolytica* hexokinase 1 and 2 (ORTNER *et al.* 1995); human hexokinase I (NISHI *et al.* 1988), II (DEEB *et al.* 1993), III (FURUTA *et al.* 1996), and glucokinase (STOFFEL *et al.* 1992); *K. lactis* hexokinase (PRIOR *et al.* 1993) and galactokinase (MEYER *et al.* 1991); mouse hexokinase I (ARORA *et al.* 1990), II (HEIKKINEN *et al.* 1999), sperm hexokinase (MORI *et al.* 1993), and glucokinase (ISHIMURA-OKA *et al.* 1995); *P. falciparum* hexokinase (OLAFSSON *et al.* 1992); rat hexokinase I (SCHWAB and WILSON 1989), II (THELEN and WILSON 1991), III (SCHWAB and WILSON 1991), and glucokinase (HUGHES *et al.* 1991); *S. cerevisiae* glucokinase (ALBIG and ENTIAN 1988), hexokinase PI (KOPETZKI *et al.* 1985), hexokinase pII (FRÖHLICH *et al.* 1985), and galactokinase (BAJWA *et al.* 1988); *S. mansoni* hexokinase (SHOEMAKER *et al.* 1995); *S. pombe* hexokinase 1 and 2 (PETIT *et al.* 1996); *S. occidentalis* hexokinase (ROSE 1995); *Y. lipolytica* hexokinase I (PETIT and GANCEDO 1999). The rooted tree was constructed using the EPROTDIST and EKITSCH programs in the PHYLIP computer package (FELSENSTEIN 1996) through the Australian National Genome Information Service.

is an inhibitor of hexokinases but not glucokinases. Thus, the level of glucose phosphorylation activity observed, when trehalose 6-phosphate is present, is an indication of glucokinase activity. No differences between the mutant and wild-type activity levels were detected.

Sequence analysis of the *A. nidulans frA* gene indicates that it encodes a hexokinase and the *frA1* mutant has been shown to have very low levels of fructose phosphorylating activity (RUIJTER *et al.* 1996). The residual fructose phosphorylating activity observed in the *frA1* mutant was not abolished in an *frA1xprF1* double mutant.

The effect of the *xprF1* mutation on carbon catabolite repression of extracellular proteases: Hexokinases are thought to play a role in triggering glucose repression in fungi (RONNE 1995). Thus, the most likely explanation for the phenotype of the *xprF1* and *xprF2* mutants is that the loss of the *xprF*-encoded hexokinase alleviates glucose repression of the extracellular proteases. However, production of extracellular proteases appeared

FIGURE 4.—DNA sequence of the *A. nidulans* sequences in pMK370 (EMBL/GenBank/DDBJ accession no. AJ251893). Restriction sites are marked in boldface type. The 5′ GTRNGT-RCTRAC-YAG consensus sequences for fungal introns (RAMBOSEK and LEACH 1986) are underlined and the intron is in lowercase letters. A potential nuclear localization sequence is double underlined and four leucine heptad repeats are circled. The base pair substitutions found in the *xprF1* and *xprF2* mutants are indicated with arrows. The oligonucleotides used in RT-PCR experiments are overlined. The sequences of oligonucleotides MK110 and MK108 are complementary to the sequences shown in the diagram.


