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Roles of Multiple Glucose Transporters in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, *TRK1* and *TRK2* are required for high- and low-affinity K^+ transport. Among suppressors of the K^+ transport defect in *trk1Δ trk2Δ* cells, we have identified members of the sugar transporter gene superfamily. One suppressor encodes the previously identified glucose transporter HXT1, and another encodes a new member of this family, HXT3. The inferred amino acid sequence of HXT3 is 87% identical to that of HXT1, 64% identical to that of HXT2, and 32% identical to that of SNF3. Like *HXT1* and *HXT2*, overexpression of *HXT3* in *snf3Δ* cells confers growth on low-glucose or raffinose media. The function of another new member of the *HXT* superfamily, *HXT4* (previously identified by its ability to suppress the *snf3Δ* phenotype; L. Bisson, personal communication), was revealed in experiments that deleted all possible combinations of the five members of the glucose transporter gene family. Neither *SNF3*, *HXT1*, *HXT2*, *HXT3*, nor *HXT4* is essential for viability. *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are unable to grow on media containing high concentrations of glucose (5%) but can grow on low-glucose (0.5%) media, revealing the presence of a sixth transporter that is itself glucose repressible. This transporter may be negatively regulated by SNF3 since expression of *SNF3* abolishes growth of *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells on low-glucose medium. *HXT1*, *HXT2*, *HXT3*, and *HXT4* can function independently: expression of any one of these genes is sufficient to confer growth on medium containing at least 1% glucose. A synergistic relationship between *SNF3* and each of the *HXT* genes is suggested by the observation that *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells and *snf3Δ HXT1 HXT2 HXT3 HXT4* cells are unable to grow on raffinose (low fructose) yet *SNF3* in combination with any single *HXT* gene is sufficient for growth on raffinose. *HXT1* and *HXT3* are differentially regulated. *HXT1::lacZ* is maximally expressed during exponential growth whereas *HXT3::lacZ* is maximally expressed after entry into stationary phase.

In *Saccharomyces cerevisiae*, glucose transport is thought to be mediated by two kinetically distinct systems. One is a glucose-repressible high-affinity system with a K_m of 1 mM, and the other is a constitutive low-affinity system with a K_m of 20 mM (1a). Three genes involved in glucose transport, *SNF3*, *HXT1*, and *HXT2*, have been previously identified. *SNF3* (sucrose nonfermenting) is glucose repressible and is required for high-affinity glucose transport; *snf3Δ* mutants are unable to grow on medium containing low concentrations of glucose (0.1%) or sugars such as raffinose and sucrose (2). *HXT1* and *HXT2* (hexose transport) were isolated as multicopy suppressors of an *snf3Δ* mutant by their ability to restore growth on medium containing raffinose (13, 14). None of these three genes essential for viability (3, 13, 14), and kinetic analysis has shown that *HXT1* and *HXT2* encode components of the high-affinity glucose transport system (13, 14).

DNA sequence analysis of *SNF3*, *HXT1*, and *HXT2* revealed that they are members of a transporter gene superfamily. Members of this superfamily include sugar transporters from mammals, bacteria, plants, and yeasts (3, 13, 14, 14a, 19, 24a, 27, and this report). Typically, members of this superfamily contain 12 putative membrane-spanning domains with a relatively large hydrophilic region between membrane-spanning domains 6 and 7. Many members of this superfamily exhibit structural conservation within membrane-spanning domains 3, 5, 7, 8, and 11, which have been postulated to form amphipathic α-helices and assemble into a pentagonal pore forming the channel through which glucose is transported (19). In addition, a majority of these members contain a leucine heptad motif (13, 14, 17, 28, and this report), although its functional significance has not been determined.

In a genetic selection scheme designed to identify suppressors of the K^+ transport defect in cells deleted for the two principal K^+ transporters, TRK1 (8) and TRK2 (12), we found that some of the suppressor loci encoded mutant alleles of glucose transporter genes (11). One locus is allelic to the previously identified *HXT1* gene. A second locus defined a previously unidentified gene, designated *HXT3*, that encodes a protein highly related to HXT1 and HXT2. Like that of *HXT1* and *HXT2*, overexpression of *HXT3* suppresses the phenotype conferred by the *snf3Δ* mutation, namely, the inability to grow on low-glucose or raffinose media.

We show that cells deleted for *SNF3*, *HXT1*, *HXT2*, *HXT3*, and *HXT4* (another new member of the *HXT* family) are viable but are unable to grow on medium containing high concentrations of glucose (5%) when respiration is inhibited. Expression of any *HXT* gene is sufficient to restore growth on medium containing high glucose as the sole carbon source, demonstrating the functional independence of these transporters. Coexpression of *SNF3* and any one of the *HXT* genes is both necessary and sufficient to confer the ability to grow on raffinose medium. Although *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are unable to grow on high-glucose medium in the presence of antimycin A, they are able to grow on media containing low concentrations of glucose (0.5%), revealing the function of a genetically unidentified glucose transporter in these cells. Unexpectedly, expression of *SNF3* in *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells abolishes the ability

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TABLE 1. *S. cerevisiae* and *E. coli* strains used

Strain	Genotype	Reference or source
HB101	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-15 mtl-14 supE44*	15
R757	*MAT***a** *his4-15 lys9 ura3-52*	8
CY152	*MAT***a** *his3Δ200 lys9 trp1Δ ura3-52 trk1Δ1 trk2Δ::HIS3*	12
CY281	*MAT***a** *MATα trp1Δ1/trp1Δ1 ura3-52/ura3-52 trk1Δ/trk1Δ trk2Δ::HIS3/trk2Δ::HIS3*	This study
CY282	*MATα his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt1Δ::TRP1Δ::hxt4Δ*	This study
CY283	*MATα his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt2Δ::LEU2*	This study
CY284	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt3Δ::URA3*	This study
CY285	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 snf3Δ::HIS3*	This study
CY286	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 snf3Δ::HIS3 hxt2Δ::LEU2*	This study
CY287	*MATα his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt1Δ::TRP1::hxt4Δ hxt3Δ::URA3*	This study
CY288	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt2Δ::LEU2 hxt3Δ::URA3*	This study
CY289	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2*	This study
CY290	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3*	This study
CY291	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt2Δ::LEU2 hxt3Δ::TRP1 snf3Δ::HIS3*	This study
CY292	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 hxt1Δ::TRP1::hxt4Δ hxt3Δ::URA3 snf3Δ::HIS3*	This study
CY293	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2*	This study
CY294	*MAT***a** *his3Δ200 leu2Δ trp1Δ1 ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3*	This study
CY295	*MAT***a**/*MATα his3Δ200/his3Δ200 trp1Δ1/trp1Δ1 ura3-52/ura3-52 leu2Δ/leu2Δ trk1Δ/trk1Δ trk2Δ::HIS3/trk2Δ::HIS3 cup1Δ::URA3/+ hxt1Δ::TRP1::hxt4Δ/+ ste12Δ::LEU2/+*	This study
CY296	*MAT***a** *leu2Δ ura3-52 trp4*	This study
CY297	*MATα leu2Δ ura3-52 ssd1Δ::LEU2 hxt3Δ::URA3*	This study
CY336	*MATα his3Δ200 leu2Δ trp1Δ1 ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1 hxt2Δ::LEU2 hxt3Δ::URA3*	This study

to grow on low-glucose medium, suggesting that SNF3 functions as a negative regulator of glucose transport.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* and *Escherichia coli* strains used in this study are listed in Table 1. YPD, YNB, sporulation media, and standard genetic techniques are described by Sherman et al. (25). Raffinose medium was prepared at a final concentration of 2% raffinose. Antimycin A (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 1 μg/ml to inhibit respiration. Respiration was inhibited to assess the ability of cells to utilize glucose as the sole carbon source. Yeast transformation was performed by the lithium acetate method (10).

