A Novel Signal Transduction Pathway in *Saccharomyces cerevisiae* Defined by Snf3-regulated Expression of *HXT6*

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We show that cells deleted for *SNF3*, *HXT1*, *HXT2*, *HXT3*, *HXT4*, *HXT6*, and *HXT7* do not take up glucose and cannot grow on media containing glucose as a sole carbon source. The expression of *Hxt1*, *Hxt2*, *Hxt3*, *Hxt6*, or *Gal2* in these cells resulted in glucose transport and allowed growth on glucose media. In contrast, the expression of *Snf3* failed to confer glucose uptake or growth on glucose. *HXT6* is highly expressed on raffinose, low glucose, or nonfermentable carbon sources but is repressed in the presence of high concentrations of glucose. The maintenance of *HXT6* glucose repression is strictly dependent on *Snf3* and not on intracellular glucose. In *snf3* Δ cells expression of *HXT6* is constitutive even when the entire repertoire of *HXT* genes is present and glucose uptake is abundant. In addition, glucose repression of *HXT6* does not require glucose uptake by *HXT1*, *HXT2*, *HXT3* or *HXT4*. We show that a signal transduction pathway defined by the *Snf3*-dependent hexose regulation of *HXT6* is distinct from but also overlaps with general glucose regulation pathways in *Saccharomyces cerevisiae*. Finally, glucose repression of *ADH2* and *SUC2* is intact in *snf3* Δ *hxt1* Δ *hxt2* Δ *hxt3* Δ *hxt4* Δ *hxt6* Δ *hxt7* Δ *gal2* cells, suggesting that the sensing and signaling mechanism for general glucose repression is independent from glucose uptake.

INTRODUCTION

Glucose is the predominant carbon and energy source for most eukaryotic cells including *Saccharomyces cerevisiae*. The transport of glucose and other hexoses in *S. cerevisiae* occurs via facilitated diffusion (Heredia *et al.*, 1968) and can display high-affinity (K_m 1–2 mM) or low-affinity (K_m ~20 mM) kinetics depending on the availability of extracellular sugar (Bisson, 1988; Bisson and Fraenkel, 1983a). At least 18 genes encoding highly related putative hexose transporters, *HXT1*–*HXT17* (hexose transporter) and *GAL2*, have been identified in *S. cerevisiae* (Kruckeberg and Bisson, 1990; Lewis and Bisson, 1991; Ko *et al.*, 1993; Theodoris *et al.*, 1994; Nelissen *et al.*, 1995; Reifengerger *et al.*, 1995; Kruckeberg, 1996). *Gal2* is able to mediate galactose transport and *gal2* Δ cells are unable to grow on galactose as the sole carbon source (Tschopp *et al.*, 1986;

Nehlin *et al.*, 1989). In contrast, none of the hexose transporter genes have been found to be essential for glucose uptake or cell growth on glucose. In cells containing null alleles of *HXT1*, *HXT2*, *HXT3*, and *HXT4*, growth on high concentrations of glucose (>2%) was abolished and expression of any one of these genes was able to restore this ability (Ko *et al.*, 1993). More recently, glucose uptake was shown to be essentially abolished in *hxt1* Δ *hxt2* Δ *hxt3* Δ *hxt4* Δ *hxt5* Δ *hxt6* Δ *hxt7* Δ cells, and the expression of *HXT1*–*HXT4*, *HXT6* or *HXT7* in this background was sufficient to restore glucose uptake (Reifengerger *et al.*, 1995).

Snf3 (sucrose nonfermenting) encodes a polytopic membrane protein that shares significant amino acid sequence identity with other members of the glucose transporter superfamily (Celenza *et al.*, 1988; Marshall-Carlson *et al.*, 1990). *snf3* mutants were identified by their inability to grow on sucrose or raffinose and high-affinity glucose transport was shown to be abolished in *snf3* cells (Neigeborn and Carlson, 1984;

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Neigeborn *et al.*, 1986; Bisson *et al.*, 1987). Based on these results, Snf3 was believed to be a high-affinity glucose transporter. This was further bolstered by studies which showed that the overexpression of other glucose transporters, encoded by *HXT1*, *HXT2*, *HXT3*, or *HXT4*, could restore the ability of *snf3* cells to grow on raffinose (Kruckeberg and Bisson, 1990; Lewis and Bisson, 1991; Ko *et al.*, 1993; Theodoris *et al.*, 1994).

Despite being a member of the sugar transporter superfamily, Snf3 harbors significant differences compared with other hexose transporters in *S. cerevisiae*. The hydrophobic core region of Snf3 containing the 12 putative membrane-spanning domains shares <28% sequence identity with that of the Hxt proteins, whereas Hxt1 through Hxt7 and Gal2 share from 68% to >99% sequence identity within that region. Furthermore, the cytoplasmic carboxyl termini of Hxt1–Hxt7 and Gal2 are highly related and are only 37–57 amino acids in length. In contrast, the analogous region of Snf3 is 303 amino acids in length and does not share any significant sequence identity with that of the Hxt or Gal2 proteins.

Several lines of evidence indicate that instead of transporting glucose as was originally believed, Snf3 might act as a regulatory protein. First, *SNF3* is very poorly expressed compared with known functional

transporter genes (Neigeborn *et al.*, 1986; Wendell and Bisson, 1994). Second, Snf3 inhibits growth of *hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells on 0.5% glucose (Ko *et al.*, 1993). Third, analysis of kinetic data suggests that the decrease in high-affinity glucose uptake in *snf3* cells could not be due to the loss of a single transporter (Coons *et al.*, 1995). Fourth, in *snf3* cells, the expression of *SUC2*, *ADH2*, and some *HXT* genes is altered (Neigeborn *et al.*, 1986; Ozcan and Johnston, 1995; and this report). In this report, we demonstrate that Snf3 is not sufficient to mediate the nutritional uptake of glucose; nevertheless, it regulates expression of *HXT6* in response to extracellular hexoses.

MATERIALS AND METHODS

Strains and Media

The *S. cerevisiae* strains used in this study are listed in Table 1. YPD, YNB, sporulation media, and standard genetic techniques were as described by Sherman *et al.* (1986). The 2% glucose in standard media was replaced by the appropriate carbon sources to make the specific media. In all of the growth tests assessing the ability of cells to utilize a specific sugar as the sole carbon source, antimycin A (Sigma Chemical, St. Louis, MO) was added at a final concentration of 1 μg/ml to inhibit respiration. Yeast transformation was performed by electroporation (Becker and Guarente, 1991) using Gene Pulser (Bio-Rad, Richmond, CA).