ATP BINDING
SUGAR BINDING
ATP BINDING
ATP
BINDING

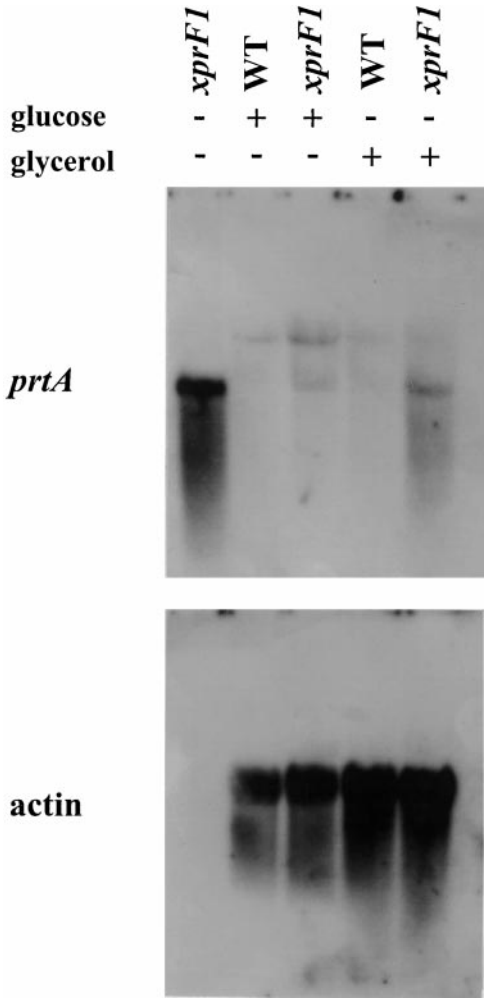


FIGURE 7.—Northern blot analysis of alkaline protease mRNA levels. Total RNA was prepared from a wild-type (MH97) and *xprF1* (MK142 and MK117) strains transferred to medium containing glucose, glycerol, or no carbon source for 16 hr. Duplicate blots were probed with ^{32}P-labeled pBC174 plasmid containing the entire coding regions of the *prtA* gene (KATZ *et al.* 1994) and, as a control, a plasmid containing nucleotides 404–1711 of the constitutively expressed γ-actin gene (FIDEL *et al.* 1988). Note that much less RNA was used in the first lane.

to be completely repressed in mycelia transferred to medium containing glucose, lactose, galactose, or glycerol (Table 3) or in mycelia grown from conidia inoculated directly into media containing these carbon sources (data not shown). The effect of the *xprF1* mutation on *prtA* mRNA levels was examined using Northern blot analysis. In medium containing glucose or glycerol, *prtA* expression was detected in the *xprF1* mutant but not in a wild-type strain (Figure 7). However, the level of *prtA* expression was very much lower than is observed in carbon-limiting conditions.

DISCUSSION

The isolation and characterization of the *xprF* gene has shown that it encodes a new type of fungal hexokinase or hexokinase-like protein. Hexokinases catalyze the first step in the metabolism of glucose and other hexose sugars. In addition, hexokinases have been shown to have a role in the uptake of glucose (BISSON and FRAENKEL 1983) and as the glucose sensor that triggers glucose repression in fungi, plants, and animals.

The hexokinases of the yeast *S. cerevisiae* have been well characterized. Three genes (HXK1, HXK2, and GLK1) that encode enzymes for glucose phosphorylation are found in *S. cerevisiae*. A functional copy of only one of the three genes is required for glucose utilization (MAITRA and LOBO 1983). The HXK2 gene product is the predominant hexokinase and plays a major role in triggering glucose repression (ENTIAN 1980). However, there is some evidence that all three genes may be involved in mediating an early glucose repression response (SANZ *et al.* 1996). Recent work suggests that the *S. cerevisiae* hexokinase pII may have a direct role in regulating gene expression. It has been shown that nuclear localization of the *S. cerevisiae* hexokinase pII protein is required to trigger glucose repression of the *SUC2* gene and hexokinase pII is a component of a DNA-protein complex that forms on *SUC2* sequences (HERRERO *et al.* 1998).

Two hexokinase genes have been isolated from the plant *A. thaliana* by complementation in a *S. cerevisiae hxk1hxk2* double mutant (JANG *et al.* 1997). The results of transformation experiments with heterologous hexokinase genes suggest that in plants and fungi (1) heterologous hexokinases are unable to activate the signal transduction pathway and (2) hexokinase catalytic activity alone is not sufficient for regulatory function (PRIOR *et al.* 1993; JANG *et al.* 1997). In mammals, the situation is different; glucose metabolism is required at least for some aspects of the glucose response (GERMAN 1993).