Cloning and sequencing of *HXT1* and *HXT3*. Plasmids pCK137 and pCK163 contain mutant alleles of *HXT1* and *HXT3* capable of suppressing the K^+ transport defect in *trk1Δ trk2Δ* cells (11). These plasmids were used to isolate the wild-type *HXT1* and *HXT3* alleles by gap repair (23). Plasmid pCK163, carrying the mutant *HXT3-1* allele, was digested with *Eco*RI and *Msc*I and used to transform a *trk1Δ trk2Δ HXT3 ura3-52* wild-type strain (CY152) to Ura^+. Plasmid pCK137 carrying the mutant *HXT1-1* allele was digested with *Eco*RI and *Hin*dIII and used to transform a *trk1Δ trk2Δ HXT1 ura3-52* wild-type strain (CY152) to Ura^+. Gap-repaired plasmids (pCK164 for *HXT3* and pCK142 for *HXT1*) were recovered from *S. cerevisiae*, amplified in *E. coli*, and sequenced by using synthetic oligonucleotide primers and Sequenase (United States Biochemical Corp., Cleveland, Ohio). The wild-type *HXT3* allele was also recovered by integrating pCK143 into CY152, digesting the genome DNA with *Xba*I, ligating with T4 ligase, and transforming *E. coli* to ampicillin resistance. The resulting plasmid pCK149 was sequenced by using synthetic oligonucleotide primers. Since the cloned DNA fragment in pCK149 is considerably larger than the other plasmids used in this study, it is not illustrated in Fig. 1B. Oligonucleotide primers were synthesized by the Northwestern University Biotechnology Center. Sequence analyses were performed with the DNA Inspector IIe software program (Textco). Protein sequence comparisons were carried out by using the Genetics Computer Group program (5).

DNA manipulations. Large-scale plasmid DNA was prepared on cesium chloride gradients, and yeast genomic DNA was prepared as described by Maniatis et al. (15). Miniprep DNA, restriction endonuclease analysis, gel electrophoresis, and Southern blot analysis were performed as described by Maniatis et al. (15). DNA probes were prepared by the random priming method (6, 7). Random hexamers were purchased from Amersham (Arlington Heights, Ill.). [α-^{32}P]-dCTP (3,000 Ci/mmol) was purchased from NEN (Boston, Mass.).

Construction of multicopy plasmids carrying *HXT1* or *HXT3*. A multicopy plasmid, pCK148, containing *HXT1* was constructed by subcloning the 4.2-kb *Cla*I-*Hin*dIII fragment of pCK142 (Fig. 1A) into the 2μm-based multicopy shuttle vector pRS426 (4). A multicopy plasmid, pCK166, containing *HXT3* was constructed by subcloning the 4.0-kb *Xho*I-*Sst*I fragment of pCK164 (Fig. 1B) into pRS426.

Construction of *S. cerevisiae* strains containing deletion mutations in *HXT1*, *HXT3*, or *HXT4*. Deletions in *HXT1*, *HXT3*, and *HXT4* were generated by the one-step integration-replacement method, known as gamma deletion (26). A plasmid designed to disrupt *HXT1* that proved capable of sometimes simultaneously disrupting *HXT1* and *HXT4* (pCK153) was constructed by subcloning the 0.6-kb *Cla*I-*Eco*RI and 0.3-kb *Kpn*I-*Apa*I fragments from pCK136 (Fig. 1A) into the integrative shuttle vector pRS304 (26). Cells deleted for *HXT1* or *HXT1* and *HXT4* were generated by first transforming a *trp1Δ/trp1Δ* homozygous diploid strain (CY281) with plasmid pCK153 (Fig. 1A), which had been linearized by digestion with *Xho*I. The resulting diploids were sporulated and dissected to obtain *hxt1Δ* or *hxt1Δ hxt4Δ* haploid strains. To construct an *HXT3*-disrupting plasmid, the 0.4-kb *Hin*dIII-*Eco*RI and 3-kb *Kpn*I-*Xho*I fragments from pCK164 were first subcloned into the integrative shuttle vector pRS306 (26). It was then digested with

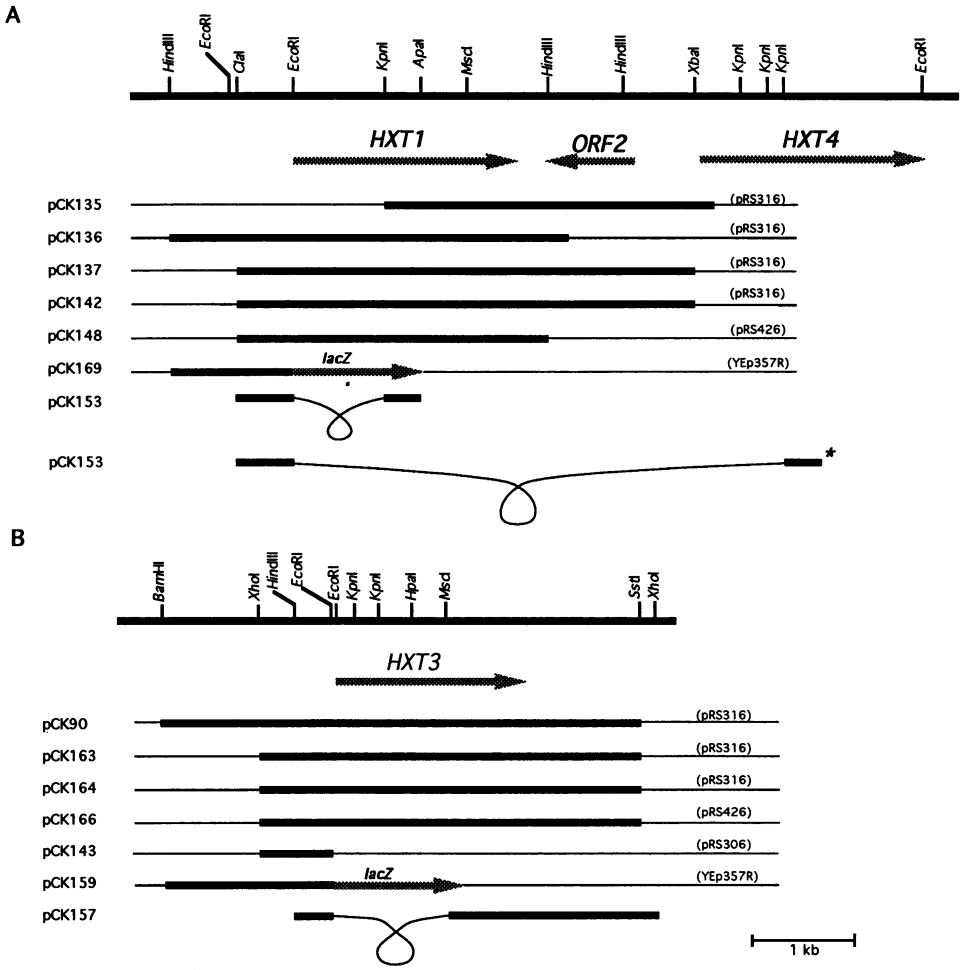


FIG. 1. Restriction maps of plasmids containing the *HXT1* gene (A) and the *HXT3* gene (B). The open reading frames of *ORF2* (14), *HXT4* (1), *HXT1*, and *HXT3* are indicated by checkered arrows. The restriction map of *HXT4* was kindly provided by L. Bisson. The thin lines indicate vector sequences, and the thick lines indicate yeast sequences. pRS316 is a single-copy vector (26) whereas pRS426 is a 2μm-derived multicopy vector (4). pCK137 and pCK163 contain point mutations in *HXT1* and *HXT3* that render them capable of suppressing the potassium transport defect in *trk1*Δ *trk2*Δ cells (11). pCK142 and pCK164 contain gap-repaired alleles of *HXT1* and *HXT3*, respectively. YEp357R is a multicopy vector used for *lacZ* fusions (20). pCK153 and pCK157 were used to construct yeast cells deleted for *HXT1* and *HXT3*, respectively. *, in some cases, integration of pCK153 mediated deletion of the *HXT1-HXT4* region because of the homology between *HXT1* and *HXT4*.

*Asp*718 and *Msc*I, treated with Klenow fragment to create blunt ends, and ligated to yield the *HXT3*-disrupting plasmid pCK157 (Fig. 1B). To generate the *hxt3*Δ null allele, plasmid pCK157 was linearized by digestion with *Cla*I and used to transform an *HXT3/HXT3 ura3-52/ura3-52* homozygous diploid strain (CY281) to Ura$^+$. A haploid strain containing the *hxt3*Δ mutation (CY284) was obtained from the meiotic progeny of this diploid.