Table 1. *S. cerevisiae* strains used in this study

Strains	Genotype	Source
R757	<i>MATα his4-15 lys9 ura3-52 gal2</i>	Gaber <i>et al.</i> , 1988
R1155	<i>MATα his4-15 lys9 ura3-52 trk1Δ gal2</i>	Gaber <i>et al.</i> , 1988
A303	<i>MATα his3Δ-200 leu2Δ1 trp1Δ1 ura3-52</i>	this laboratory
HY135	<i>MATα his3Δ-200 leu2Δ1 trp1-1 ura3-52 snf3Δ::TRP1</i>	this study
CY263	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::TRP1 HXT6/7 gal2</i>	this laboratory
CY280	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 HXT6/7 gal2</i>	Ko <i>et al.</i> , 1993
CY316	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 HXT6/7 gal2</i>	Ko <i>et al.</i> , 1993
CY327	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt2Δ::LEU2 hxt3Δ::URA3 HXT6/7 gal2</i>	this laboratory
HY101	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt2Δ::LEU2 hxt3Δ::URA3 hxt6/7Δ::TRP1 gal2</i>	this study
HY102	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 HXT6/7 gal2</i>	this study
HY103	<i>MATα his3Δ-200 leu2Δ trp1Δ ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 hxt6/7Δ::TRP1 gal2</i>	this study
HY133	<i>MATα his3Δ-200 leu2Δ1 trp1Δ ura3-52 snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::TRP1 hxt6/7Δ::TRP1 gal2</i>	this study
YM4127	<i>MATα ade2-10 his3Δ-200 leu2-3,2-112 lys2-801 trp1-901 tyr1-501 ura3-52</i>	Ozcan and Johnston, 1995
YM2955	<i>MATα ade2-10 his3Δ-200 leu2-3,2-112 lys2-801 ura3-52 grr1Δ::LEU2</i>	Ozcan and Johnston, 1995
YM4512	<i>MATα ade2-10 his3Δ-200 leu2-3,2-112 lys2-801 trp1-901 tyr1-501 ura3-52 hxt2Δ::LEU2</i>	Ozcan and Johnston, 1995
YM4513	<i>MATα ade2-10 his3Δ-200 leu2-3,2-112 lys2-801 trp1-901 tyr1-501 ura3-52 tup1Δ::TRP1</i>	Ozcan and Johnston, 1995
YM4554	<i>MATα ade2-10 his3Δ-200 leu2-3,2-112 lys2-801 trp1-901 tyr1-501 ura3-52 ssn6Δ::ura3::Leu2</i>	Ozcan and Johnston, 1995

DNA Manipulations

Standard techniques for DNA purification and cloning were as described in Maniatis *et al.* (1982). DNA sequencing analysis was performed using the dideoxy method (Sanger *et al.*, 1977) and synthetic oligonucleotide primers. Database searches were performed using BLAST (National Center for Biotechnology Information). DNA and protein sequence alignments and analyses were performed using the GeneWorks software (IntelliGenetics, Inc., Mountain View, CA) and the DNA Inspector IIe (Textco, W. Lebanon, NH). Polymerase chain reaction (PCR) was performed on a GeneAmp PCR system 9600 (Perkin Elmer-Cetus, Norwalk, CT) as described by Innis *et al.* (1990). Oligonucleotide primers were either obtained from Northwestern University Biotechnology Facility or synthesized from Oligo Synthesizer 1000 (Beckman, Arlington Heights, IL). DNA hybridization analyses were performed using QuickHyb solution (Stratagene, La Jolla, CA) according to the manufacturer's instructions. DNA probes were prepared with the random priming method (Feinberg and Volgelstein, 1983) using [α -³²P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA).

Cloning of *HXT6/7*

Southern blot analysis was performed on genomic DNA isolated from *S. cerevisiae* strain CY280 (*snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ gal2*) and probed with a 783-bp *EcoRI-HpaI* DNA fragment within the *HXT3* open reading frame isolated from pCK164 (Ko *et al.*, 1993). The filters were hybridized at 60°C for 60 min with the labeled probe in QuickHyb solution (Stratagene), washed three times for 15 min in 0.1× SSC/0.1% SDS at 55°C (high stringency), and exposed to Kodak X-OMAT AT film for 12 to 24 h. A 2.3-kb *NsiI* DNA fragment and a 2.8-kb *HindIII* DNA fragment were detected by the *HXT3* probe. *NsiI* DNA fragments ranging from 2 to 2.6 kb and *HindIII* fragments ranging from 2.5 to 3.2 kb were retrieved from agarose gel and cloned into plasmid pGEM7Z (Promega, Madison, WI). The resulting two DNA pools were subjected to colony hybridization as described in Maniatis *et al.* (1982) using the same *HXT3* probe and hybridization conditions as described above. Two overlapping positive clones (pHL112 and pHL114), one from each of the DNA pools, were obtained.

Plasmid Construction

pHL112 and pHL114 were isolated using the colony hybridization experiments described above, carrying a 2.3-kb *NsiI* fragment and a 2.8-kb *HindIII* fragment, respectively. The full-length *HXT6/7* gene was constructed and cloned in a centromeric yeast expression vector pRS316 (Sikorski and Heiter, 1989; pHL132) as follows: The 1.8-kb *NsiI-HindIII* fragment from pHL112 was subcloned in pRS316 (digested with *HindIII* and partially digested with *PstI*) to produce pHL147. The 2.8-kb *HindIII* fragment from pHL114 was subcloned into pHL147 at the correct orientation to produce pHL132. The multicopy plasmid pHL133 containing *HXT6/7* was constructed by subcloning the 4.6-kb *SalI-BamHI* fragment from pHL132 into a 2 μ m-based vector pRS426 (Christianson *et al.*, 1992). Centromeric and 2 μ m-based plasmids harboring the *HXT6* gene, pHL157 and pHL158, respectively, were constructed as follows: The 1-kb DNA fragment encompassing the 5' half of *HXT6* was amplified by PCR using *HXT6-3* (AATTATACCTTCTATTGGCAACATCTTGCCCT) and *HXT6-4* (AAGATCGAAGAAGCCAAACGT) as primers and the yeast genomic DNA (strain R1155; Gaber *et al.*, 1988) as templates and treated with endonucleases *BamHI* and *HindIII*. The 1.6-kb DNA fragment encompassing the 3' half of *HXT6* was obtained from pHL133 with *BamHI* and *HindIII* digestion. Both halves of *HXT6* were cloned in *BamHI*-treated pRS316 (centromeric) or pRS426 (2 μ m-based) in a one-step ligation. The correct sequence of the plasmids was verified by DNA sequence analysis.

Centromeric (pHL136) and 2 μ m-based multicopy (pHL137) plasmids harboring *SNF3* were constructed as follows: The 2.5-kb *SalI-EcoRI* fragment from p*SNF3A-10* (gift from M. Carlson; Marshall-

Carlson *et al.*, 1990) was subcloned into pRS316 to produce pHL150. The 400-bp DNA fragment amplified by PCR using *SNF3-1* (ATAACAGAGGTCCACCTTCAA) and *SNF3-2* (GACGAATTCGTCGACATGTAATTGAATGTGTGTCAAGA) as primers and yeast genomic DNA (strain R1155) as template was treated with endonuclease *EcoRI* and subcloned into pHL150 to produce pHL151. The ~3-kb *SalI* fragment from pHL151 was cloned into pRS316 and pRS426 to create plasmids pHL136 and pHL137, respectively.

The *HXT2*-containing plasmid pSC2-1C3 was kindly provided by L. Bisson (Kruckeberg and Bisson, 1990). Single-copy plasmid pHL144 harboring *HXT2* was constructed by subcloning the *XhoI-SalI* fragment from pSC2-1C3 into pRS316.

Centromeric plasmids pCK142 and pCK164 (Ko *et al.*, 1993) were used to express *HXT1* and *HXT3*, respectively.

Plasmid pHL125 (p*ADH1p-GAL2*) expresses *GAL2* under control of the constitutive promoter of the *ADH1* gene and was constructed as follows: The 2.7-kb *EcoRI* fragment encompassing the *GAL2* gene from plasmid pTUG4 (gift from R. Schekman; Tschoopp *et al.*, 1986) was subcloned into pRS316 in both orientations to produce pAM2 and pTH318. The 2.3-kb *HindIII-XbaI* fragment from pAM2 was introduced into vector pVT100-U (Vernet *et al.*, 1987) to create plasmid pHL125.