No regulatory role for hexokinases has been established in Aspergillus. The *A. nidulans frA* gene encodes the major hexokinase found in this fungus. In contrast

FIGURE 6.—Alignment of the putative XprF amino acid sequence with the sequences of the *A. niger* glucokinase (A.nig.glk; PANNEMAN *et al.* 1996) and hexokinase (A.nig.hex; PANNEMAN *et al.* 1998); the *S. cerevisiae* glucokinase (S.cer.glk; ALBIG and ENTIAN 1988), hexokinase PI (S.cer.hex1; KOPETZKI *et al.* 1985), and hexokinase PII (S.cer.hex2; FRÖHLICH *et al.* 1985); human glucokinase (H.sap.glk; STOFFEL *et al.* 1992); and *A. thaliana* hexokinase 1 (A.tha.hex; JANG *et al.* 1997). Amino acids that are conserved in all eight proteins are shaded in black, those conserved in at least six of the proteins are shaded in dark gray, and those conserved in at least half of the proteins are shaded in light gray. The ATP-binding sites and sugar-binding sites, identified by BORK *et al.* (1993), are boxed. The alignment was generated with the ECLUSTALW (THOMPSON *et al.* 1994) and the GeneDoc (NICHOLAS *et al.* 1997) computer programs.

TABLE 2

Hexokinase and glucokinase activity in *xprF*⁻ mutants

Strain	Relevant genotype[a]	Hexose phosphorylating activity[b]			
		Glucose		Fructose	
		−T6P	+T6P	−T6P	+T6P
MH97	Wild type	430 (19)	140 (55)	466 (88)	73 (20)
MK142	*xprF1*	385 (39)	142 (10)	527 (0)	74 (10)
MK82	*xprF2*	371 (0)	135 (6)	444 (25)	66 (0)
G47	*frA1*	195 (11)	157 (22)	25 (5)	19 (0)
MK282	*frA1 xprF1*	143 (16)	107 (0)	40 (0)	40 (0)

[a] The full genotypes of the strains are given in Table 1.

[b] Phosphorylation of 1 mM glucose or 10 mM fructose was measured as described in MATERIALS AND METHODS. The phosphorylating activity is given in nmol/min/mg protein. The assays were performed in the presence (+T6P) or absence (−T6P) of the hexokinase inhibitor trehalose 6-phosphate. Each assay was performed in triplicate except in the assays of the wild-type strain in which six replicates were performed. The averages of the three (or six) results are given. The standard deviations are shown in parentheses.

to the situation in *S. cerevisiae*, loss of hexokinase activity in the *frA1* mutant does not appear to affect glucose repression (RUIJTER *et al.* 1996).

The similarity of the *xprF* gene product to hexokinases suggests that it may be involved in carbon catabolite repression of the extracellular proteases. The *xprF1* and *xprF2* mutations have a clear effect on extracellular protease production as measured on medium containing milk as a sole source of carbon. Yet, in enzyme assays of the *xprF1* mutant, the production of extracellular proteases appears to be fully repressed by glucose, lactose, galactose, and glycerol and only marginal derepression of the alkaline protease gene *prtA* was detected through Northern blot analysis. This discrepancy may be due to the presence of exogenous protein in milk, which could act as an inducer under these conditions, or it may reflect a greater sensitivity of milk plates in detecting low levels of extracellular protease.

Extracellular proteases are produced only if no source of carbon other than protein is present. Thus, the production of extracellular proteases may be a response to carbon *starvation*, which may involve a regulatory mechanism that is distinct from carbon catabolite repression. The *xprF1* and *xprF2* mutants exhibit higher levels of extracellular protease in response to carbon-limiting nutrient conditions suggesting that the *xprF* gene may have a role in the response to carbon starvation.

The demonstration that mutations in the *xprG* gene can suppress mutations in *xprF* provides further evidence for the interaction of these two genes. The data presented in this article are consistent with a model in which the *xprG* gene is a positive regulator of extracellu-

TABLE 3

Effect of carbon source on extracellular protease levels

Strain	Relevant genotype[a]	Carbon source[b]	Protease activity[c]
MH97	Wild type	None	55.3 (13.8)
MK142	*xprF1*	None	158.1 (50.9)
MH97	Wild type	1% glucose	9.4 (1.4)
MK142	*xprF1*	1% glucose	6.8 (0.7)
MH97	Wild type	1% lactose	10.9 (1.8)
MK142	*xprF1*	1% lactose	10.4 (3.6)
MH97	Wild type	1% galactose	9.1 (2.0)
MK142	*xprF1*	1% galactose	3.4 (1.6)
MH97	Wild type	0.5% glycerol	13.2 (2.4)
MK142	*xprF1*	0.5% glycerol	11.2 (2.8)

[a] The full genotypes of the strains are given in Table 1.