Construction of *S. cerevisiae* strains containing deletion mutations in *HXT1*, *HXT2*, *HXT3*, *HXT4*, and *SNF3*. The *hxt2*Δ and *snf3*Δ null alleles used in this study have been previously described (13, 22). *hxt1*Δ *hxt3*Δ *hxt4*Δ cells (CY287) were obtained as recombinant spore colonies from a diploid generated by crossing strain CY282 (*hxt1*Δ *hxt4*Δ) with CY284 (*hxt3*Δ). Cells containing *snf3*Δ and *hxt2*Δ mutations (CY286) were obtained as recombinant spore colonies from a diploid generated by crossing strains CY285 (*snf3*Δ) and CY283 (*hxt2*Δ). Cells containing multiple combinations of deletion mutations were generated as meiotic recombinants of a cross between CY287 (*hxt1*Δ *hxt3*Δ *hxt4*Δ) and CY286 (*snf3*Δ *hxt2*Δ).

Construction of plasmids containing *HXT1*::*lacZ* and *HXT3*::*lacZ* fusions. *HXT1*::*lacZ* (pCK169) and *HXT3*::*lacZ* (pCK159) fusion plasmids contain the appropriate *HXT* promoter fused to the coding region of *lacZ*. Plasmids pCK169 and pCK159 were constructed by subcloning the 1.2-kb *Hin*dIII-*Eco*RI fragment from pCK136 (*HXT1*) and the 2.0-kb *Bam*HI-*Eco*RI from pCK90 (*HXT3*) into the 2μm-based multicopy *lacZ* fusion shuttle vector, YEp357R (20) (Fig. 1). These fusion plasmids contain the upstream regulatory regions and the first codon of the open reading frames of *HXT1* and *HXT3*. β-Galactosidase activity was measured as described by Guarente (9) and expressed in Miller units (18). *O*-Nitrophenyl-β-D-galactopyranoside was purchased from Sigma Chemical Co.

Genetic mapping experiments. A blot containing electrophoretically separated yeast chromosomes (Clonetech, Palo Alto, Calif.) was probed with a ^{32}P-labeled 1.3-kb *Eco*RI-*Msc*I fragment from pCK142 (Fig. 1A) for chromosomal assignment of *HXT1* or with a ^{32}P-labeled 1.2-kb *Eco*RI-*Msc*I fragment from pCK164 (Fig. 1B) for assignment of *HXT3*. The blot was hybridized at 65°C for 12 h and washed

three times with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–sodium dodecyl sulfate (SDS) at 65°C for 15 min. To determine map positions of *HXT1* and *HXT3* relative to those of other markers, Prime Lambda Grid Filters containing large fragments derived from yeast chromosomes (a gift of M. Olson) were probed with a ^{32}P-labeled 0.6-kb *Cla*I-*Eco*RI fragment of pCK142 (*HXT1*) or with a ^{32}P-labeled 0.8-kb *Xho*I-*Eco*RI fragment of pCK164 (*HXT3*). These probes were derived from the unique 5′ nontranslated regions of *HXT1* and *HXT3*. Gene order was determined by multipoint crosses as described in the legend to Fig. 4. Standard genetic linkage values were derived from tetrad data by the equation X [in centimorgans (cM)] = 50 [tetratype asci + 6 (nonparental ditype asci)]/total asci (24).

Nucleotide sequence accession number. The nucleic acid sequences of *HXT1* and *HXT3* have been deposited in GenBank (accession no. L07079 and L07080, respectively).

RESULTS

Cloning of *HXT1* and *HXT3*. Mutant alleles of *HXT1* and *HXT3* capable of suppressing the K^+ uptake defect in cells deleted for the high- and low-affinity K^+ transporters, *TRK1* and *TRK2*, were previously isolated (11). The wild-type alleles of *HXT1* and *HXT3* were cloned by the gap-repair method (23) by using plasmids containing the *HXT1-1* and *HXT3-1* suppressor alleles. Plasmids containing *HXT1-1* and *HXT3-1* were treated with endonucleases to remove a large portion of the coding regions which included the mutant (suppressor) sites and were used to transform a *trk1*Δ *trk2*Δ *ura3-52* recipient strain (CY152) containing the wild-type *HXT1* and *HXT3* alleles (see Materials and Methods). Since plasmids containing *HXT1-1* or *HXT3-1* confer upon CY152 cells the ability to grow on medium containing low concentrations of potassium (7 mM), transformants that did not exhibit such suppression of the *trk1*Δ *trk2*Δ phenotype were likely to contain plasmids carrying the wild-type *HXT* alleles. Gap-repaired plasmids pCK142 (*HXT1*) and pCK164 (*HXT3*) were recovered from such transformants. Reintroduction of the gap-repaired plasmids into the *trk1*Δ *trk2*Δ recipient (CY152) did not suppress the *trk1*Δ *trk2*Δ phenotype, suggesting the presence of the wild-type *HXT1* and *HXT3* alleles. In addition to the *HXT3* wild-type allele recovered by gap repair, another *HXT3* wild-type allele (pCK149) was obtained by the integration-excision method (see Materials and Methods). Maps of relevant restriction endonuclease sites of *HXT1* and *HXT3* are shown in Fig. 1.

Molecular characterization of *HXT1*. DNA sequence analysis of the 2.1-kb region of pCK142 revealed a single open reading frame capable of encoding a protein of 570 amino acids with a predicted molecular mass of 63 kDa. A search of the GenBank data base for similar sequences revealed that the sequence is essentially identical to the sequence of *HXT1*, a putative glucose transporter gene (14). The only discrepancy between the two sequences is the presence of an additional residue (valine) found at position 70 of our sequence. Whether or not this represents a polymorphic difference between strains of *S. cerevisiae* remains to be determined. However, this valine residue is conserved in HXT3.

Molecular characterization of *HXT3*. DNA sequence analysis of the 2.0-kb fragment encompassing the coding region of *HXT3* contained in pCK164 and pCK149 revealed a single open reading frame capable of encoding a 567-amino-acid protein with a predicted molecular mass of 62 kDa (Fig. 2). Four TATA sequences are found at positions −111, −177, −265, and −280. Hydropathy analysis of the inferred protein sequence predicted that the protein contains 12 putative membrane-spanning domains (M1 to M12). Comparison of the inferred sequences of HXT3 and HXT1 revealed 87% identity, indicating that HXT3 is a member of the sugar transporter gene superfamily. HXT3 is related to other members of the sugar transporter superfamily including glucose (HXT2 and SNF3), galactose (GAL2), and maltose (MAL61) transporters. The inferred amino acid sequence of HXT3 is 64% identical to that of HXT2, 32% identical to that of SNF3, 66% identical to that of GAL2, and 21% identical to that of MAL61.

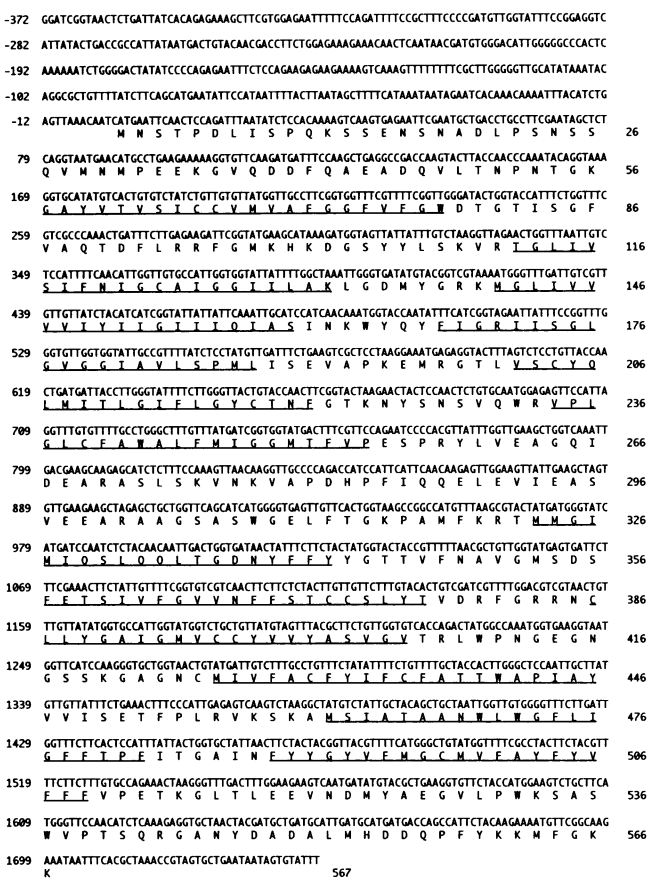


FIG. 2. Nucleotide and deduced amino acid sequences of *HXT3*. Nucleotides are numbered on the left, and amino acids are numbered on the right. Putative membrane-spanning domains are underlined.