Plasmid pHL183 expresses the *HIS3* reporter gene under control of the *HXT6* promoter (p*HXT6p-HIS3*). The sequence of the *HIS3* open reading frame was amplified by PCR using *HIS3-1* (GCCGAATTCCCAAGAGCAGAAAGCCCTAGTAAA) and *HIS3-2* (GACGGATCCCACACCGCATAGATCCGTCGA) as primers and pRS423 (Christianson *et al.*, 1992) as template. The DNA fragment obtained from PCR was treated with restriction endonucleases *BamHI* and *EcoRI* and subcloned into pRS316 to produce pHL182. The 1.2-kb *HXT6* promoter region was amplified by PCR using *HXT6p-1* (GCCAAGCTTTGCATGATGACCAGCCATTCTA) and *HXT6p-2* (GCCGAATTCCAATAGCGTCTTGTGACAT) as primers and a plasmid carrying *HXT6/7* (pHL133) as template. The DNA fragment obtained from PCR was treated with restriction endonucleases *HindIII* and *EcoRI* and subcloned into pHL182 to create pHL183.

Plasmid pHL115 was generated to disrupt the chromosomal *HXT6/7* gene using the one-step integration method (Sikorski and Heiter, 1989). It was constructed by subcloning the 1.8-kb *NsiI-ApaI* fragment and the 286-bp *HpaII-NsiI* fragment into an integrative shuttle vector pRS304 (Sikorski and Heiter, 1989) treated with *ApaI* and *Clal* in a one-step ligation. The resulting plasmid pHL115 was linearized at the *NsiI* site prior to be transformed into host cells.

Strain Construction

Deletion of *HXT6/7* was achieved with the one-step integration method (Sikorski and Heiter, 1989) using plasmid pHL115. Haploid strain CY327 (this laboratory; *snf3Δ hxt2Δ hxt3Δ gal2*) was transformed to a TRP⁺ phenotype with pHL115 linearized at the *NsiI* site. The appropriate recombination at the *HXT6/7* locus in the resulting strain (HY101) was verified by Southern blot hybridization analysis (our unpublished results). Strain HY103 (*snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 hxt6/7Δ::TRP1 gal2*) was one of the two TRP⁺ spores in a nonparental ditype tetrad from the following cross: strain HY101 (*snf3Δ::HIS3 hxt2Δ::LEU2 hxt3Δ::URA3 hxt6/7Δ::TRP1 gal2*) and strain HY102 (*snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::URA3 gal2*). Strain HY102 was obtained as a meiotic segregant from a cross between strain CY316 (Ko *et al.*, 1993) and strain CY327. Strain HY133 (*snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::TRP1 hxt6/7Δ::TRP1 gal2*) was obtained as a meiotic segregant from a cross between strain HY103 and CY263 (*snf3Δ::HIS3 hxt1Δ::TRP1::hxt4Δ hxt2Δ::LEU2 hxt3Δ::TRP1 gal2*, this laboratory).

Strain HY135 contains a deletion of the entire open reading frame of *SNF3* and was constructed according to the method described by Baudin *et al.* (1993). Hybrid DNA fragments containing the *TRP1* gene flanked by the 50-bp sequences immediately adjacent to the

SNF3 open reading frame on each end were generated by PCR. *snf3d1* (TTTAAACAGAAGGATATGCCTTTGTTGGCATAGAAA-GAAGAATTTATAAATTGTACTGAGAGTGCACCAT) and *snf3d2* (CACTTTATGGCGTGTCTACGCTACATAAATTAATTCGCTG-CACGTAATATTAAAATTAGTACACTTTCTTTA) were used as primers and plasmid pRS304 was used as template in the PCR. DNA fragments obtained were purified and introduced into a haploid strain A303 to produce the TRP⁺ phenotype. The appropriate recombination at the *SNF3* locus was verified by PCR.

Glucose Uptake Assays

The glucose uptake assay protocol used was kindly provided by L. Bisson (Bisson and Fraenkel, 1983b) with slight modifications suggested by K. van Dam. Yeast cells were grown to mid-log or late log phase in appropriate media, harvested by filtration, washed three times with potassium phosphate buffer (100 mM, pH 6.5), and resuspended in the phosphate buffer to a final cell density of OD₅₈₀ 15. Cells were stored at 4°C while waiting and preincubated for 20 min at 30°C before the assay. Eighty microliters of cell suspension were mixed with 20 μ l of radiolabeled glucose ([U-¹⁴C]D-glucose, 242 mCi/mmol; ICN Biomedicals, Costa Mesa, CA) solution for 5 s, washed twice with ice-cold 5% glucose, and the radioactivity of the filters was measured with a scintillation counter. The final glucose concentrations in the cell suspensions ranged from 0.2 to 200 mM. A zero second control was performed with each cell sample at a certain glucose concentration. Uptake was plotted as V [nmoles glucose/min/mg cells (dry wt)] vs. V/S where S is mM glucose.

RNA Hybridization Analysis

RNA samples were purified from yeast cells and Northern blot hybridization analysis was performed as described by Ausubel *et al.* (1987). Approximately 20 μ g of total RNA were loaded in each lane. Antisense synthetic oligonucleotides were labeled with [γ -³²P]dATP (6000 Ci/mmol, New England Nuclear) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Because of the extreme high sequence identity between *HXT6* and *HXT7*, it was not possible to design a probe that could easily distinguish *HXT6* from *HXT7* RNA. An antisense oligonucleotide probe *HXT6*-p (CCACAG-GAGTTTGCTCTGCAATAGCAGCTTGTGACAT) complementary to *HXT6* RNA from positions +1 to +37 indistinguishably recognizes RNA products from *HXT6*, *HXT7* and *HXT6/7*, since all three contain identical sequences at those positions. Synthetic oligonucleotides *ACT1*-p (AGGACAAAAGGCTTGGATGGAAGTA-GAAGGCTGGA ACG), *SUC2*-p (AATCAACCACCATGGAGACA-GAGAAAGCACCTGAAT CGTTACGCTTGGGAG), *ADH2*-p (TGCCGTTGGATTTCGTAGAAGATAATGGCTTTTCTGG-AATAGAC), and *HIS3*-p (AGGCCTCTTTAAAAGCTTGAC-CGAGAGCAATCCCGCAGTCTT CAGTGGT) were used to detect transcripts from *ACT1*, *SUC2*, *ADH2*, and *HXT6p-HIS3* (*HIS3* open reading frame expressed from the *HXT6* promoter), respectively. Filters were hybridized for 1 h at 60°C with the labeled probe in QuickHyb solution (Stratagene) and washed in 0.1 $\times$ SSC/0.1% SDS solution once at room temperature for 30 min and twice at 55°C for 15 min. The filters were exposed to Kodak X-OMAT AR film for 12 to 72 h.

RESULTS

Cloning of *HXT6/7* and *HXT6*

We previously showed that *S. cerevisiae* cells deleted for *SNF3*, *HXT1*, *HXT2*, *HXT3*, and *HXT4* retain the ability to grow on media containing 0.5% glucose as the sole carbon source (Ko *et al.*, 1993). To identify the glucose transporters that are still present in *snf3* Δ *hxt1* Δ *hxt2* Δ *hxt3* Δ *hxt4* Δ cells (strain CY280), a colony

hybridization experiment was performed in which a library of genomic DNA prepared from these cells was probed with a segment of the *HXT3* coding sequence. Under stringent hybridization conditions, a single class of cross-hybridizing DNA fragments was identified. DNA sequence analysis revealed that these fragments harbored a chimeric gene consisting of the 5' half of *HXT6* (including the 5' nontranslated region) and the 3' half of *HXT7* (including the 3' nontranslated region). The coding sequence of the chimera (designated as *HXT6/7*) differs from that of *HXT6* and *HXT7* by two and three nucleotides, respectively. The inferred amino acid sequence of *Hxt6/7* differs from that of *Hxt6* or *Hxt7* by only a single amino acid: *HXT6/7* contains a threonine codon at position 556 whereas *HXT6* contains an alanine codon at this position. A valine codon corresponding to amino acid position 293 in *Hxt7* is replaced by a isoleucine codon in *HXT6/7* (Figure 1).