[b] Mycelia were grown for 24 hr in medium containing 1% glucose as a carbon source and then transferred to media containing the indicated carbon source for 16 hr.

[c] The protease enzyme assays were performed as described in MATERIALS AND METHODS. The results were calculated as total absorbance units per gram (dry weight) of mycelium and are expressed in arbitrary units. The results are the average for three cultures and the standard deviations are given in parentheses.

lar protease production and XprF prevents activation of extracellular protease gene expression by XprG, possibly through direct protein-protein interaction of the *xprF* and *xprG* gene products. The *xprF1* and *xprF2* mutations are both predicted to lead to the production of a truncated protein lacking the putative nuclear localization sequence. Thus, in the *xprF1* and *xprF2* mutants, activation of protease gene expression by XprG could not be blocked by XprF as no XprF protein would be present in the nucleus. Similarly, the reduced levels of extracellular protease in transformants containing more than one copy of the wild-type *xprF* gene could be due to increased inhibition of XprG activity.

If this model is correct, the *xprG2* and *xprG3* mutations would represent loss-of-function mutations in the positively acting *xprG* gene and the *xprG1* mutation would represent a gain-of-function mutation, which might prevent the regulation of XprG by XprF. Three lines of evidence support this hypothesis. (1) The *xprG1* mutation, which leads to increased extracellular protease activity, is partially dominant as would be expected of a gain-of-function mutation. (2) The *xprG2* and *xprG3* protease-deficient phenotype is recessive, which is consistent with loss-of-function mutations. (3) An *xprG1/xprG2* diploid resembles an *xprG1* haploid strain in phenotype. In addition, we have preliminary evidence that *xprG1* revertants, which are identical in phenotype to the *xprG2* and *xprG3* protease-deficient mutants, carry a second mutation in the *xprG* gene.

The *xprF1*, *xprF2*, and *xprG1* mutations were first identified as suppressors of the recessive *xprE1* mutation, which leads to a protease-deficient phenotype. It has been proposed that the *xprE* gene could encode a protease-specific, positive-acting, regulator of extracellular protease production (Katz *et al.* 1996). The *xprE* gene product could be an antagonist of XprF, preventing XprF-mediated inhibition of protease production. Alternatively, the *xprE* gene may be involved in the activation of a separate regulatory circuit, the loss of which is circumvented by the activation of XprG. An understanding of the roles of the *xprG* and *xprE* genes in the regulation of extracellular protease production will depend on the isolation and molecular characterization of these two genes.

Some of the phenotypic effects of *xprF1* and *xprF2* mutations are not completely recessive, which is unusual for loss-of-function mutations, but could be explained if the concentration of XprF in the nucleus is critical or if the truncated XprF1 and XprF2 proteins exert a dominant-negative effect through protein-protein interactions. The close proximity of the *xprF1* and *xprF2* nonsense mutations may not be a coincidence.

Mutations in both the *xprF* and *xprG* genes can affect nitrogen source utilization as well as extracellular protease production. The phenotype of the *xprF1* and *xprF2* mutants suggests a link between hexokinases and nitrogen regulation. In plants, sugars are known to affect many processes including nitrogen metabolism (Lam *et al.* 1994). In *A. nidulans*, a phenomenon that has been called reverse carbon catabolite repression leads to the repression of some enzymes involved in nitrogen metabolism under conditions of carbon starvation (Davis and Hynes 1991). Thus mutations in the *xprF* and *xprG* genes may affect enzymes that participate in nitrogen metabolism, if these genes are involved in triggering the response to carbon starvation.