An amino acid sequence alignment of HXT1, HXT2, HXT3, and SNF3 reveals that the highest degree of sequence identity (27%) occurs in the putative membrane-spanning regions (Fig. 3). This is consistent with the prediction that membrane-spanning domains 3, 5, 7, 8, and 11 are involved in forming a pore through which glucose is transported (19). Amino acid sequence identity outside of the putative transmembrane domains between all four transporters is only 17% (Fig. 3). HXT3 also contains a relatively large (68-amino-acid) hydrophilic region between M6 and M7, typical of sugar transporters. Sites for potential N-linked glycosylation are found at amino acid positions 24, 225, and 416. The site at position 225 is conserved between HXT3 and HXT1. A leucine heptad repeat motif is found in the region containing amino acids 107 to 128. This motif is found in HXT1 and HXT2 and in human glucose transport-

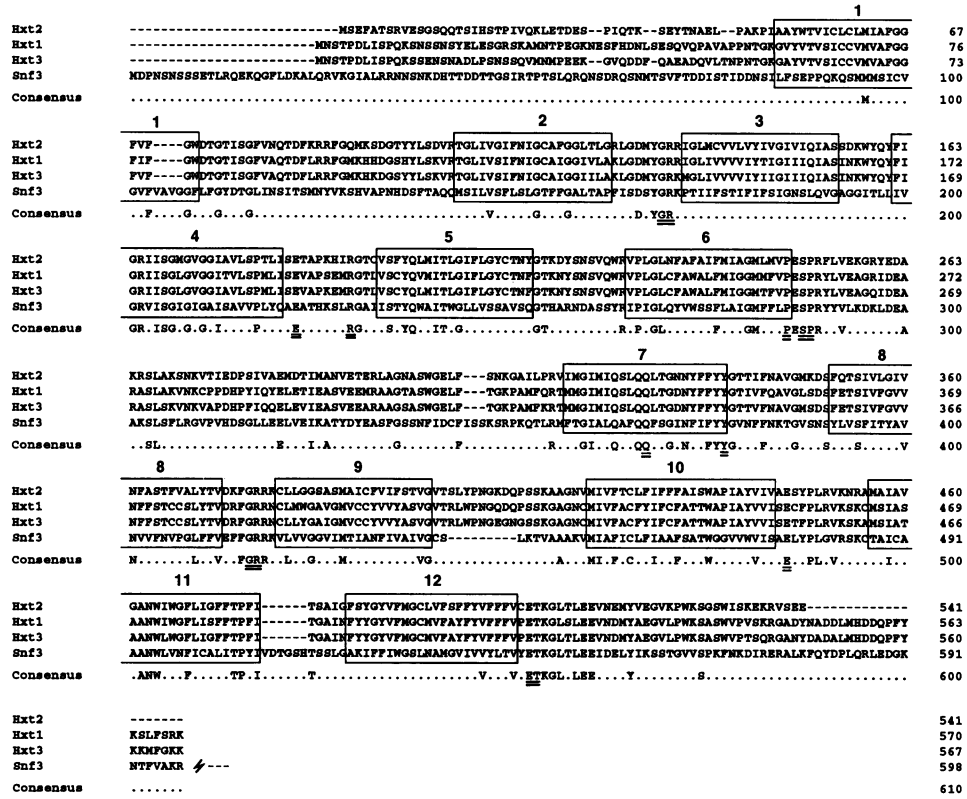


FIG. 3. Alignment of deduced amino acid sequences of HXT1, HXT2, HXT3, and SNF3. The alignment was generated by the GeneWorks program. Putative membrane-spanning domains are boxed. Amino acid positions are numbered on the right side. Sequences conserved between HXT1, HXT2, HXT3, and SNF3 are shown in the consensus line. Sequences conserved in virtually all known sugar transporters are double underlined. Carboxy-terminal SNF3 sequences unrelated to the HXT sequences are deleted as indicated (dashed lines after lightning bolt).

ers but is not found in SNF3 (3, 13, 14, 17, 28). Although the coding regions of *HXT1* and *HXT3* are highly related, the 5′ untranslated regions of these genes are unrelated, consistent with results showing that these genes are differentially regulated (see below).

Genetic mapping of *HXT1* and *HXT3*. *HXT1* was mapped to chromosome VIII by probing a blot containing electrophoretically separated intact yeast chromosomes with the 1.2-kb *Eco*RI-*Msc*I fragment of pCK142 (Fig. 1A). To reduce cross-hybridization of homologs, a probe containing 5′ untranslated sequences unique to *HXT1* was used to hybridize the Prime Lambda Grid Filters which contain large yeast genomic DNA fragments in lambda phage clones (M. Olson, Washington University). *HXT1* was localized to a region between the *CUP1* and *cdc12* loci (data not shown). A three-point mapping cross involving *HXT1*, *CUP1*, and *ste12* established that *HXT1* is 0.63 cM distal from *ste12* (Fig. 4A). The most likely gene order in this region is *CUP1 ste12 HXT1*.

HXT3 was mapped to chromosome IV by using the blot containing yeast chromosomes mentioned above. Hybridization of the Prime Lambda Grid Filters with an *HXT3*-specific probe containing 5′ untranslated sequences localized *HXT3* to a genomic segment containing the *SSD1* and *trp4* loci. Tetrad analysis of meiotic progeny of a three-point cross involving *HXT3*, *SSD1*, and *trp4* showed that *HXT3* maps 0.76 cM proximal from *trp4* and 43 cM distal from *SSD1* (Fig. 4B). The most likely gene order in this region is *SSD1 HXT3 trp4*.

Suppression of the *snf3*Δ mutation phenotype by overexpression of *HXT1* and *HXT3*. Growth of *S. cerevisiae* on media containing raffinose as the sole carbon source depends on the ability of the cells to take up fructose, a product of invertase-mediated cleavage of the trisaccharide. Cells deleted for *SNF3* cannot transport fructose with high affinity and therefore fail to grow on raffinose medium (22). As a test of the likelihood that *HXT1* and *HXT3* encode glucose transporters, we assessed their ability to suppress the *snf3*Δ mutation phenotype on raffinose medium. Multicopy (pCK148 and pCK166) and single-copy (pCK142 and pCK164) plasmids containing *HXT1* and *HXT3*, respectively, were introduced into an *snf3*Δ *ura3-52* recipient (CY285) by transformation. Only the multicopy plasmids carrying *HXT1* or *HXT3* restored the ability of these cells to grow on raffinose medium (data not shown). Thus, like that of *HXT1* and *HXT2* (13, 14), overexpression of *HXT3* can suppress the loss of high-affinity glucose (fructose) uptake.

Effect of *hxt1*Δ and *hxt3*Δ mutations. To further assess the role of *HXT3* in glucose transport, cells containing an *hxt3*Δ null mutation were constructed. An *HXT3/HXT3 ura3-52/ura3-52* homozygous diploid (CY281) was transformed with linearized pCK157 to Ura$^+$ to generate a 1.2-kb deletion in *HXT3* in one chromosome (see Materials and Methods) (Fig. 1B). The resulting diploids were sporulated, and tetrads were dissected for analysis. Among 10 four-spored tetrads examined, all showed a 2 Ura$^+$:2 Ura$^-$ segregation pattern and all spores grew normally on 2% glucose YPD medium, suggesting that *HXT3* is not essential for growth. When tested for growth on media containing raffinose or low glucose concen-

