High-Copy Suppression of Glucose Transport Defects by *HXT4* and Regulatory Elements in the Promoters of the *HXT* Genes in *Saccharomyces cerevisiae*

George Theodoris, Noel M. Fong, David M. Coons and Linda F. Bisson

Department of Viticulture and Enology, University of California, Davis, California 95616

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ABSTRACT

HXT4, a new member of the hexose transporter (*HXT*) family in *Saccharomyces cerevisiae* was identified by its ability to suppress the *snf3* mutation in multicopy. Multicopy *HXT4* increases both high and low affinity glucose transport in *snf3* strains and increases low and high affinity transport in wild-type strains. Characterization of *HXT4* led to the discovery of a new class of multicopy suppressors of glucose transport defects: regulatory elements in the promoters of the *HXT* genes. We have designated these sequences DDSEs (DNA sequence dependent suppressing element). Multicopy *HXT4* and DDSEs in the *HXT1*, *HXT2*, *HXT3* and *HXT4* promoters were found to restore growth to *snf3* and *grr1* strains on low glucose media. The DDSE in the *HXT4* promoter was refined to a 340-bp sequence 450 bp upstream of the *HXT4* translational start. This region was found to contain an 183-amino acid open reading frame. Extensive analysis indicates that the DNA sequence itself and not the encoded protein is responsible for suppression. The promoters of *SNF3* and of other glycolytic genes examined did not suppress *snf3* in multicopy. Suppression of *snf3* by DDSE is dependent on the presence of either *HXT2* or *HXT3*.

SEVERAL genes involved in glucose transport in *Saccharomyces* have previously been identified. These include *SNF3* (sucrose non fermenting) and the *HXT* (hexose transport) genes (*HXT1*–*4*) (Ko *et al.* 1993; KRUCKEBERG and BISSON 1990; LEWIS and BISSON 1991; BISSON *et al.* 1987; CELENZA *et al.* 1988). Sequence analysis revealed that these genes belong to a large family of sugar transporter proteins found in mammalian and avian systems, fungi, plants and prokaryotes (reviewed in BISSON *et al.* 1993). Given the importance of glucose as a carbon and energy source, and of transport as a site of control for glycolytic flux, the presence of a multigene family of glucose transporters is readily explainable.

Glucose transport in yeast has both a high and low affinity component (BISSON and FRAENKEL 1983). Low affinity transport is constitutive and high affinity transport is glucose repressible. High affinity transport is defective in strains carrying mutations preventing derepression of glucose-repressible functions (*snf1*, *snf4* and *snf6*) (BISSON 1988). Mutations in *snf3* result in greatly diminished high affinity glucose transport and an inability to grow on low glucose media when respiration is inhibited (BISSON *et al.* 1987; CELENZA *et al.* 1988). These defects can be suppressed by multiple copies of any of the *HXT* genes. The *HXT* genes appear to encode bona fide transporters, thus high affinity transport activity is the result of the expression of one or more distinct genes. The precise mechanism by which the *HXT* genes interact with *SNF3* and other genes involved in carbohydrate metabolism to regulate glucose transport is unclear. Recent work by Ko *et al.* (1993) suggests

that a synergistic relationship exists between *SNF3* and each of the *HXT* genes.

We describe herein the identification of a new member of the *HXT* family *HXT4*. Additionally, a new group of high-copy *snf3* suppressors which appear to function by titrating transcription factors has been identified. We have found that regulatory regions in the promoters of the *HXT* genes can suppress *snf3* in multicopy. We have designated these multicopy suppressors DDSEs (DNA sequence dependent suppressing elements). The first DDSE was identified by virtue of its location between *HXT1* and *HXT4*. This is the same region previously identified as *ORF2* by LEWIS and BISSON (1991) and has also been identified by another group (OZCAN *et al.* 1993) as a multicopy suppressor of *HTR1* (hexose transport regulation), a dominant mutation defective in glucose transport. Although DDSE lies within an 183-amino acid open reading frame (ORF), our analysis reveals that the mechanism of multicopy suppression is mediated by the DDSE DNA sequence and appears to be autonomous of the ORF. Both *HXT4* and DDSE suppress growth defects on low glucose in *snf3* strains.