Using primers that could specifically hybridize to the noncoding sequences of *HXT6* or *HXT7*, we determined that strain CY280 from which *HXT6/7* was cloned contains the *HXT6/7* chimera rather than the separate *HXT6* and *HXT7* genes. In fact, most of the *hxt* strains used in this study harbor the *HXT6/7* chimera gene. However, strains A303 and R757, which were used as wild-type controls, each contain the distinct *HXT6* and *HXT7* loci (our unpublished results). DNA sequence analysis of a cloned genomic DNA fragment (pSG256, this laboratory) that encompasses both the *HXT3* and *HXT6* genes revealed that they are oriented in the same direction and separated by 1644 nucleotides. The sequence of this region is essentially identical to that determined as part of the *S. cerevisiae* genome sequencing project (accession number

Figure 1. Relationship between the genomic regions encompassing *HXT6*, *HXT7*, and *HXT6/7*. The white areas represent identical sequences among *HXT6*, *HXT7*, and *HXT6/7*. The black regions represent the *HXT6* locus (accession number Z31691) and the gray regions represent the *HXT7* locus (accession number Z31692). Arrows denote open reading frames. The two sites where *HXT6* and *HXT7* encode different amino acids are indicated.

YSCD9651). *HXT6* was cloned from strain R1155 (*trk1Δ HXT6 HXT7*). The extensive nucleotide sequence identity between *HXT6* and *HXT7* along with the observation that they are linked in tandem on chromosome IV (Reifenberger *et al.*, 1995) suggest that the *HXT6/7* chimera arose as the result of an intrachromosomal recombination event.

Overexpression of *HXT6* or *HXT6/7* Can Suppress the Inability of *snf3Δ* Cells to Grow on Raffinose Media

snf3Δ cells are defective for high-affinity glucose uptake and are unable to grow on raffinose when respiration is blocked by antimycin A (Bisson *et al.*, 1987; Celenza *et al.*, 1988). Many members of the *HXT* gene family including *HXT1*, *HXT2*, *HXT3*, and *HXT4* have the ability, when overexpressed, to suppress the *snf3Δ* phenotypes (Kruckeberg and Bisson, 1990; Lewis and Bisson, 1991; Ko *et al.*, 1993; Theodoris *et al.*, 1994). To determine whether *HXT6* and *HXT6/7* also have this ability, 2 μ m-based plasmids containing *HXT6* or *HXT6/7* were introduced into a *snf3Δ* strain (HY135) and the ability of the resulting cells to utilize raffinose was tested. Overexpression of either *HXT6* or *HXT6/7* is sufficient to confer growth of *snf3Δ* cells on 2% raffinose (Figure 2). Furthermore, consistent with the fact that *HXT6* and *HXT6/7* are essentially identical, suppression of the *snf3Δ* phenotype by *HXT6/7* was indistinguishable from that by *HXT6*. Thus, *HXT6* and *HXT6/7* are capable of conferring the typical *HXT* overexpression phenotype.

Deletion of *HXT6/7* from *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* Cells Abolishes Glucose Uptake

To generate a glucose transport deficient strain and to determine whether *HXT6/7* is responsible for the

growth of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ* cells on glucose media, we constructed a strain in which *HXT6/7* was deleted from this background. Since the strains used in this study were derived from S288C which contains a *gal2* mutation, the full relevant genotype of the resulting strain is *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ hxt6Δ hxt7Δ gal2* (abbreviated as multi- Δ in this study). The multi- Δ cells failed to grow on medium containing raffinose, sucrose, glucose, or fructose (concentrations ranging from 0.1 to 5%) as sole carbon sources (Figure 3A and our unpublished results). No growth was observed even after 2 wk of incubation at 30°C, indicating that glucose uptake in these cells is severely impaired. This result also implicates *HXT6/7* as the main source of glucose uptake in *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ gal2* cells.

[¹⁴C]glucose uptake assays were performed to assess the glucose uptake activity of the multi- Δ cells. Under the growth conditions tested (medium containing glycerol/ethanol, glycerol/ethanol supplemented with 0.5% or 5% glucose), the multi- Δ cells showed virtually no [¹⁴C]glucose uptake. By comparison, a wild-type control (strain R757) which contains the full complement of *HXT* genes exhibited robust [¹⁴C]glucose uptake under the same growth conditions (Figure

Figure 2. Suppression of *snf3Δ* phenotype by overexpression of *HXT6* or *HXT6/7*. *snf3Δ* cells (strain HY135) harboring vector (pRS316), pSNF3 (pHL136), p2μ-*HXT6/7* (pHL133), or p2μ-*HXT6* (pHL158) were patched on glycerol/ethanol medium (left panel), replica plated onto raffinose medium, and incubated at 30°C for 3 days (right panel).

Figure 3. (A) Inability of multi- Δ cells to grow on glucose media. Multi- Δ (*snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ hxt6/7Δ gal2*, strain HY133) and WT (*gal2*, strain R757) cells were grown on glycerol/ethanol medium (left panel), replica plated to 0.5% (middle panel) or 5% (right panel) glucose medium, and incubated at 30°C for 3 days. (B) Eadie-Hofstee plot derived from [¹⁴C]glucose uptake assays showing the inability of the multi- Δ cells to take up glucose. Multi- Δ cells (strain HY133, $\square$) and WT cells (R757, $\blacklozenge$) were grown in glycerol/ethanol media before the uptake assays.

3B and our unpublished results). Our results show that both low- and high-affinity uptake can be abolished by deletion of glucose transporter genes, supporting the view that low-affinity glucose uptake is mediated through transporter proteins (Bisson and Fraenkel, 1983a; Coons *et al.*, 1995; Gamo *et al.*, 1995) instead of simple diffusion across the membrane bilayer (Fuhrmann and Volker, 1993). Our results also demonstrate that glucose uptake in *S. cerevisiae* requires one or more of the genes disrupted in the multi- Δ strain and strongly suggest that other *HXT*-related genes in *S. cerevisiae*, of which there are at least 11 (Kruckeberg, 1996), are insufficient to confer glucose uptake under typical growth conditions. These observations are consistent with a recent report by Reifemberger *et al.* (1995), which suggested that *HXT1*, *HXT2*, *HXT3*, *HXT4*, *HXT6*, and *HXT7* are able to mediate glucose uptake and that the remaining *HXT* genes are not functional under the conditions tested.

Glucose Transport by Gal2

Gal2 was identified as a galactose transporter in *S. cerevisiae* (Nehlin *et al.*, 1989). The inferred amino acid sequence of Gal2 is highly related to those of the Hxt family (for review, see Bisson *et al.*, 1993). It has been implicated that in addition to galactose transport, Gal2 is also capable of transporting glucose (Nishizawa *et al.*, 1995). The inability of the multi- Δ cells to take up glucose provided the opportunity to directly assess glucose uptake by Gal2. Plasmids expressing *GAL2* from either its own promoter (p*GAL2*) or the constitutive promoter of *ADH1* (p*ADH1p-GAL2*) were introduced into the multi- Δ strain, and the abilities of the resulting cells to utilize glucose were tested. Since the activity of its promoter is repressed by glucose, expression of *GAL2* in the multi- Δ cells was not able to confer growth on glucose (Figure 4A). However, expression of Gal2 from p*ADH1p-GAL2* in the multi- Δ cells allowed growth that was indistinguishable from wild-type cells on either 0.5% or 5% glucose (Figure 4A). In fact, these cells exhibited luxurious growth on either glucose, fructose, raffinose, or sucrose (our unpublished results). Furthermore, constitutive expression of Gal2 in the multi- Δ cells resulted in [¹⁴C]glucose uptake activity comparable to that seen with the wild-type control (Figures 3B and 4B). Interestingly, data from the uptake assays displayed a pattern consistent with both low- and high-affinity transport kinetics, indicating that glucose uptake mediated by Gal2 mimics both uptake components that were observed in wild-type cells.