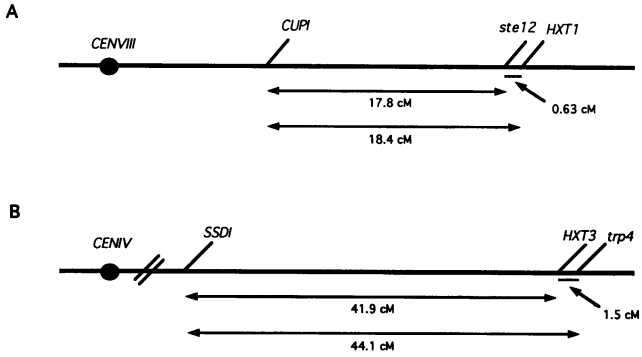


FIG. 4. (A) Genetic mapping of *HXT1* on the right arm of chromosome VIII (CENVIII). The values for genetic linkage between *HXT1* and linked markers are indicated in centimorgans. The *HXT1* locus was marked with the *TRP1* gene by integration of the *HXT1* deletion plasmid described in Materials and Methods. The *CUP1* and *ste12* loci were marked with the *URA3* gene and the *LEU2* gene, respectively. The CY295 strain was transformed with an *ste12*-disrupting plasmid, and the resulting diploids were sporulated for tetrad analysis. The *CUP1*::*URA3-hxt1*Δ::*TRP1* marker pair segregated 48 parental ditype (PD):0 nonparental ditype (NPD):28 tetratype (T) (18.4 cM). The *hxt1*Δ::*TRP1-ste12*Δ::*LEU2* marker pair segregated 78P:0NPD:1T (0.63 cM). The *CUP1*::*URA3-ste12*Δ::*LEU2* marker pair segregated 49P:0NPD:27T (17.8 cM). Among 28 tetrads that were tetratype for the *CUP1*::*URA3-hxt1*Δ::*TRP1* marker pair, 27 remained tetratype and 1 was parental ditype for the *hxt1*Δ::*TRP1-ste12*Δ::*LEU2* marker pair. The combined results indicate that the most likely order of genes is *CUP1 ste12 HXT1*. (B) Genetic mapping of *HXT3* on the right arm of chromosome IV (CENIV). The *HXT3* locus was marked with the *URA3* gene by integration of the *HXT3* deletion plasmid described in Materials and Methods. The *SSD1* locus was marked by an insertion of the *LEU2* gene within the coding region. CY295 and CY296 strains were mated and sporulated for tetrad analysis. The *ssd1*Δ::*LEU2-hxt3*Δ::*URA3* marker pair segregated 38PD:4NPD:69T (41.9 cM). The *hxt3*Δ::*URA3-trp4* marker pair segregated 100P:0NPD:3T (1.5 cM). The *ssd1*Δ::*LEU2-trp4* marker pair segregated 38P:5NPD:68T (44.1 cM). Among 69 tetrads that were tetratype for the *ssd1*Δ::*LEU2-hxt3*Δ::*URA3* marker pair, 68 remained tetratype and 1 was nonparental ditype for the *ssd1*Δ::*LEU2-trp4* marker pair. The combined results indicate that the most likely order of genes is *SSD1 HXT3 trp4*.

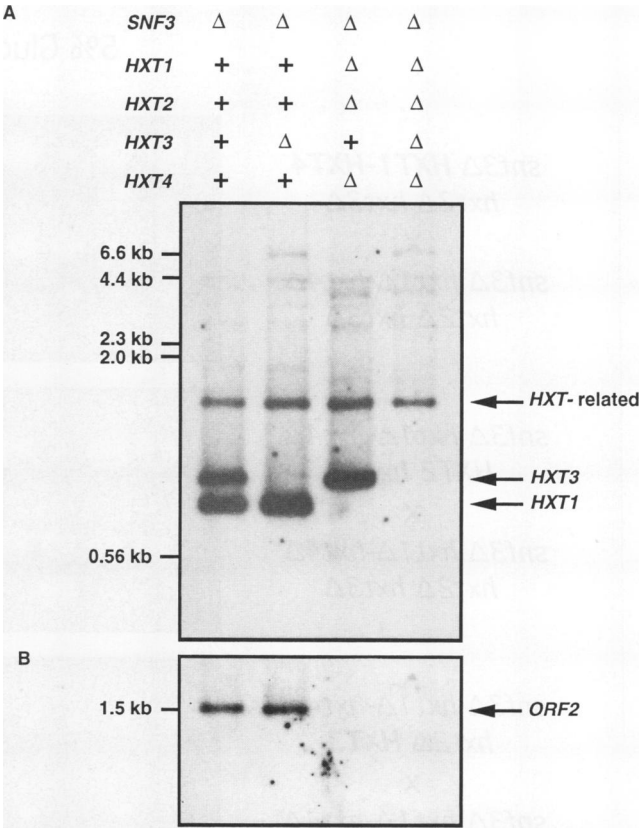


FIG. 5. (A) Genomic Southern blot analysis of *HXT1*, *HXT3*, and *HXT4*. Genomic DNA of spore colonies of a tetrad from a cross between an *snf3*Δ *hxt2*Δ strain (CY286) and an *hxt1*Δ *hxt3*Δ *hxt4*Δ strain (CY287) was prepared; digested with *Eco*RI, *Hpa*I, and *Acc*I; and transferred to a filter. The presence of wild-type genes (+) and deletion alleles (Δ) is indicated. The blot was probed with the ^{32}P-labeled 834-bp *Eco*RI-*Hpa*I fragment of *HXT1* and the 784-bp *Eco*RI-*Hpa*I fragment of *HXT3*. The blot was washed three times with 0.1× SSC–0.1% SDS for 15 min at 65°C. The 654-bp fragment detected is the *Acc*I-*Hpa*I fragment of *HXT1*, and the 784-bp fragment detected is the *Eco*RI-*Hpa*I of *HXT3*. (B) The same blot was stripped and reprobed with the *Hin*dIII fragment of *ORF2* shown in Fig. 1A. The absence of the 1.5-kb fragment indicates that *HXT1* and *HXT4* (1) were simultaneously deleted.

trations, the *hxt3*Δ mutants did not exhibit a discernible difference in growth compared with wild-type cells.

Cells containing an *hxt1*Δ mutation were constructed in a similar manner. Integration of plasmid pCK153 generated a 1.1-kb deletion within the coding region of *HXT1*. An *HXT1/HXT1 trp1*Δ*/trp1*Δ homozygous diploid strain (CY281) was transformed with linearized pCK153 to Trp$^+$, and the transformants were sporulated to obtain meiotic progeny containing the *hxt1*Δ alleles. Among 10 four-spored tetrads examined, all showed a 2 Trp$^+$:2 Trp$^-$ segregation pattern and all spores grew normally on YPD medium, indicating that *HXT1* is not essential for growth. Similar results were previously reported by Lewis and Bisson (14).

During the course of this study, we were informed that a fifth member of the glucose transporter gene family, *HXT4*, is very closely linked to *HXT1* (1). Southern blot analysis of the putative *hxt1*Δ transformants analyzed above revealed a mixture of *hxt1*Δ::*TRP1* and *hxt1*Δ::*TRP1*::*hxt4*Δ mutants (data not shown and Fig. 5A). Both *hxt1*Δ and *hxt1*Δ *hxt4*Δ deletion mutants are viable and show growth on high- or low-glucose media that is indistinguishable from that of the wild type.

To determine the effect of a triple mutation on glucose transport, an *hxt1*Δ *hxt3*Δ *hxt4*Δ strain (CY287) was generated as a Trp$^+$ Ura$^+$ meiotic recombinant from a cross between the *hxt1*Δ::*TRP1*::*hxt4*Δ strain CY282 and the *hxt3*Δ::*URA3* strain CY284. The growth of *hxt1*Δ *hxt3*Δ *hxt4*Δ recombinants among the progeny from this cross was not discernibly slower than that of the wild-type (*SNF3 HXT1 HXT2 HXT3*) segregants, on high- or low-glucose medium containing antimycin A (data not shown), indicating that the transporters remaining in the triple mutants are able to take up sufficient glucose for normal growth. Southern blot analysis of these mutants confirmed that deletion mutations were generated at the *HXT1*, *HXT3*, and *HXT4* loci (Fig. 5A).