grr1 (glucose repression resistant), a pleiotropic mutation isolated in several laboratories (FLICK and JOHNSTON 1991; VALLIER and CARLSON 1991), encodes a 1,151-amino acid protein that is weakly expressed and contains 12 tandem leucine-rich repeats. Mutations in *grr1* are pleiotropically defective in glucose repression and also have defects in cellular morphology, aromatic amino acid metabolism, cobalt and glucose transport (FLICK and JOHNSTON 1991; CONKLIN *et al.* 1993; VALLIER *et al.* 1994). It has been suggested that the *GRR1* protein

TABLE 1
Yeast strains

Strain	Genotype ^a	Source
YPH500	<i>MATa</i>	P. HEITER
LBV403	<i>MATa snf3-Δ4::HIS3</i>	This laboratory
LBV311	<i>MATa snf3::TRP1</i>	This laboratory
LBV313	<i>MATa snf3::TRP1 hxt1::TRP1::hxt4^b hxt2::LEU2 hxt3::URA3</i>	This laboratory
LBV203	<i>MATa snf3-Δ4::HIS3 hxt1::URA3</i>	This laboratory
LBV416	<i>MATa snf3-Δ4::HIS3 hxt2::LEU2</i>	This laboratory
LBV901	<i>MATa hxt4::URA3</i>	This work
LBV903	<i>MATa snf3-Δ4::HIS3 hxt4::URA3</i>	This work
LBV904	<i>MATa DDSE-ORF::LEU2</i>	This work
LBV920	<i>MATa grr1::URA3</i>	This work
LBV921	<i>MATa grr1::LEU2</i>	This work
CY290	<i>MATa hxt1::TRP1::hxt4 hxt2::LEU2 hxt3::URA3</i>	R. GABER
CY291	<i>MATa snf3-Δ4::HIS3 hxt2::LEU2 hxt3::URA3</i>	R. GABER
CY292	<i>MATa snf3-Δ4::HIS3 hxt1::TRP1::hxt4 hxt3::URA3</i>	R. GABER
CY293	<i>MATa snf3-Δ4::HIS3 hxt1::TRP1::hxt4 hxt2::LEU2</i>	R. GABER
MCY2605	<i>MATa grr1Δ</i>	M. CARLSON
YM2953	<i>MATa grr1::LEU2</i>	M. JOHNSTON
YM2996	<i>MATa sup4-o grr1::LEU2</i>	M. JOHNSTON

^a All strains also carry *ura3-52 lys2-801 ade2-100 trp1-Δ63 his3-Δ200 leu2-Δ1*.

^b *hxt1::TRP1::hxt4* indicates a knockout of *HXT1*, *HXT4* and the intervening DNA including *DDSE*.

may mediate protein-protein interactions. It is paradoxical that *grr1* strains are defective in glucose transport since high affinity glucose transport is under general glucose repressional control and glucose repression is absent in *grr1* strains. The effect of *grr1* on glucose repression may be indirect and a consequence of the defect in glucose transport. If that is true then multicopy expression of transporters may suppress the *grr1* phenotype. Therefore, the effect of *HXT* genes and *DDSE* sequences on *grr1* suppression was also examined.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains used in this study are shown in Table 1. Yeast were grown in standard media (GUTHRIE and FINK 1991), except that URA and TRP dropout media was supplemented with 0.2% casamino acids. Selecting for URA auxotrophy on casamino acids plates was found to greatly enhance the transformation frequency and vigor of strains compared to minimal SC-URA plates. Yeast were transformed by the lithium acetate method (ITO *et al.* 1983; SHIELT and GIETZ 1989). Genetic manipulations were carried out using standard techniques (GUTHRIE and FINK 1991). *snf3* and *grr1* suppression was tested by streaking yeast strains onto YEP plates containing 0.05% glucose or 2% raffinose with antimycin A (2 µg/ml) added to inhibit respiration. Growth after 2 days was scored as ++ or +++ depending on the size of the colonies formed. Growth after 3 days was scored as +. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates were made by adding 100 ml of 1 M phosphate buffer (pH 7.0) and 1 ml of X-gal (20 mg/ml) to 1 liter of YNB media containing the appropriate supplements and carbon source.