The predicted XprF1 and XprF2 truncated proteins lack highly conserved hexokinase functional domains and would be nonfunctional with respect to sugar phosphorylating activity, yet no reduction in hexokinase or glucokinase activity was detected in the *xprF1* and *xprF2* mutants. There are two possible explanations for this observation: (1) the *xprF* gene may encode a minor hexokinase that is expressed at low levels or (2) the *xprF* gene may encode a protein with no hexose phosphorylating activity.

The *xprF* gene product contains some unusual structural features. Plant genes encoding atypical hexokinase-like proteins have been identified (GenBank accession nos. CAB38932 and CAA63966). These putative proteins lack some of the consensus sequences in the sugar- and ATP-binding sites of hexokinases, so these genes may have a primarily regulatory function. It seems likely that additional atypical hexokinase-like proteins will be brought to light through genome sequencing projects.

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LITERATURE CITED

Albig, W., and K.-D. Entian, 1988 Structure of yeast glucokinase, a strongly diverged specific aldo-hexose-phosphorylating isoenzyme. Gene **73:** 141–152.

Andrianopoulos, A., and M. J. Hynes, 1988 Cloning and analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. Mol. Cell. Biol. **8:** 3532–3541.

Arora, K. K., M. Fanciulli and P. L. Pedersen, 1990 Glucose phosphorylation in tumor cells. Cloning, sequencing, and overexpression in active form of a full-length cDNA encoding a mitochondrial bindable form of hexokinase. J. Biol. Chem. **265:** 6481–6488.

Arst, H. N., Jr., and D. J. Cove, 1973 Nitrogen metabolite repression in *Aspergillus nidulans*. Mol. Gen. Genet. **126:** 111–141.

Bajwa, W., T. E. Torchia and J. E. Hopper, 1988 Yeast regulatory gene *GAL3*: carbon regulation; *UAS-gal* elements in common with *GAL1, GAL2, GAL7, GAL10, GAL80,* and *MEL1*; encoded protein strikingly similar to yeast and *Escherichia coli* galactokinases. Mol. Cell. Biol. **8:** 3439–3447.

Berka, R. M., M. Ward, L. J. Wilson, K. J. Hayenga, K. H. Kodama *et al.*, 1990 Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. Gene **86:** 153–162.

Bisson, L. F., and D. G. Fraenkel, 1983 Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80:** 1730–1734.

Bork, P., C. Sander and A. Valencia, 1993 Convergent evolution of similar enzymatic function on different protein folds: the hexokinase ribokinase, and galactokinase families of sugar kinases. Protein Sci. **2:** 31–40.

Brody, H., J. Griffith, A. J. Cuticchia, J. Arnold and W. E. Timberlake, 1991 Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*. Nucleic Acids Res. **19:** 3105–3109.

Clutterbuck, A. J., 1974 *Aspergillus nidulans*, pp. 447–510 in *Handbook of Genetics: Bacteria, Bacteriophages and Fungi*, Vol. 1, edited by R. C. King. Plenum Press, New York.

Clutterbuck, A. J., 1993 *Aspergillus nidulans* (nuclear genes), pp. 3.71–3.84 in *Locus Maps of Complex Genomes: Lower Eukaryotes*, Vol. 3, edited by S. J. O'Brien. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Cohen, B. L., 1972 Ammonium repression of extracellular protease in *Aspergillus nidulans*. J. Gen. Microbiol. **71:** 293–299.

Cohen, B. L., 1973 Regulation of intracellular and extracellular neutral and alkaline proteases in *Aspergillus nidulans*. J. Gen. Microbiol. **79:** 311–320.

Cove, D. J., 1966 The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim. Biophys. Acta **113:** 51–56.

Davis, M. A., and M. J. Hynes, 1991 Regulatory circuits in *Aspergillus nidulans*, pp. 151–189 in *More Gene Manipulations in Fungi*, edited by J. W. Bennett and L. L. Lasure. Academic Press, San Diego.

Deeb, S., M. Malkki and M. Laakso, 1993 Human hexokinase II: sequence and homology to other hexokinases. Biochem. Biophys. Res. Commun. **197:** 68–74.

Entian, K.-D., 1980 Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression. Mol. Gen. Genet. **178:** 633–637.