***snf3*Δ *hxt1*Δ *hxt2*Δ *hxt3*Δ *hxt4*Δ cells are unable to grow on media containing high concentrations of glucose.** To assess the roles of the known glucose transporters individually, we generated an *snf3*Δ *hxt1*Δ *hxt2*Δ *hxt3*Δ *hxt4*Δ strain (CY294) by meiotic recombination from a cross between an *snf3*Δ *hxt2*Δ strain (CY286) and an *hxt1*Δ *hxt3*Δ *hxt4*Δ strain (CY287). Tetrad analysis of the meiotic progeny from this

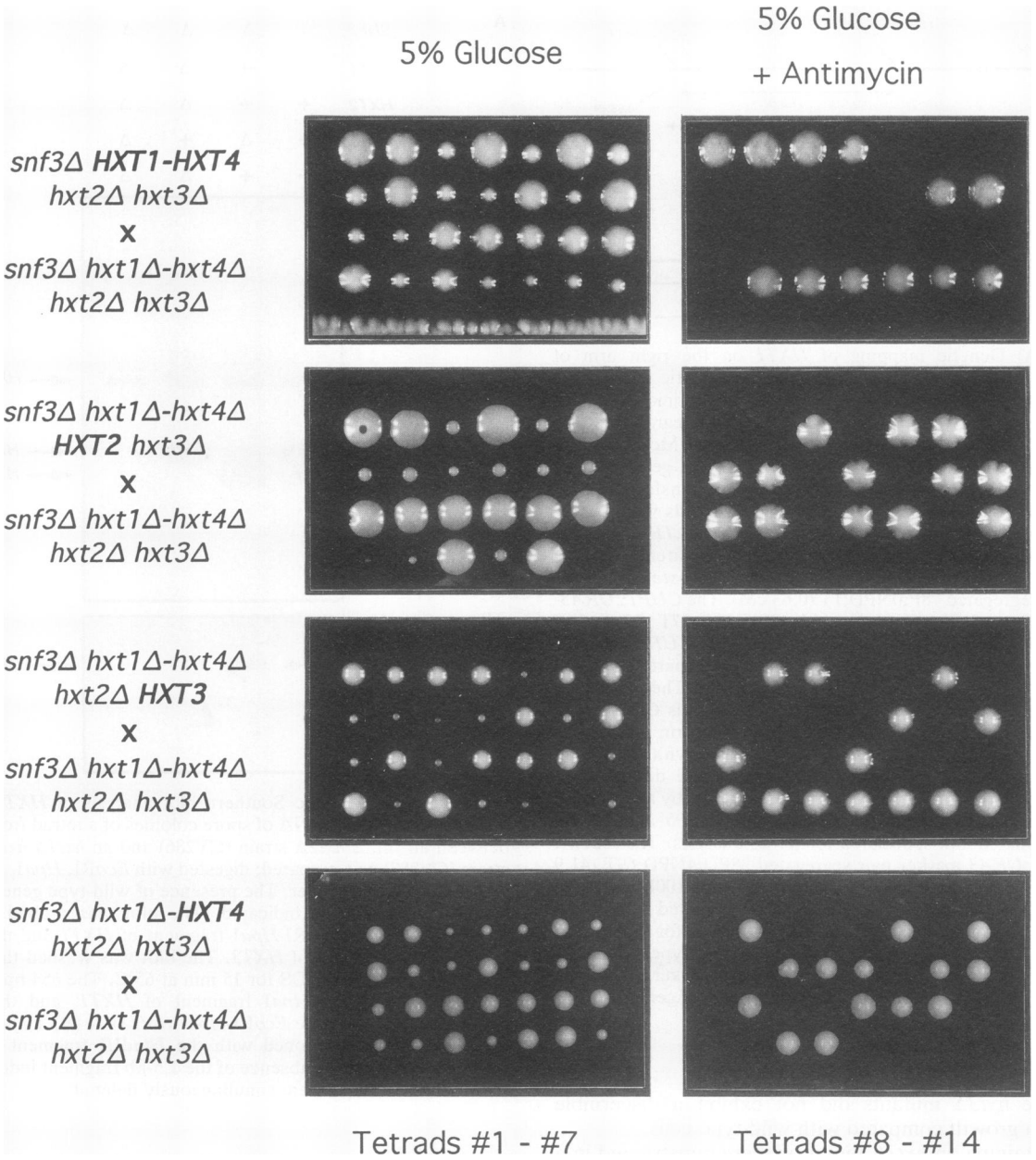


FIG. 6. Tetrads showing segregation of *HXT1-HXT4*, *HXT2*, *HXT3*, and *HXT4* genes in an *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* background. The left panels show growth on 5% glucose YPD medium. In all cases, the two smaller spores exhibited the prototrophy associated with the *hxt* deletion mutations. The right panels show growth on 5% glucose YPD medium supplemented with 1 μg of antimycin A per ml. The two growing spore colonies harbor the wild-type *HXT* genes indicated. A hyphen is used to indicate physical linkage between the *HXT1* and *HXT4* loci (Fig. 1).

cross showed that all spores were viable on 2% glucose YPD medium. Glucose transport appears to be impaired in *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* quadruple mutants and *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* quintuple mutants since these colonies developed more slowly than did colonies of cells containing other combinations of *hxt* deletion mutations on 2% glucose medium. This interpretation was supported by the observation that expression of any one of the *HXT* genes is sufficient for normal growth on 2% glucose YPD medium. The left panel in Fig. 6 shows a 2 large:2 small segregation pattern among tetrads derived from crosses in which wild-type *HXT* genes are segregating in the quintuple deletion background. In each case, the two larger spore colonies carry the wild-type *HXT1-HXT4*, *HXT2*, *HXT3*, or *HXT4* gene as indicated by the absence of the selective markers associated with the *hxtΔ* alleles. The smaller spore colonies carried the *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* mutations.

The slow growth of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* segregants on YPD medium is probably due to the ability of these cells to utilize amino acids as carbon sources via respiration. To better assess the ability of the spore colonies from these crosses to utilize glucose, antimycin A was added to medium containing 5% sugar. Fresh ascospores were then dissected and allowed to develop into colonies. Under these

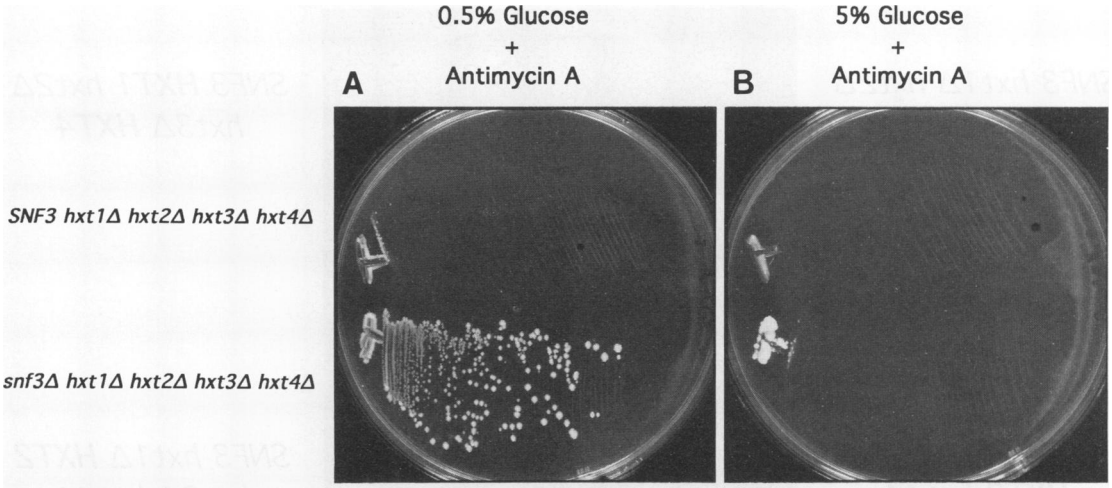


FIG. 7. Growth phenotypes of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells (CY294) and *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells (CY290) on 0.5% (A) and 5% (B) glucose YPD medium with 1 μg of antimycin A per ml. Cells were spread on a plate and incubated at 30°C for 3 days before being photographed.

conditions, *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* spores were unable to develop into colonies even after 4 days of incubation at 30°C. However, spores containing *HXT2*, *HXT3*, *HXT4*, or the linked *HXT1-HXT4* double combination rapidly developed into colonies (Fig. 6, right panel), demonstrating the functional independence of these transporters. In other experiments, *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells transformed with a wild-type *HXT1* gene carried on a centromeric plasmid were capable of luxuriant growth on 5% glucose with antimycin A (data not shown), confirming the functional independence of *HXT1* as well. The negative growth phenotype of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells suggests that HXT1, HXT2, HXT3, and HXT4 are the only transporters capable of supporting rapid growth on a high-glucose medium. Upon continued incubation, however, *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells ultimately show growth even in the presence of the respiration inhibitor. We do not yet understand the basis of this latent phenotype.

The glucose transporter(s) remaining in *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells is glucose repressible. Surprisingly, although *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells fail to grow on medium containing 5% glucose with antimycin A, they are capable of growth on media containing lower concentrations of glucose (≤0.5%) even in the presence of the inhibitor (Fig. 7). This suggests the existence of at least one additional glucose transporter. The inability of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells to grow on high concentrations of glucose is not due to a sensitivity to glucose since these cells grew well on medium containing 5% glucose supplemented with 3% glycerol and 2% ethanol (data not shown). Thus, either the expression or the function of this sixth glucose transporter is glucose repressible.