Plasmids: Plasmids used in this study are shown in Table 2. Standard methods for DNA manipulation were used in this study (SAMBROOK *et al.* 1989; AUSUBEL *et al.* 1992). Plasmids were propagated in the *Escherichia coli* strain DH5a and purified using the Magic miniprep kit from Promega. DNA modifying enzymes were purchased from New England Biolabs, U. S. Biochemical Corp. (USB) or Boehringer Mannheim. Plasmids were constructed in the yeast-*E. coli* shuttle vectors

TABLE 2
Plasmids

Plasmid name	Description ^a	Source
pSC7	Original <i>HXT1-HXT4</i> clone	This laboratory
pGT7, pGT2, pGT8	<i>HXT4</i> subclones	This work
pGT7R, pGT2R, pGT8R	<i>HXT4::lacZ</i>	This work
pGT9, pGT9X ^b	<i>DDSE4</i> subclones	This work
pDL502	<i>HXT1::lacZ</i>	This laboratory
pGTD1	<i>DDSE1</i> subclone	This work
pSC2-1C3	<i>HXT2</i> subclone	This laboratory
pGTD2, pGT1C4	<i>DDSE2</i> subclones	This work
pGT1C5	<i>HXT2</i> subclone	This work
pAK12	<i>HXT2::lacZ</i>	This laboratory
pCK166	<i>HXT3</i>	R. GABER
pGT166, pGTD3	<i>DDSE3</i> subclones	This work
pLN19	<i>SNF3</i>	M. CARLSON
pAK15	<i>SNF3::lacZ</i>	This laboratory
pTLG2	<i>GAL2</i>	V. CIRILLO
pRB58	<i>SUC2</i>	M. CARLSON
pBM1829	<i>grr1::LEU2</i>	M. JOHNSTON
pBM2101	<i>grr1::URA3</i>	M. JOHNSTON
pBM2081	<i>GRR1</i>	M. JOHNSTON
pBW111	<i>HXX1</i>	D. FRAENKEL
pBW112	<i>HXX2</i>	D. FRAENKEL
pBW113	<i>GLK1</i>	D. FRAENKEL
pENO2	<i>ENO2</i>	M. HOLLAND

^a All plasmids are multicopy except for pBM1829 and pBM2101. *DDSEs* are numbered such that they correspond to the downstream *HXT* genes, i.e.: *DDSE4* is upstream of *HXT4*, etc. All plasmids constructed for this study are in the yeast-*E. coli* shuttle vector YEp352 (2µ, *URA3*) (HILL *et al.* 1986) except for the *HXT4::lacZ* fusions which were constructed in YEp357R (MYERS *et al.* 1986).

^b Subclones of *DDSE-4* were also made in the multicopy vectors YEp351 (*LEU2*), pRS423 (*HIS3*) and pRS424 (*TRP1*).

YEp351, YEp352, pRS423, pRS424, pRS425 or pRS426 (HILL *et al.* 1986; CHRISTIANSON *et al.* 1992).

The Sequenase 2.0 kit (USB) was used for all sequencing reactions. Sequencing procedures were carried out as described in the USB DNA Sequencing Guide. Both strands of *HXT4* were sequenced using exonuclease III-generated nested deletions, restriction enzyme generated subclones and

FIGURE 1.—Restriction maps of *HXT* and *DDSE* bearing multicopy plasmids. Vector sequences are not shown. Sources of the plasmids are shown in Table 1. The ability of each multicopy plasmid to suppress the *snf3* growth defect on YEP 0.05% glucose/antimycin A is denoted at right. Vector sequences alone were unable to suppress *snf3*.

custom primers from Operon. Nucleic acid and amino acid sequence data were analyzed using the Wisconsin Genetics Computer Group software (GCG) (DEVERAUX *et al.* 1984).

Glucose transport assays: Glucose transport assays were performed as previously described (LEWIS and BISSEON 1991). Briefly, the yeast strains were grown to the appropriate growth conditions in SC-casamino acids medium, harvested, washed with phosphate buffer (pH 7.0) and incubated for 5-sec intervals with 14 C-labeled glucose and then harvested onto glass fiber filters.