Felsenstein, J., 1996 Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods, pp. 418–427 in *Methods in Enzymology*, Vol. 266, edited by R. F. Dolittle. Academic Press, San Diego.

Fidel, S., J. H. Doonan and N. R. Morris, 1988 *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a γ-actin. Gene **70:** 283–293.

Fröhlich, K.-U., K.-D. Entian and D. Mecke, 1985 The primary structure of the yeast hexokinase PII gene (*HXK2*) which is responsible for glucose repression. Gene **36:** 105–111.

Furuta, H., S. Nishi, M. M. Le Beau, A. A. Fernald, H. Yano *et al.*, 1996 Sequence of human hexokinase III cDNA and assignment of the human hexokinase III gene (HK3) to chromosome band 5q35.2 by fluorescence *in situ* hybridization. Genomics **36:** 206–209.

German, M. S., 1993 Glucose sensing in pancreatic islet β cells: the key role of glucokinase and the glycolytic intermediates. Proc. Natl. Acad. Sci. USA **90:** 1781–1785.

Griffin, L. D., B. D. Gelb, D. A. Wheeler, D. Davison, V. Adams *et al.*, 1991 Mammalian hexokinase 1: evolutionary conservation and structure to function analysis. Genomics **11:** 1014–1024.

Heikkinen, S., M. Pietila, M. Halmekyto, S. Suppola, E. Pirinen *et al.*, 1999 Hexokinase II-deficient mice. Prenatal death of homozygotes without disturbances in glucose tolerance in heterozygotes. J. Biol. Chem. **274:** 22517–22523.

Herrero, P., C. Martínez-Campa and F. Moreno, 1998 The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. FEBS Lett. **434:** 71–76.

Hughes, S. D., C. Quaade, J. L. Milburn, L. Cassidy and C. B. Newgard, 1991 Expression of normal and novel glucokinase mRNAs in anterior pituitary and islet cells. J. Biol. Chem. **266:** 4521–4530.

Hull, E. P., P. M. Green, H. N. Arst, Jr. and C. Scazzocchio, 1989 Cloning and physical characterization of the L-proline catabolism gene cluster of *Aspergillus nidulans*. Mol. Microbiol. **3:** 553–559.

Hynes, M. J., and J. M. Kelly, 1977 Pleiotropic mutants of *Aspergillus nidulans* altered in carbon metabolism. Mol. Gen. Genet. **150:** 193–204.

Inoue, H., T. Kimura, O. Makabe and K. Takahashi, 1991 The gene and deduced protein sequences of the zymogen of *Aspergillus niger* acid proteinase A. J. Biol. Chem. **266:** 19484–19489.

Ishimura-Oka, K., M. Nakamuta, M. J. Chu, M. Sullivan, L. Chan *et al.*, 1995 Partial structure of the mouse glucokinase gene. Genomics **29:** 751–754.

Jang, J.-C., P. Léon, L. Zhou and J. Sheen, 1997 Hexokinase as a sugar sensor in higher plants. Plant Cell **9:** 5–19.

Jarai, G., and F. Buxton, 1994 Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. Curr. Genet. **26:** 238–244.

Kalderon, D., W. D. Richardson, A. F. Markham and A. E. Smith, 1984 Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature **311:** 33–38.

Katz, M. E., R. N. Rice and B. F. Cheetham, 1994 Isolation and characterization of an *Aspergillus nidulans* gene encoding an alkaline protease. Gene **150:** 287–292.

Katz, M. E., P. K. Flynn, P. A. vanKuyk and B. F. Cheetham, 1996 Mutations affecting extracellular protease production in the filamentous fungus *Aspergillus nidulans*. Mol. Gen. Genet. **250:** 715–724.

Kelly, J. M., 1994 Carbon catabolite repression, pp. 355–367 in *Aspergillus: 50 Years On: Progress in Industrial Microbiology*, Vol. 28, edited by S. D. Martinelli and J. R. Kinghorn. Elsevier Science, Amsterdam.

Kopetzki, E., K.-D. Entian and D. Mecke, 1985 Complete nucleotide sequence of the hexokinase PI gene (*HXK1*) of *Saccharomyces cerevisiae*. Gene **39:** 95–102.