Consistent with the presence of a sixth glucose transporter, we detected DNA sequences on chromosome V by using probes derived from the coding regions of *HXT1* and *HXT3* (data not shown). These sequences are not *HXT2*, *GAL2*, or *SNF3* since these genes have been mapped to chromosomes XIII, XII, and IV, respectively (13, 16, 27). High-stringency Southern blot analysis of the tetrad obtained from a diploid (CY286/CY287) heterozygous for the *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* mutations detected a common 1.3-kb fragment hybridizing to the *HXT1* and *HXT3* probes (Fig. 5). This sequence might correspond to the putative sixth glucose transporter; the sequence identity between the 1.3-kb fragment and *HXT1/HXT3* is likely to be great since *HXT2* (61% identical to *HXT1*) was not detectable under the stringent washing conditions of this experiment.

***SNF3* abolishes the ability of *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells to grow on low-glucose media.** When tetrads from a cross between an *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* strain (CY290) and an *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* strain (CY294) were dissected on high-glucose medium (2 or 5%) with antimycin A, none of the segregants developed into colonies. We anticipated this result because *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are unable to grow on high-glucose medium and rescue by *SNF3* was not likely since *SNF3* expression is itself glucose repressible (3). Unexpectedly, when tetrads from this cross were dissected on low-glucose medium (0.5%) with antimycin A, a 2:0 segregation pattern for growth was observed and the viable spores contained the *snf3Δ* allele (His$^+$; *snf3Δ::HIS3*). Thus, although cells containing all five deletion mutations are capable of growth on low-glucose medium with antimycin A, the expression of *SNF3* abolishes this ability (Fig. 7). These results reveal that the putative sixth glucose transporter that allows the growth of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells on low glucose may be negatively regulated by *SNF3*. Alternative conclusions are presented in the Discussion.

***SNF3* is insufficient for growth on glucose.** Taken together, the requirement for *SNF3* for growth on raffinose (2) and the inferred structure of the SNF3 protein (3) strongly suggest that *SNF3* is a glucose transporter. However, our results show that expression of *SNF3* prevents the growth of *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells on low-glucose medium. To determine directly the contribution by *SNF3* to sugar uptake, we tested *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells for growth on raffinose medium and media containing 0.1 to 5% glucose, each supplemented with antimycin A. *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells failed to grow under any of these conditions. Thus, *SNF3* is insufficient for growth on high or low glucose.

Neither the entire complement of four *HXT* genes nor *SNF3* alone is sufficient for growth on raffinose. However, growth on raffinose is restored by any one of the *HXT* genes

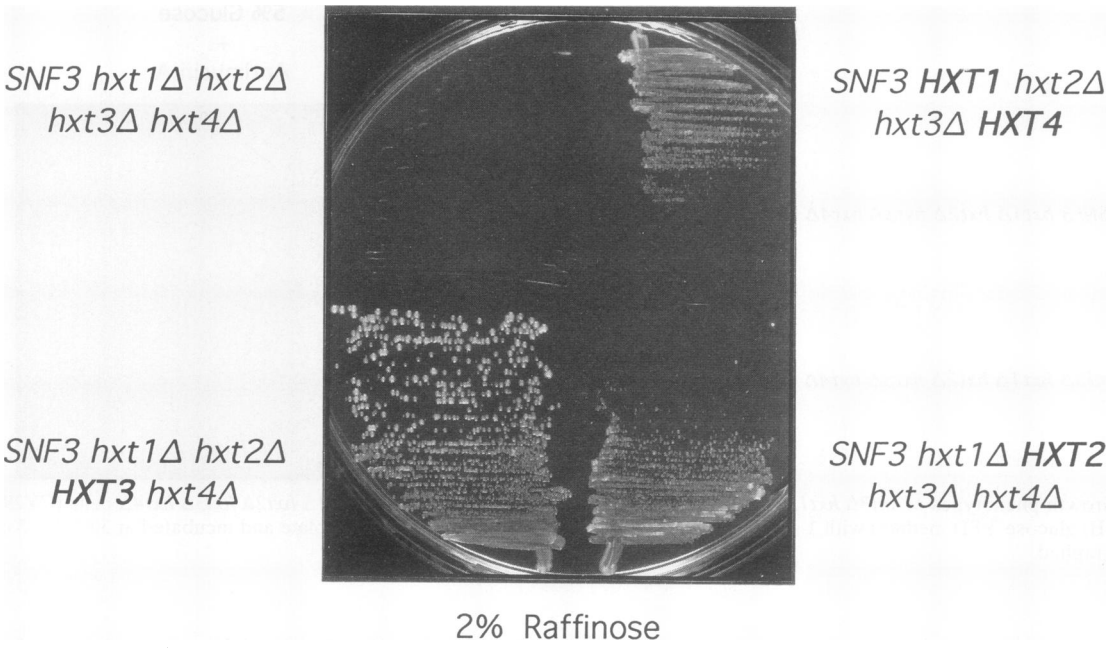


FIG. 8. Growth phenotypes of *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* (CY290), *SNF3 HXT1 hxt2Δ hxt3Δ HXT4* (CY288), *SNF3 hxt1Δ hxt2Δ HXT3 hxt4Δ* (CY287), and *SNF3 hxt1Δ HXT2 hxt3Δ hxt4* (CY289) cells on raffinose medium containing 100 mM KCl. Cells were spread on a plate and incubated at 30°C for 3 days before being photographed. *SNF3Δ hxt1Δ hxt2Δ hxt3Δ HXT4* cells were capable of growth on raffinose medium (data not shown).

in the presence of *SNF3* (Fig. 8). This suggests a synergistic relationship between *SNF3* and the members of the *HXT* family.

***HXT1* and *HXT3* are expressed under different growth conditions.** Since the 5′ untranslated regions of the *HXT1* and *HXT3* genes contain unrelated sequences, we wished to determine the conditions under which *HXT1* and *HXT3* are maximally expressed. The *HXT1*::*lacZ* and *HXT3*::*lacZ* fusion plasmids described in Materials and Methods were used to measure *HXT1*- and *HXT3*-dependent β-galactosidase activity under different growth conditions. pCK169 and pCK159 containing the *HXT1* and *HXT3* promoters, respectively, fused to the *lacZ* coding region were introduced into wild-type recipients (R757) by transformation to Ura^+. The expression of *HXT1* and *HXT3* was inferred by measuring β-galactosidase activity at different points during the growth of the culture. Figure 9 shows the β-galactosidase activity profiles of the *HXT1*::*lacZ* and *HXT3*::*lacZ* fusions in conjunction with their respective growth curves. The *HXT1*::*lacZ* fusion was primarily expressed during the exponential phase of growth. These data are in disagreement with the earlier conclusion of Lewis and Bisson (14) that *HXT1* is expressed at a very low level and only during the lag phase of growth. This discrepancy could originate from the unusually large degree of oscillation that we repeatedly observed for *HXT1*::*lacZ* expression. This oscillation was not observed for the *HXT3*::*lacZ* fusion.