Construction of *HXT4* promoter-*lacZ* translational fusions: The pGT7R *HXT4* protein- β -galactosidase fusion was created by ligating the 242-bp *XbaI*-*AccI* fragment containing the *HXT4* promoter and first 20 amino acids of *HXT4* from pGT1 into the *XbaI*-*SmaI* sites of YEP357R (MYERS *et al.* 1986) to yield an inframe translational fusion of *HXT4* and β -galactosidase. The pGT2R and pGT8R *HXT4* protein- β -galactosidase fusions were created by using polymerase chain reaction (PCR) primers to generate *HXT4* promoter fragments which were ligated into YEP357R. The lengths of the promoters contained in these fusions corresponds to the length of plasmids pGT2 and pGT8. Primer "GTfusion" (CGGATTCAACTGGTACCGACAAAGC), contains an engineered *KpnI* site and primers "GT8" (CCGCATGCAGGAAAACCTTGGGG) and "GT2" (CGTCATTATGCATGCAGGAGAACCG) contain engineered *SphI* sites. PCR fragments generated with GTfusion and GT8 or GT2 were cleaved with *KpnI* and *SphI* and ligated into the *KpnI*-*SphI* site of YEP357R generating in frame fusions of *HXT4* protein and β -galactosidase at the *KpnI* junction.

Growth of cultures for β -galactosidase activity: Colonies of the yeast transformants were maintained on ura⁻ plates. A single colony was used to start an inoculum in liquid ura⁻ with 4% glucose, and grown to stationary phase at 30° for 24–48 hr at 250 rpm. One-liter cultures of ura⁻ 4% were inoculated to an initial OD₅₈₀ of 0.030. Samples of 20 OD₅₈₀ units were taken before inoculation, at *t* = 0 and at other intervals for measurement of β -galactosidase activity, OD₅₈₀, and plasmid stability.

β -Galactosidase activity assay: Cell extracts were made from 20 OD₅₈₀ units using the glass bead method as prescribed (ROSE *et al.* 1990) to give a final volume of 200 μ l. Samples were taken in triplicate for protein concentration determination using the Bradford reagent (Bio-Rad, Richmond, Cali-

fornia). Samples were taken in triplicate for β -galactosidase activity assay (GUARENTE 1983). Activity was expressed in nanomoles of 2-nitrophenyl- β -D-galactopyranoside (ONPG)/min/mg total protein.

PCR site-directed mutagenesis: PCRs were performed using the Gene Amp kit from Perkin-Elmer Cetus. Site-directed PCR mutagenesis was used to construct nonsense mutations of the ORF in *DDSE*, converting the ATG codons in the ORF to ATT. The protocol used was obtained from ERDMAN and BURTIS (1993). Mutagenesis was confirmed by sequencing.

Gene disruptions: The *PstI*-*EcoRI* *HXT4* containing fragment of pGT2 was subcloned into pUC19. The internal *HindIII* fragment was removed and replaced with *URA3*. The construct was used to transform yeast strains YPH500 and LBY403. For the ORF2 disruption the 750-bp *HindIII* fragment containing ORF2 was replaced with *LEU2*. The *grr1*-disrupted strains LBY920 and LBY921 were generated using the *grr1* disruption plasmids pBM2101 (*grr1::URA3*) and pBM1829 (*grr1::LEU2*), respectively (FLICK and JOHNSTON 1991). Gene disruptions were confirmed by Southern blot.

Southern and Northern analysis: Southern and Northern analysis were performed using standard techniques (SAMBROOK *et al.* 1989; AUSUBEL *et al.* 1992). Poly A RNA was obtained using oligo(dT)-cellulose obtained from Sigma. Probes were made using the random prime method.

RESULTS

Isolation of *HXT4*: The pSC7 plasmid was previously isolated as a multicopy suppressor of the *snf3* growth defect. Initial characterization of pSC7 by LEWIS and BISSEON (1991) revealed that in addition to the region containing *HXT1*, pSC7 contained an uncharacterized region capable of suppressing *snf3* (LEWIS and BISSEON 1991). We have found that another *HXT* gene, designated *HXT4*, lies 2.1 kb downstream of *HXT1* (Figure 1). The *HXT4* gene as well as a short sequence in the *HXT4* distal promoter were found to restore growth to *snf3* and *grr1* strains on YEP 0.05% glucose and 2% raffinose when respiration is inhibited. The suppressor

in the promoter of *HXT4*, designated DDSE, will be discussed shortly. Disruption of *HXT4* with *URA3* revealed that *HXT4* is not an essential gene. The *hxt4* null strain had no obvious or easily detectable phenotype.