Kudla, B., M. X. Caddick, T. Langdon, N. M. Martinez-Rossi, C. F. Bennett *et al.*, 1990 The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. EMBO J. **9:** 1355–1364.

Lam, H.-M., S. S.-Y. Peng and G. M. Coruzzi, 1994 Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. Plant Physiol. **106:** 1347–1357.

Maitra, P. K., and Z. Lobo, 1983 Genetics of yeast glucokinase. Genetics **105:** 501–515.

Meyer, J., A. Walker-Jonah and C. P. Hollenberg, 1991 Galactokinase encoded by *GAL1* is a bifunctional protein required for induction of the *GAL* genes in *Kluyveromyces lactis* and is able to suppress the *gal3* phenotype in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 5454–5461.

Mori, C., J. E. Welch, K. D. Fulcher, D. A. O'Brien and E. M. Eddy, 1993 Unique hexokinase messenger ribonucleic acids lacking the porin-binding domain are developmentally expressed in mouse spermatogenic cells. Biol. Reprod. **49:** 191–203.

Nicholas, K. B., H. B. Nicholas, Jr. and D. W. Deerfield II, 1997 GeneDoc: analysis and visualization of genetic variation. EMBNEW.NEWS **4:** 14.

Nishi, S., S. Seino and G. I. Bell, 1988 Human hexokinase: sequences of amino- and carboxyl-terminal halves are homologous. Biochem. Biophys. Res. Commun. **157:** 937–943.

Oakley, C. E., C. F. Weil, P. L. Kretz and B. R. Oakley, 1987 Cloning of the *riboB* locus of *Aspergillus nidulans*. Gene **53:** 293–298.

Olafsson, P., H. Matile and U. Cesta, 1992 Molecular analysis of *Plasmodium falciparum* hexokinase. Mol. Biochem. Parasitol. **56:** 89–102.

Ortner, S., B. Plaimauer, M. Binder, O. Scheiner, G. Wiedermann *et al.*, 1995 Molecular analysis of two hexokinase isoenzymes from *Entamoeba histolytica*. Mol. Biochem. Parasitol. **73:** 189–198.

Panneman, H., G. J. G. Ruijter, H. C. van den Broeck, E. T. M. Driever and J. Visser, 1996 Cloning and biochemical characterization of an *Aspergillus niger* glucokinase. Evidence for the presence of separate glucokinase and hexokinase enzymes. Eur. J. Biochem. **240:** 518–525.

Panneman, H., G. J. G. Ruijter, H. C. van den Broeck and J. Visser, 1998 Cloning and biochemical characterization of *Aspergillus niger* hexokinase. The enzyme is strongly inhibited by physiological concentrations of trehalose 6-phosphate. Eur. J. Biochem. **258:** 223–232.

Petit, T., and C. Gancedo, 1999 Molecular cloning and characterization of the gene *HXK1* encoding the hexokinase from *Yarrowia lipolytica*. Yeast **15:** 1573–1584.

Petit, T., M. A. Blazquez and C. Gancedo, 1996 *Schizosaccharomyces pombe* possesses an unusual and a conventional hexokinase: biochemical and molecular characterization of both hexokinases. FEBS Lett. **378:** 185–189.

Polkinghorne, M., and M. J. Hynes, 1975 Mutants affecting histidine utilisation in *Aspergillus nidulans*. Genet. Res. **25:** 119–135.

Prade, R. A., J. Griffith, K. Kochut, J. Arnold and W. E. Timberlake, 1997 *In vitro* reconstruction of the *Aspergillus* (= *Emericella*) *nidulans* genome. Proc. Natl. Acad. Sci. USA **94:** 14564–14569.

Prior, C., P. Mamessier, H. Fukuhara, X. J. Chen and M. Wesolow-

SKI-LOUVEL, 1993 The hexokinase gene is required for transcriptional regulation of the glucose transporter gene-*RAG1* in *Kluyveromyces lactis*. Mol. Cell. Biol. **13:** 3882–3889.

RAMBOSEK, J. A., and J. LEACH, 1986 Recombinant DNA in filamentous fungi: progress and prospects. Crit. Rev. Biotechnol. **6:** 357–391.