Expression of *SNF3* in Multi- Δ Cells Does not Increase Glucose Uptake or Confer Growth on Glucose

As mentioned earlier, although *snf3* Δ cells exhibit defects in high-affinity glucose uptake and are unable to grow on media containing low concentrations of glucose or fructose, several reports suggest that Snf3 may not be a high-affinity glucose transporter (Neugeborn *et al.*, 1986; Bisson *et al.*, 1993; Ko *et al.*, 1993; Coons *et al.*, 1995). The multi- Δ strain allowed us to test the glucose transport ability of Snf3 in a background in which glucose transport activity by other transporters is essentially absent. Growth tests and [¹⁴C]glucose uptake assays were performed on multi- Δ cells containing either single-copy or multicopy plasmids expressing *SNF3*. Neither of the plasmids were able to confer growth of multi- Δ cells on glucose (Figure 4A), fructose, mannose, or raffinose (our unpublished results) as sole carbon sources. Furthermore, when grown in either glycerol/ethanol or glycerol/ethanol supplemented with various concentrations of glucose, expression of *SNF3* in the multi- Δ cells did not confer a measurable increase in [¹⁴C]glucose uptake activity beyond that exhibited by multi- Δ cells harboring the vector control (Figure 4C and our unpublished results). Expression of functional *SNF3* genes from the p*SNF3* plasmids was confirmed by their ability to suppress *snf3*[−] phenotypes in a *snf3* Δ strain (Figure 2 and our unpublished results). Our results revealed that Snf3 is not sufficient to confer metabolic glucose uptake under normal growth conditions.

Expression of *HXT1*, *HXT2*, *HXT3*, *HXT6*, or *HXT6/7* Is Sufficient to Confer Glucose Uptake in Multi- Δ Cells

To assess the glucose transport activity of individual hexose transporters, single-copy plasmids containing the *HXT* genes were introduced into the multi- Δ cells and the ability of the resulting strains to utilize glucose was then tested. Expression of *HXT1*, *HXT2*, *HXT3*, *HXT6*, or *HXT6/7* enabled the multi- Δ cells to grow on glucose to various degrees, suggesting that the products of these *HXT* genes are capable of mediating glucose transport (Figure 4A). *HXT4* was not tested in this study.

The capability of *HXT6/7* to confer growth of multi- Δ cells on glucose confirmed that the chimeric gene is functional. Furthermore, multi- Δ cells expressing either *HXT6/7* or *HXT6* exhibited stronger growth on 0.5% glucose than on 5% glucose (Figure 4A), suggesting that the glucose regulation of *HXT6/7* mimics that of *HXT6*. These results also showed that *HXT6* and *HXT6/7* are negatively regulated by high concentrations of glucose.

Figure 4. (A) Growth tests on glucose media showing the ability of individual genes to confer glucose transport. Multi-Δ cells (*snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ hxt6/7Δ gal2*, strain HY133) harboring vector (pRS316), pHXT1 (pCK142), pHXT2 (pHL144), pHXT3 (pCK164), pHXT6 (pHL157), pHXT6/7 (pHL132), pSNF3 (pHL136), p2μ-SNF3 (pHL137), pGAL2 (pTH318), or pADH1p-GAL2 (pHL125) were grown and tested as described in Figure 3A. WT cells (strain R757) harboring vector (pRS316) was used as a positive control. (B and C) Eadie-Hofstee plots derived from [14 C]glucose uptake assays showing glucose uptake (B) mediated by multi-Δ (HY133) cells harboring pSNF3 (pHL136, $\blacklozenge$) or (C) multi-Δ cells harboring pADH1p-GAL2 (pHL125, $\blacklozenge$). Multi-Δ cells harboring vector (pRS316, $\square$) were used as a control. Cultures were grown in glycerol/ethanol media before the uptake assays.

Expression of *HXT6* Is Repressed by Glucose

To investigate the putative glucose regulation of *HXT6*, hybridization experiments were performed to monitor steady-state levels of *HXT6* RNA. Because of the extreme sequence identity existing between *HXT6* and *HXT7*, we were unable to design a probe that could reliably distinguish *HXT6* transcripts from *HXT7* transcripts. Using a *HXT6* probe that hybridizes to identical sequences of *HXT6* and *HXT7* (see MATERIALS AND METHODS), transcription of one or both of these genes was found to be repressed by glucose in a wild-type strain (A303). When glucose (0.5–5%) was added to wild-type cells pregrown in glycerol/ethanol medium, steady-state levels of *HXT6* and/or *HXT7* mRNAs were dramatically decreased in <15 min (Figure 5A and our unpublished results). The transcripts reappeared by 2 h when only 0.5% glucose was added to the medium, due to the depletion of

extracellular glucose. In contrast, recovery failed to occur even after 4 h when the medium was made to 5% glucose, indicating that as long as glucose was abundant transcription of *HXT6* and/or *HXT7* was repressed. Quantification of the rate of disappearance of *HXT6* and/or *HXT7* RNA upon the addition of glucose indicated that its half-life is <3 min.

The glucose regulation of *HXT6* transcription was also examined by analyzing expression of the *HIS3* reporter gene from the *HXT6* promoter. A single-copy plasmid containing the *HXT6p-HIS3* construct was introduced into a wild-type strain (A303) and Northern blot analysis was performed using an oligonucleotide probe that hybridizes to the *HIS3* RNA. Results shown in Figure 5B demonstrate that the 1.2-kb region immediately upstream of the *HXT6* coding sequence is sufficient to drive expression and confer glucose repression of the *HIS3* reporter gene. An abundant signal of

Figure 5. RNA hybridization analysis of *HXT6*, *HXT7*, and *HXT6/7*. Cells were cultured in glycerol/ethanol (glyc/EtOH) media to OD_{600} 0.4–0.6. Samples were collected at the indicated time points after the addition of glucose. The level of *ACT1* RNA was used to indicate the relative amount of RNA present in each lane. (A) Glucose repression of *HXT6* and/or *HXT7*. Wild-type cells (A303) were grown in glycerol/ethanol and glucose was added to a final concentration of 5% or 0.5% as indicated. (B and C) *Snf3*-regulated glucose repression of *HXT6* and *HXT6/7*. *SNF3* (left panel) or *snf3Δ* (right panel) cells either expressing or deleted for *HXT1-HXT4* (as indicated under strain background) were grown in glycerol/ethanol, and 5% (B) or 0.5% glucose (C) was added at 0 time point. Wild-type cells (A303), *snf3Δ* cells (HY135), A303 harboring *pHXT6p-HIS3* (pHL183), HY135 harboring *pHXT6p-HIS3* (pHL183), *snf3Δ hxt1Δ-hxt4Δ HXT6/7 gal2* cells (CY263) harboring *pSNF3* (pHL136) or CY263 harboring vector (pRS316) were used.

1.2-kb *HIS3* RNA was observed when cells were grown in glycerol/ethanol media but decreased rapidly upon the addition of glucose. These results also suggest that although the *HXT6* probe could recognize transcripts from both *HXT6* and *HXT7*, the signals it detected represent the expression pattern of *HXT6*. If *HXT7* was expressed, it too underwent glucose repression. To facilitate discussion, reference is limited to *HXT6* transcription in this report.