Sequence analysis of *HXT4*: *HXT4* encodes a 576-amino acid protein, with homology to sugar transporters (Figure 2). Kyte-Doolittle hydropathy analysis revealed that *HXT4* contains two sets of six putative membrane spanning regions separated by a long hydrophilic loop. Sequence comparisons using the Fasta program (GCG) revealed that *HXT4* was highly homologous to other members of the HXT family but only weakly homologous to *SNF3*: 74.3% identical to *HXT1*, 71% identical to *GAL2*, 68% identical to *RAG1*, 67.5% identical to *HXT2* and 28.5% identical to *SNF3* on the amino acid level.

***HXT4* plasmids with different promoter lengths exhibit different levels of *snf3* suppression:** Initially we observed differences in the ability of *HXT4* plasmids containing different promoter lengths to suppress *snf3*. pGT7 contains the shortest promoter (145 bp) and was found to strongly suppress *snf3*. pGT8 contains a slightly longer promoter (440 bp) but only weakly suppresses *snf3*. pGT2 contains the longest promoter (1 kb) and also contains DDSE. pGT2 suppressed *snf3* almost as strongly as pGT7 (Figure 3). Suppression of *snf3* by DDSE alone was similar to the suppression seen with pGT8.

Glucose transport assays revealed that these plasmids also differed with respect to their ability to increase glucose transport as well (Figure 4). pGT7 had the greatest effect on glucose transport increasing both high and low affinity transport to *snf3* strains. Additionally, pGT7 was able to increase low and high affinity glucose transport in a wild-type *SNF3* strain, whereas pGT2 and pGT8 could not (Figure 5). pGT2 increased high affinity transport to the *snf3* null as strongly as pGT7. pGT8 only marginally increased transport.

Multicopy DDSE alone was found to also only slightly increase glucose transport to *snf3* strains (Figure 6), similar to the marginal increase seen with plasmid pGT8 (Figure 4). These data suggest the presence of multiple regulatory elements in the *HXT4* promoter that effect expression of *HXT4* in multicopy. Promoter regions allowing the greatest expression of *HXT4* display the highest degree of suppression.

Expression of *HXT4*: To understand the differences in *snf3* suppression observed with different promoter lengths, β -galactosidase expression from *HXT4* protein- β -galactosidase fusions to the same set of lengths was examined (Figure 3). It was expected that increased expression would correlate with increased suppression. However, this was not the case. β -Galactosidase expression was found to decrease with increasing promoter length. This finding implied that regulatory elements in the promoter region may be playing a role in suppres-

sion independent of their role in *HXT4* regulation. The presence of DDSE on the pGT2 plasmid appears to decrease expression of *HXT4* while increasing the ability of the plasmid to suppress *snf3*. The pGT2R fusion plasmid was able to suppress *snf3* while pGT7R and pGT8R could not, again implying a regulatory element plays a role in the suppression. β -Galactosidase activity was expressed in stationary phase in cells carrying all three *HXT4* protein- β -galactosidase fusions, when growth curves were examined (Figure 7).

When patched onto solid media containing X-gal, the *HXT4* protein- β -galactosidase fusions appeared to be repressed (white) on 2% glucose, 2% fructose and 2% galactose media. *HXT4* was de-repressed (blue) on 0.05% glucose and 2% raffinose (low fructose) (data not shown). Glucose repression of pGT7R, with the shortest promoter length, appeared to be diminished in comparison to pGT2R and pGT8R, as strains bearing this plasmid were light blue on the X-gal/2% glucose plates. This loss of glucose repression of *HXT4* from pGT7R could account for the increase in low affinity transport seen with multicopy pGT7.

Identification of DDSE as an independent multicopy suppressor of *snf3*: Deletion analysis revealed that the region between *HXT1* and *HXT4* contained a suppressor of the *snf3* growth defect on low glucose. This region contains a short (183 amino acids) open reading frame. Deletion of the ORF had no easily apparent phenotype. A fusion of the ORF to lacZ revealed a low level of β -gal expression (data not shown). However, Northern analysis revealed no transcript from the ORF even when the ORF was present in multicopy and cells were grown under conditions where suppression is observed (data not shown).