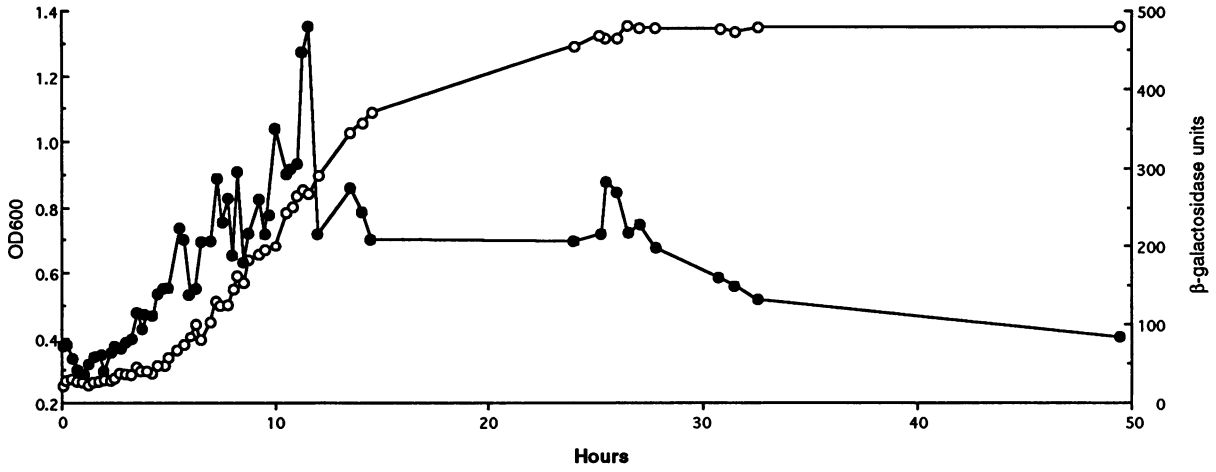
The *HXT3*::*lacZ* fusion expressed maximal β-galactosidase activity after entry into stationary phase (Fig. 9B). *HXT3*::*lacZ* expression decreased as cells progressed further into stationary phase. To determine whether expression of the *HXT3*::*lacZ* fusion was induced by low glucose, cultures in the exponential phase of growth were shifted to low-glucose medium and β-galactosidase activity was monitored for 3 h after the shift. The *HXT3*::*lacZ* fusion was not induced by low glucose (data not shown). Since induction of *HXT3* may take longer than 3 h, no definitive conclusions can be drawn concerning low-glucose inducibility of *HXT3*. However, our results clearly demonstrate that *HXT1* and *HXT3* are differentially regulated.

DISCUSSION

We have identified mutant alleles of two members of the sugar transporter gene superfamily, *HXT1* and *HXT3*, among suppressors that restore potassium uptake in cells deleted for *TRK1* and *TRK2* (11). *HXT1* was previously identified as a probable glucose transporter while *HXT3* is a new member of this family in *S. cerevisiae* that also includes *HXT2* (13) and *SNF3* (3). HXT1 and HXT3 share 87% sequence identity, making them the most highly related members of the sugar transporter superfamily. HXT1 and HXT3 share significant sequence identity with the other glucose transporters as well as with the galactose transporter (GAL2).

The presence of any one (or more) of the *HXT* genes is sufficient for growth on high-glucose medium. Thus, each of these putative transporters is functionally independent. In contrast, our results suggest that at least the normal expression of SNF3 is insufficient for glucose transport. In the presence of antimycin A, *SNF3 hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are unable to grow on media containing glucose or raffinose as the sole carbon source regardless of their concentrations. Thus, the normal expression of *SNF3* alone cannot provide the cell with sufficient glucose for growth. Furthermore, since *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are able to grow on low glucose, our results suggest that SNF3 can function as a negative regulator of glucose transport. Perhaps SNF3 has undergone evolutionary changes in which it acquired regulatory function at the expense of transport capability. In this regard, we note that there is significantly

A *HXT1::lacZ*



B *HXT3::lacZ*

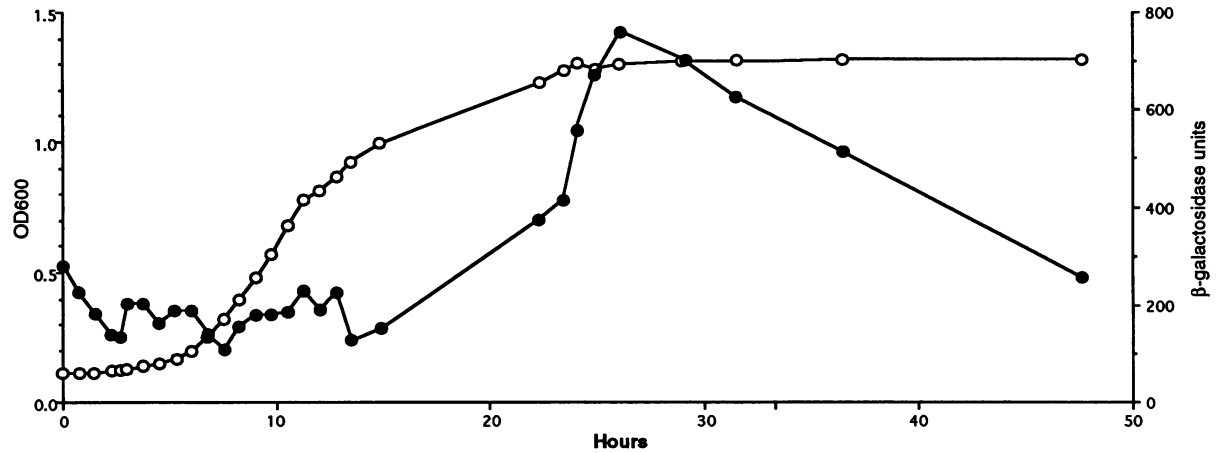


FIG. 9. β-Galactosidase activity profiles of the *HXT1::lacZ* fusion (A) and the *HXT3::lacZ* fusion (B) in wild-type cells (R757). Yeast transformants were grown to stationary phase in 2% glucose synthetic complete medium lacking uracil to maintain plasmid selection. Triplicate samples were drawn during the growth cycle, and β-galactosidase activity was measured as described by Guarente (9). β-galactosidase activity, in Miller units (18), is indicated by closed circles, and optical density at 600 nm (OD600) is indicated by open circles.

greater sequence identity between each of the HXT transporters (62 to 88%) than there is between them and SNF3 (22%). Even GAL2, a galactose transporter, contains greater sequence identity with the HXT transporters (62 to 66%) than does SNF3. The overexpression of *SNF3* in *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells may provide a test to determine whether SNF3 can transport glucose in the absence of other transporters.

SNF3 was previously shown to play a positive role in transport: *snf3* cells are unable to grow on raffinose or low-glucose media because of the loss of a component of the high-affinity uptake system (2). Our results show that *SNF3* is not sufficient for growth on raffinose or low-glucose media: at least one of the *HXT* genes is also required. Several nonexclusive possibilities are consistent with these results; (i) SNF3 may be incapable of independent transport of fructose or glucose; (ii) full expression of *SNF3* or activity of the SNF3 protein might depend on the presence of at least one functional HXT protein, or (iii) in the absence of an HXT transporter, interaction between SNF3 and another protein might interfere with its transport capability.

Although *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells presumably cannot take up glucose when the sugar is present in high concentrations, these cells grow well on medium containing low concentrations of glucose (≤0.5%) even in the presence of antimycin A. This result reveals the presence of an additional glucose transporter in *S. cerevisiae*. Since *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells are capable of growth on low-glucose medium, their inability to grow on high-glucose medium suggests that the remaining glucose transporter(s) is itself glucose repressible. We speculate that this transporter is encoded by the *HXT1/3*-related sequence detected in *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells by Southern blot analysis with *HXT1* and *HXT3* probes.

We have discovered that *SNF3* may actually play a

negative role in transport. Although *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells grow on low-glucose medium, *SNF3* abolishes this ability. *SNF3* apparently inhibits the function (or expression) of a glucose-repressible transporter that otherwise allows growth of *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells. Several possibilities can explain the negative effect that *SNF3* has on glucose transport. First, SNF3 could interact directly with other glucose transporters. Compared with other sugar transporters, SNF3 contains unusually long amino and carboxy termini. Either of these domains could be involved in interactions with the putative sixth glucose transporter, resulting in negative effects on transport. Alternatively, although unlikely, *SNF3* could regulate the transcription of other transporter genes. It is also possible that SNF3 transports glucose in a manner that does not supply the cell with metabolic glucose but mediates a regulatory signal that in turn controls the expression or function of other transporters (1).

We have shown that cells containing null alleles of five genes encoding putative glucose transporters are viable. *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells grow normally when galactose, maltose, or glycerol plus ethanol are supplied as the sole carbon source. In contrast, these cells are unable to grow on high-glucose (>1%) medium when the ability to use other carbon sources such as amino acids is precluded by the presence of the respiration inhibitor antimycin A. As far as we are aware, this is the first time that a eukaryotic cell has been rendered unable to take up glucose when the sugar is abundant. *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells should be useful for the identification and characterization of heterologous glucose transporters by providing a null background in which even partial function could be detected. In addition, the availability of cells expressing individual *S. cerevisiae* glucose transporters should allow direct measurements of their kinetic parameters. Such analyses have, up to now, been complicated by the expression of multiple transporters. Finally, the availability of a negative phenotype may allow the isolation of intergenic and extragenic suppressors that restore glucose uptake to cells expressing mutant transporters.

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