As expected, expression of the *HXT6/7* chimera is also glucose repressible. Northern blot analysis of RNA prepared from *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ HXT6/7 gal2/pSNF3* cells (strain CY263 harboring a single-copy plasmid expressing *SNF3*) revealed that the amount of *HXT6/7* transcript rapidly decreased upon exposure to glucose in a manner similar to *HXT6* RNA in wild-type cells (Figure 5, B and C). However, in *hxt1Δ-hxt4Δ HXT6/7 gal2* cells, repression of *HXT6/7* was not released even 4 h after the addition of 0.5% glucose (Figure 5C) as was observed with *HXT6* in wild-type cells (Figure 5A), due to their deficiency in glucose utilization. Along with the observations described earlier that *HXT6* and *HXT6/7* confer identical growth phenotypes in either *snf3Δ* cells or multi- Δ cells (Figures 2 and 4B), our results strongly suggest

that they are essentially identical genes. That *HXT6/7* is repressed by glucose in cells deleted for *HXT1*, *HXT2*, *HXT3*, and *HXT4* also shows that this repression does not depend on the glucose transporters encoded by these *HXT* genes.

Glucose Repression of *HXT6* Requires *Snf3*

RNA hybridization experiments revealed that *Snf3* is required for the glucose repression of *HXT6*. Although transcription of *HXT6* is repressed by glucose in wild-type cells, it is essentially constitutive in *snf3Δ* cells (Figure 5B). The dependence of glucose repression on *Snf3* was confirmed by monitoring the expression of the *pHXT6p-HIS3* reporter construct. Steady-state mRNA levels of *HIS3* expressed from the *HXT6* promoter were repressed by glucose in wild-type cells but found to be constitutive in *snf3Δ* cells (Figure 5B).

Not only is glucose uptake by other members of the *Hxt* family found to be insufficient to confer glucose repression of *HXT6*, it is not even required. RNA hybridization experiments showed that *HXT6/7* expression was constitutive in *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ HXT6/7 gal2* cells but glucose repression was fully restored upon introduction of a single-copy plasmid expressing *SNF3* (Figure 5, B and C). These results strongly suggest that even though *Snf3* is not able to take up glucose, it can mediate glucose repression of *HXT6*.

A closer inspection revealed that *Snf3* is required for the maintenance of glucose repression of *HXT6*. Even in *snf3Δ* cells, steady-state levels of *HXT6* RNA decreased within 15–30 min after the addition of glucose, although this reduction was usually less than that observed in *SNF3* cells (Figure 5, B and C). Whereas repression was complete and permanent in *SNF3* cells, the levels of *HXT6* RNA recovered after 2 h in the *snf3Δ* cells. Although some variation was observed from experiment to experiment, in every case, *HXT6* RNA consistently and substantially recovered from glucose repression by 2 h after the addition of glucose (2–5%) in *snf3Δ* cells, whereas it was not detectable even after 6 h under similar conditions in *SNF3* cells (Figure 5B and our unpublished results).

We previously reported that *Snf3* could play a negative role in glucose transport. A *snf3Δ hxt1Δ-hxt4Δ* strain was able to grow on 0.5% glucose, but a *hxt1Δ-hxt4Δ* strain harboring the *SNF3* gene did not have this ability (Ko *et al.*, 1993). [14 C]glucose uptake assays confirmed that the *SNF3* cells (*hxt1Δ-hxt4Δ*) exhibit decreased uptake activity compared with *snf3Δ* cells (*snf3Δ hxt1Δ-hxt4Δ*; our unpublished observations). The inability of the *SNF3* cells (*hxt1Δ-hxt4Δ*) to take up glucose was not because of differences in strain backgrounds. As shown in Figure 6, the parental strain CY263 (*snf3Δ hxt1Δ-hxt4Δ HXT6/7 gal2*) harboring vector sequence only was able to grow on 0.5% glucose

media. Expression of *SNF3* from a single-copy plasmid in this strain inhibits such growth. The target of the inhibitory effect of Snf3 appears to be *HXT6/7*, since, as shown earlier, deletion of *HXT6/7* from *snf3Δ hxt1Δ-hxt4Δ* cells abolishes growth on glucose media (Figure 3A). This negative effect of Snf3 on *HXT6/7*-dependent growth is consistent with our finding that Snf3 is required for glucose repression of *HXT6/7*. As shown in Figure 5C, glucose repression of *HXT6/7* is relieved in *snf3Δ hxt1Δ-hxt4Δ HXT6/7 gal2* cells harboring vector sequence only and constitutive expression of *HXT6/7* was observed. Expression of *SNF3* from a single-copy plasmid in these cells restores the glucose repression, and *HXT6/7* RNA was not detected in 0.5% glucose.

Snf3 Plays a Positive Role in HXT6 Expression on Raffinose

In addition to its requirement in glucose repression of *HXT6*, Snf3 also plays a positive role in *HXT6* transcriptional regulation. Full expression of *HXT6* on raffinose or very low concentrations of glucose is dependent on Snf3 (Figure 7A). Since raffinose is converted into fructose and melibiose by invertase before transport into yeast cells and the strains used in this study are *mel⁻*, the presence of raffinose in the growth media provides a constant and very low source of fructose, which is believed to share the same transport system as glucose. Although *HXT6* is repressed by moderate and high concentrations of glucose, its expression is unaffected or perhaps slightly induced (Figure 7A) by very low levels of glucose. Maximal expression of *HXT6* on raffinose requires Snf3. As shown in Figure 7A, although the steady-state level of *HXT6* RNA is high on raffinose in wild-type cells, it is significantly reduced in *snf3Δ* cells.

It has been reported that Snf3 is essential for raffinose induction of some *HXT* genes, including *HXT2*, *HXT3*, and *HXT4* (Ozcan and Johnston, 1995). This is consistent with the observation that *snf3Δ* cells failed to grow on raffinose or sucrose as sole carbon sources

Figure 6. Negative effect of Snf3 on *HXT6/7*-dependent growth. *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ HXT6/7 gal2* (CY263) cells harboring p*SNF3* (pHL136) or vector (pRS316) were patched on glycerol/ethanol medium, replica plated to 0.5% glucose media, and incubated at 30°C for 3 days.

(Neugeborn and Carlson, 1984; Neugeborn *et al.*, 1986; Figure 7B). To determine whether Snf3 could mediate this positive effect in the absence of *HXT1*, *HXT2*, *HXT3*, and *HXT4*, the ability of a *hxt1Δ-hxt4Δ HXT6/7* strain harboring either the vector sequence or a single-copy plasmid expressing Snf3 was tested for growth on raffinose. As shown in Figure 7B, expression of *SNF3* in these cells modestly improved their ability to grow on raffinose. This effect appeared to be mediated at least in part through *HXT6/7*, since, as described earlier, deletion of *HXT6/7* from *hxt1Δ-hxt4Δ HXT6/7* cells abolishes the ability of Snf3 to confer growth on raffinose. Thus, Snf3 plays a positive role in *HXT6/7*-dependent growth on raffinose.

Expression of HXT6 Is under the Control of General Glucose Regulators

To determine whether *HXT6* is under the control of the general glucose repression pathway, RNA hybridization experiments were performed to study *HXT6* expression in *hxx2Δ*, *ssn6Δ*, and *tup1Δ* cells. Hexokinase Hxk2 and general repressors Ssn6 and Tup1 are known to be required for the glucose repression of many genes. Repression of *HXT6* by glucose was abolished in *ssn6Δ* and *tup1Δ* cells, and only partial repression was observed in *hxx2Δ* cells (Figure 8). The level of *HXT6* basal expression during growth on nonfermentable carbon sources could not be tested in *ssn6Δ* and *tup1Δ* cells since these cells are unable to grow in

Figure 7. Snf3-dependent expression of *HXT6* and *HXT6/7* on raffinose. (A) RNA hybridization analysis of *HXT6* and *HXT7*. *SNF3* cells (strain A303) and *snf3Δ* cells (strain HY135) were cultured in glycerol/ethanol media to OD₆₀₀ 0.4–0.6. Samples were collected at the indicated time points after addition of 2% raffinose. The level of *ACT1* RNA was used to indicate the relative amount of RNA present in each lane. (B) Positive effect of Snf3 on *HXT*-dependent growth. *snf3Δ* cells (strain HY135) and *snf3Δ hxt1Δ-hxt4Δ HXT6/7* cells (strain CY263) harboring either p*SNF3* (pHL136) or vector (pRS316) were patched on glycerol/ethanol medium, replica-plated onto raffinose medium, and incubated at 30°C for 2 days.

glycerol/ethanol media. Thus, some components known to be required for glucose repression of other genes are also required for glucose repression of *HXT6*.