REINERT, W. R., V. B. PATEL and N. H. GILES, 1981 Genetic regulation of the *qa* gene cluster of *Neurospora crassa*: induction of *qa* messenger RNA and dependency on *qa-1* function. Mol. Cell. Biol. **1:** 829–835.

RONNE, H., 1995 Glucose repression in fungi. Trends Genet. **11:** 12–17.

ROSE, M., 1995 Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast *Schwanniomyces occidentalis*. Curr. Genet. **27:** 330–338.

RUIJTER, G. J. G., H. PANNEMAN, H. C. VAN DEN BROECK, J. M. BENNETT and J. VISSER, 1996 Characterization of the *Aspergillus nidulans frA1* mutant: hexose phosphorylation and apparent lack of involvement of hexokinase in glucose repression. FEMS Microbiol. Lett. **139:** 223–228.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANZ, P., A. NIETO and J. A. PRIETO, 1996 Glucose repression may involve processes with different sugar kinase requirements. J. Bacteriol. **178:** 4721–4723.

SCHWAB, D. A., and J. E. WILSON, 1989 Complete amino acid sequence of rat brain hexokinase, deduced from the cloned cDNA, and proposed structure of a mammalian hexokinase. Proc. Natl. Acad. Sci. USA **86:** 2563–2567.

SCHWAB, D. A., and J. E. WILSON, 1991 Complete amino acid sequence of the type III isozyme of rat hexokinase, deduced from the cloned cDNA. Arch. Biochem. Biophys. **285:** 365–370.

SHOEMAKER, C. B., S. REYNOLDS, G. WEI and D. HARN, 1995 *Schistosoma mansoni* hexokinase: cDNA cloning and immunogenicity studies. Exp. Parasitol. **80:** 36–45.

SIRAKOVA, T. D., A. MARKARYAN and P. E. KOLATTUDKUDY, 1994 Molecular cloning and sequencing of the cDNA and gene for a novel elastinolytic metalloproteinase from *Aspergillus fumigatus* and its expression in *Escherichia coli*. Infect. Immun. **62:** 4208–4218.

STOFFEL, M., P. FROGUEL, J. TAKEDA, H. ZOUALI, N. VIONNET *et al.*, 1992 Human glucokinase gene: isolation, characterization and identification of two missense mutations linked to early-onset non-insulin-dependent (type 2) diabetes mellitus. Proc. Natl. Acad. Sci. USA **89:** 7698–7702.

TATSUMI, H., Y. OGAWA, S. MURAKAMI, Y. ISHIDA, K. MURAKAMI *et al.*, 1989 A full length cDNA clone for the alkaline protease from *Aspergillus oryzae*: structural analysis and expression in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **219:** 33–38.

TATSUMI, H., S. MURAKAMI, R. F. TSUJI, Y. ISHIDA, K. MURAKAMI *et al.*, 1991 Cloning and expression in yeast of a cDNA clone encoding *Aspergillus oryzae* neutral protease II, a unique metalloprotease. Mol. Gen. Genet. **228:** 97–103.

THELEN, A. P., and J. E. WILSON, 1991 Complete amino acid sequence of the type II isozyme of rat hexokinase, deduced from the cloned cDNA: comparison with a hexokinase from novikoff ascites tumor. Arch. Biochem. Biophys. **286:** 645–651.

THOMPSON, J. D., D. G. HIGGINS and T. J. GIBSON, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

TILBURN, J., C. SCAZZOCCHIO, G. G. TAYLOR, J. H. ZABICKY-ZISSMAN, R. A. LOCKINGTON *et al.*, 1983 Transformation by integration in *Aspergillus nidulans*. Gene **26:** 205–211.

VAN DEN HOMBERGH, J. P. T. W., G. JARAI, F. P. BUXTON and J. VISSER, 1994 Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. Gene **151:** 73–79.

VANKUYK, P. A., B. F. CHEETHAM and M. E. KATZ, 2000 Analysis of two *Aspergillus nidulans* genes encoding extracellular proteases. Fungal Genet. Biol. **29:** 201–210.

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