Initially it was believed that the ORF protein was responsible for suppression of *SNF3*. To test this possibility, site directed mutagenesis was used to create alleles of the ORF lacking the first (pGT902) or both (pGT903) start codons in the ORF. Mutagenesis was confirmed by sequencing. The double mutant was still able to function as a multicopy *snf3* suppressor. Further deletion analysis using restriction enzymes revealed that a 340-bp fragment within the ORF was able to suppress *snf3*. The minimal suppressing fragment is located between -795 and -435 from the *HXT4* ATG (Figure 2). The DDSE minimal suppressing sequence has no promoter region and lacks any apparent consensus splicing sites. The minimal DDSE suppressing region may be fairly large, as initial attempts to shorten the sequence have produced shorter fragments that give only weak suppression (data not shown).

Promoters of other *HXT* genes contain DDSEs: To determine if other promoter regions might also contain DDSEs we examined upstream regulatory regions of several other genes for their ability to suppress *snf3*. We found that the promoters of *HXT1*, *HXT2* and *HXT3*

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1  AAAATCGTTGAAAAAGCATTATGACACTATGTTTGGCGCCAAATAGGAATAGGAATACTAC
61  GTCATTATGCAATGGAGGAGAACCGAATATTTTTTTTATCCAGCCCTCTTCATGTAA
121  TGTCTGATTGATAAAGAAATGAAGCCCTAGGGTTATTAATCCAAACCTAGGCTGCC
181  AACCTGACATTTGAGACAGACGACAGCGGTAAGAGAGATCTGACAGCTGACGCCA
241  GTACCCAGATATTTTCCAACTTTTACGGGTTTTTGTCTAATAGGAGGAGGTGGGA
301  ATCATGCTTTAGTTTTCACCTTTGAGGTTTAGCTTTGACAGCAGAAACCTAGAGGAACA
361  TTATGTTGTTTACCTTTTCATTTTGGACAGAACTACGCTACCGCATCAATTTGCGGAA
421  TTTGATTTGGCAACGCGACGATTTTTCAGCGATGCGTAAGTAAGTAACAGTCAGATA
481  GTCTCATCTAGATGGTGAAGGTTAGGACCGAAACCGGTTCAACGATGTTGCCAAT
541  AGTCGTACCTGAAGAAGCTTAGGTTTCCATTTATGCGAATATCTCTATGCAAAATAATTT
601  TAGTACCTCAAAATTTTTTGCACCGATCAGATATAATATCAGATGAGATGAATAA
661  ACAGAGCGCAGATGAAGGCTGGGCTCCACGAAACCCAGTACACTGCAGTATTTCCAC
721  CAGAAACGCTTGGCCTTCGTAAGTCACTTGCAGCAGCATAGCGAAACAGGAAGAA
781  AATATCCGAGTCAGCTGGTTCGGGATTTTTTTTGCCTATAGGAACCAATGCCCAA
841  ACCGAACCATTCCCGTTTTTCTTTCGGTTATCCGCTTTCTTCCAAAAATCCGTA
901  CAAAATGGAAACCAATGTTATATAATATCCACATTTTCTGGCCTGCGTCTCTTTC
961  TGTGGAGAGAGATATTTCCCTGAGCAGTTTTTTTTTCTGACCCCTTTTTTTTCCAC
1021 CCCCCGGTTTTTTTTTCCATGGGCCCCATATTTCCCGCCTGACGAAACCTTGGGA
1081 AGAGGAAAAACACTTCGGATAAAACGGTCAGAGGCTCTTCGACGATTAGTGCCACC
1141 TTTCATGAATAATCCAGAGTTTTTCCAGCTGCTTTGATTTACAGTCCATTATTCGGCG
1201 CTAATGCTTCTGATTAGGAACACGGAGGAAACCTCAATTTCAATATAATATTTT