Expression of some *HXT* genes, namely *HXT1*, *HXT2*, *HXT3*, and *HXT4*, is significantly decreased in *grr1* cells when grown in glucose media (Flick and Johnston, 1991; Ozcan *et al.*, 1994). We found that expression of *HXT6*, too, requires *GRR1*. No *HXT6* message was detected in *grr1Δ* cells when either glucose, raffinose, galactose, or glycerol/ethanol was supplied as carbon sources (Figure 8 and our unpublished observations). Our results show that *Grr1* is not only essential for the induction of *HXT* genes in glucose media, it is also required for the basal expression of *HXT6*, which is thus far the only *HXT* gene that has been found to be highly expressed on alternative carbon sources.

Snf3 Is Required for Raffinose-mediated but not Glucose-mediated Transcriptional Regulation of ADH2 and SUC2

To determine whether other glucose-repressible genes showed a dependency on *Snf3* for glucose repression, transcriptional regulation of the glucose-repressible genes, *ADH2* and *SUC2*, was examined by RNA hybridization analysis. In both *SNF3* and *snf3Δ* cells, transcription of *ADH2* and *SUC2* was fully repressed in glucose-grown cultures or upon the addition of glucose to glycerol/ethanol-grown cultures (Figure 9A and our unpublished observations). In fact, glucose repression of *SUC2* and *ADH2* is not even altered in

multi- Δ cells. As shown in Figure 9B, expression of both genes is repressed by glucose in multi- Δ cells just as that observed in the wild-type cells. This indicates that the sensing process for general glucose repression does not require *SNF3* or the *HXT* genes deleted in the multi- Δ cells, nor does it need the influx and metabolism of glucose or the accumulation of its metabolic products. However, the *snf3Δ* mutation abolished the ability of raffinose to repress *ADH2* and to induce *SUC2*, revealing a complex connection between the *HXT6*, *ADH2*, and *SUC2* regulatory pathways.

DISCUSSION

We have constructed a multi- Δ strain (*snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ hxt6Δ hxt7Δ gal2*) in which glucose uptake is essentially abolished. These cells exhibit normal growth on glycerol/ethanol, but are unable to grow on glucose, fructose, raffinose, or sucrose when respiration is inhibited by antimycin A. [14 C]glucose uptake experiments have confirmed that the glucose uptake in our multi- Δ cells is virtually abolished compared with that in wild-type cells. By testing for the ability to restore glucose uptake and for growth on glucose in these cells, we have confirmed that *HXT1*, *HXT2*, *HXT3*, *HXT6*, and *HXT6/7* are functional glucose transporter genes. We also have direct evidence to show that *Gal2* is able to conduct glucose transport, and we have demonstrated that *SNF3* is not sufficient to confer nutritional uptake of glucose, even when expressed from a multicopy plasmid.

The inability of the multi- Δ cells to take up glucose indicates that glucose transport in *S. cerevisiae* requires one or more of the genes deleted in these cells and that

Figure 8. RNA hybridization analysis of *HXT6* and *HXT7*. WT (YM4127), *grr1Δ* (YM2955), *hxx2Δ* (YM4512), *ssn6Δ* (YM4554), and *tup1Δ* (YM4513) cells were cultured in 2% glucose, glycerol/ethanol (glyc/EtOH), or 2% raffinose media overnight to OD₆₀₀ 0.8–1.1. The level of *ACT1* RNA was used to indicate the relative amount of RNA present in each lane.

Figure 9. RNA hybridization analysis of *SUC2* and *ADH2*. Cells were harvested at OD₆₀₀ 0.8–1.1. The level of *ACT1* RNA was used to indicate the relative amount of RNA present in each lane. (A) RNA was isolated from WT (A303) and *snf3Δ* (HY135) cells were grown in 5% glucose, 2% raffinose, or glycerol/ethanol (glyc/EtOH) media overnight. (B) RNA was isolated from strains A303 (WT) and multi- Δ (HY133) cells were grown in either glycerol/ethanol media or glucose (5%) + glycerol/ethanol media overnight.

the remaining *HXT* genes (*HXT5* and *HXT8-HXT17*) appear to be nonfunctional or insufficient to confer growth under the conditions tested. Reifengerger *et al.* (1995) have recently reached similar conclusions regarding the apparent inability of *HXT5* and *HXT8* through *HXT17* to mediate growth on glucose. However, the glucose transport-deficient strain they described harbored a wild-type copy of *SNF3*. Since we show that *SNF3* can act as a negative regulator of transcription, the multideletion strain we generated provided a genetic background free of the inhibitory effects of *SNF3*. Thus, the inability of *snf3Δ hxt1Δ hxt2Δ hxt3Δ hxt4Δ hxt6Δ hxt7Δ* cells to grow on glucose or take up glucose is not simply due to the repression by *SNF3* of the remaining *HXT* genes.

It is possible that one or more of the *HXT* genes remaining in the multideletion cells is in fact repressed by as yet unidentified factors. The protein product of the *RGT2* gene in *S. cerevisiae* is a candidate for such regulation. Because Rgt2 shares significantly greater sequence identity with *SNF3* (60%) than it does with the Hxt proteins (26–30%; Kruckeberg, 1996) and because *SNF3* plays a regulatory role in glucose transport (Ko *et al.*, 1993 and this report), Rgt2, too, may provide regulatory functions. In fact, *RGT2* mutants were identified as dominant suppressors that remedy the growth defect of *snf3* cells on low concentrations of glucose or fructose (Marshall-Carlson *et al.*, 1991), and Ozcan *et al.* (1996) have recently shown that the dominant *RGT2-1* mutation and an analogous mutation in *SNF3* result in constitutive induction of *HXT1* and *HXT2*. It will be of interest to determine whether or not a null allele of *RGT2* results in derepression of any of the *HXT* genes.

Two models have been proposed regarding the low-affinity glucose uptake in yeast cells. One suggested that glucose transport in *S. cerevisiae* consists of both low- and high-affinity systems (Coons *et al.*, 1995; Gamo *et al.*, 1995), whereas others acknowledged the existence of the high-affinity uptake system but considered the high K_m uptake as the result of simple diffusion through membrane bilayer (Fuhrmann and Volker, 1993). We show that the multi- Δ cells do not exhibit either high- or low-affinity glucose uptake, demonstrating that both components may be eliminated from yeast cells by deleting glucose transporter genes. Moreover, kinetic data indicated that when constitutively expressed from the *ADH1* promoter, Gal2-mediated [14 C]glucose uptake exhibits both low- and high-affinity components. This result suggests that transporter proteins are able to mediate low-affinity glucose uptake. Our results strongly support the view that the component that kinetically resembles low-affinity glucose transport in *S. cerevisiae* cells is mediated by transporters.

HXT6, *HXT7*, and *HXT6/7* are nearly identical genes (Reifengerger *et al.*, 1995 and this report). They share

>99% sequence identity within the coding regions and it is likely that they undergo similar regulation. *HXT6/7* and *HXT6* not only confer indistinguishable growth phenotypes, but also display identical regulatory pattern when their expression was monitored in RNA hybridization experiments. *HXT7* may be regulated in the same manner as *HXT6*. The RNA signals detected by a probe that recognizes transcripts from both *HXT6* and *HXT7* exhibited the same regulatory pattern as the expression of the *HIS3* reporter gene driven by the *HXT6* promoter. This suggests that *HXT7* either exhibits the same expression pattern as *HXT6* or its expression level is so low that its mRNA was not detected in the hybridization experiments performed. The former is more likely to be the case, since Reifengerger *et al.* (1995) have reported that *HXT7* is a functional glucose transporter.

Expression of *HXT6* displays a novel pattern of regulation. Three different mechanisms for the transcriptional regulation of *HXT* genes in response to glucose have been described previously as follows: 1) induction only by high concentrations of glucose, as in the case of *HXT1*; 2) induction by low levels of glucose and repression by high levels, as in the case of *HXT2* and *HXT4*; and 3) induction by glucose independent of sugar concentration, as in the case of *HXT3* (Wendell and Bisson, 1994; Ozcan and Johnston, 1995). Similar to the high-affinity glucose transporters *HXT2* and *HXT4*, *HXT6* is highly expressed on raffinose or very low concentrations of glucose or fructose and completely repressed by higher concentrations of glucose (>0.5%). However, although the expression of *HXT2* and *HXT4* is almost not detectable in nonfermentable carbon sources like glycerol and ethanol (Ozcan and Johnston, 1995), *HXT6* is highly expressed under these growth conditions. To our knowledge, *HXT6* (possibility also *HXT7*) is the only glucose transporter gene that has a high basal expression level on nonfermentable carbon sources. This is consistent with the proposal that as a single-celled organism, *S. cerevisiae* has evolved multiple hexose transporters to adapt to a quickly changing environment (Bisson *et al.*, 1993). High expression of one or two high-affinity glucose transporters on alternative carbon sources enables yeast cells to immediately take up glucose and stimulate growth upon exposure to glucose.

We found that glucose repression of *HXT6* shares some common requirements with other glucose repressible genes, namely, Hxk2, Ssn6, and Tup1. Hxk2 is a glucokinase that has been implicated in playing a central role in glucose sensing and it is considered to be involved in the early steps in the glucose repression pathway, if not the initial sensing (Entian *et al.*, 1984; Rose *et al.*, 1991). Ssn6 and Tup1, on the other hand, are believed to form a global repressor complex that is recruited to promoters by DNA-binding proteins and therefore act on the final step in the repression path-

way (Komachi *et al.*, 1994; Treitel and Carlson, 1995). The fact that these proteins are required for glucose repression of *HXT6* suggests that *HXT6* is under the control of the general glucose repression pathway. Furthermore, *Grr1*, a protein that is required for the induction of other *HXT* genes on glucose media (Ozcan *et al.*, 1994; Vallier *et al.*, 1994; Ozcan and Johnston, 1995), is also required for the expression of *HXT6*. Transcripts of *HXT6* were not detected in *grr1Δ* cells, regardless of the carbon source provided. These results show that *Grr1* is not only essential for the induction of *HXT* genes in glucose media, but is also required for the basal expression of *HXT6*. *grr1* cells also exhibit pleiotropic defects regarding glucose sensing (Flick and Johnston, 1991; Ozcan *et al.*, 1994; Vallier *et al.*, 1994), which could result from the lack of glucose transporter or initial sensing. Our results support the model of *Grr1* as a universal positive regulator of hexose transporters.

We also found that the regulation of *HXT6* expression requires *Snf3*. Although the basal level of *HXT6* expression was not significantly altered in *snf3Δ* cells, glucose repression of *HXT6* is essentially abolished in the absence of *SNF3*. Although completely repressed in wild-type cells, the expression of *HXT6* is essentially constitutive in *snf3Δ* cells in the presence of high concentrations of glucose. This was surprising since *Snf3* itself can be repressed about 10-fold by high glucose (Neigeborn *et al.*, 1986). However, although its expression is low, it is still sufficient to sense the high concentrations of glucose and repress *HXT6*. Interestingly, in *snf3Δ* cells, *HXT6* repression still occurs within 15 min after the addition of glucose, but the steady-state RNA level soon recovers. This suggests that a glucose signal to repress *HXT6* can be generated independently from *Snf3*, but in the absence of *Snf3*, the signal is either ineffective or no longer present, and *HXT6* expression is derepressed within 30 min. *Snf3* is also required for expression of *HXT6* during growth on raffinose. In *snf3Δ* cells, *HXT6* expression is significantly lower on raffinose media compared with that on glycerol/ethanol media, whereas it is abundant or even slightly induced by raffinose in *SNF3* cells.

In addition to transcriptional repression, *Hxt6* might also be down-regulated posttranscriptionally by high concentrations of glucose (>2%). Although steady-state levels of *HXT6* or *HXT6/7* mRNA are constitutive in *snf3Δ hxt1Δ-hxt4Δ* cells under all conditions tested, high concentrations of glucose are inhibitory to *HXT6*- or *HXT6/7*-dependent growth (Ko *et al.*, 1993, and this report). In this regard, Wendell and Bisson (1994) have shown that the expression of *Hxt2* protein does not necessarily result in *Hxt2*-dependent glucose transport.

It has been reported that the induction of *HXT2*, *HXT3*, and *HXT4* on low concentrations of glucose is

dramatically reduced in *snf3* cells (Ozcan and Johnston, 1995). In addition, although the repression of other glucose-repressible genes, *SUC2* and *ADH2*, was not altered in *snf3Δ* cells, induction of *SUC2* and repression of *ADH2* by raffinose requires *SNF3* (Neigeborn *et al.*, 1986 and this report). However, whether the loss of induction directly resulted from lack of glucose signaling or was a consequence of the lack of glucose transport mediated by *Snf3* was not clear. Our results strongly suggest that the *Snf3*-dependent expression of *HXT6* on raffinose and its repression on glucose is independent of the nutritional uptake of glucose or its metabolic products for the following reasons. First, *Snf3* is incapable of mediating nutritional glucose transport. Second, uptake of glucose by *HXT1*, *HXT2*, *HXT3*, and *HXT4* is neither sufficient nor necessary for the glucose regulation of *HXT6*.

We propose *Snf3* as a key component in a signal transduction pathway that regulates the expression of the *HXT* genes. If *Snf3* is at all capable of mediating glucose uptake, this activity is likely to serve as a regulatory signal rather than for nutritional uptake. It is possible that as a plasma membrane protein, *Snf3* is able to sense the availability of extracellular glucose or fructose and transmit the signals inside the cell to regulate the expression of *HXT* genes. The sensing process could involve the modifications of the *Snf3* structure triggered by the binding or transport of glucose. The extraordinarily long intracellular carboxyl terminus of *Snf3* is a good candidate for transmitting the signals to downstream targets. Alternatively, the glucose molecules transported by *Snf3* can serve as the regulatory signal upon differential modification compared with those transported by the *Hxt* proteins. Our results lead to the following two possibilities: 1) The glucose sensing mediated by *Snf3* is *HXT* gene specific, and *Snf3* is not directly involved in the regulation of *SUC2* and *ADH2*. The altered expression patterns of both genes observed in *snf3Δ* cells on raffinose could result from the lack of induction of glucose transporter genes. 2) *Snf3* is involved in the general sensing mechanism for very low concentrations of glucose to regulate the expression of a variety of target genes, including the *HXT* genes, *SUC2* and *ADH1*. However, when extracellular concentrations of glucose reach 0.5% or higher, *Snf3* is able to generate a specific signal to repress *HXT6*.

We have also shown that glucose repression of *SUC2* and *ADH2* is intact in the multi- Δ cells where nutritional glucose uptake is essentially abolished, suggesting that the general glucose sensing is not through the influx of glucose or its metabolic products.

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