1261 AGTTTATGAAGGTGGGTTGTAAGAAAGCACTAAATAATCTTCAGTCAATTAGTGG
1321 TGAAGGCTTCAACCTGGGGAATGAATAATGTCATCTAGAAAAATTTATATAATAC
1381 TCAGTGTTCATATCTCGGATCATCTCATCTCTCATGAGTAATAGAAACATAAAG
1441 AAAAGATATATCAAGCCTTATCAAGGTTGGTTTTGAACACTTTACATAAATCTGCC
1501 AAAATGTCTGAAGAGCTGCTATCAAGAGGATACAGCAGTCCAAATACTCCAGCTGA
    M S E E A R V Q E D T A U Q N T P A D
1561 TGCTTTGTCGCGAGTGATTCGGATTTCAATTCGGCTTTGCTACTCCATCCACAAAGC
    A L S P U E S D S N S A L S T P S N K A
1621 TGAAGAGATGACATGAAGATTTTCGACGAGATCAGGAGATCTAATACTACGTTGA
    E R D D M K D F D E N H E E S N N Y U E
1681 AATTCGAAGAGAGCCCGCTCCGCTACGTTACAGTTTCCATCTGTGTTAATGGTTGC
    I P K K P A S A Y U T U S I C C L M U A
1741 TTTCGGTGGTTTTTTCGGTTGGGATACGTTTCCGTTTTTGTGTTTGTCTCAAC
    F G G F U F G U D T G T I S G F U A Q T
1801 TGATTTATCAGAGATTTGGTATGAAGCACCAGATGGTACTTATTATTGTCTAAGT
    D F I R R F G M K H D G T Y Y L S K U
1861 TAGAAGTGGTTAATGTCTCCATTTCAACATGGTTGTCATGGTGGTATTATTT
    A T G L I U S I F N I G C A I G G I I L
1921 AGCAAAATAGGTGATATGATGGTCGTAATAGGTTGATGCTGTGTTGTCATCTA
    A K L G D M Y M A K M G L I U U U I Y
1981 CATTATCGGTATCATATCCAAATTCCTCAACAGTGGTACCAATTTTCATGG
    I I G I I I Q I A S I N K M Y Y Q V F I G
2041 TAGAATATCTCTGGTTAGGTGTCGGTGGTATTGCCGTTTATCTCCTATGTTGATTC
    R I I S G L G U G G I A U L S P M L I S
2101 TGAAGTCTCCAAAGCATATAGAGGTAATTTGGTTTCACTGTTACCACTTATGATAC
    E U S P K H I R G T L U V S C Y O L M I T
2161 TTTGGTATTTCTTGGTTACTGTACAACTACGTTACCAAGACCTACCAATTCGT
    L G I F L G Y C T N Y G T K T Y T N S U
2221 CCAAATGAAGATTCATAGGTTAGGTTTCGCTTGGGCTTTGTTATGATGGTGGTAT
    Q W R U P L G L G F A W A L F M I G G M
2281 GACATTCGTTCCAGAACTCCACGTTATTTAGTTGAAGTCGGTAATTAAGAGAGCTAA
    T F U P E S P M Y L U E U G K I E E A K
2341 GCGTTCTATTGCTCTTCAAAATAGGTCAGCGACGATCCAGCTGTTATGGCTGAAGT
    A S I A L S N K U S A D D P A U M A E U
2401 CGAAGTGTTCAGACTACAGTTGAAGCTGAAGAAATAGCTGGTATGCTCCTGGGGTGA
    E U U Q A T U E A E K L A G N A S W G E
2461 AATATTTAGCACTAAGACTAAGGTTTCCAACTTTGATCATGGGCTATGATTCATC
    I F S T K T K U F Q A L I M G A M I Q S
2521 ATTCGAACCAATGACAGGTGATAACTATTTCTTCTATTCGGTACTACCGTTTCTACTGC
    L Q O L T G D N Y F F Y V G T T U F T A
2581 TGTCGGTTTGAAGATTTCTTGAACCTTCTATTGCTTGGGTTATGTCACACTTGTCTC
    U G L E O S F E T S I U L G I U N F A S
2641 CACCTTTGTTGGTATTTCTTATGTCGAAGATATGTCGTCGATGTTTATTATGGGG
    T F U G I F L U E R Y M M R C L L W G
2701 TGCTGCTTCATGACAGCTTGATGGTTGTTTCGCTTCTGTTGGTGTACAGATTGTG
    A A S M T A C M U U F A S U G U T R L W
2761 GCCAAATGGTAGAAGACGGGCTTCTAAGGGTCTGGTAACTGTATGATGTTCTTAC
    P H G K K N G S S K G A G N C M I U F T
2821 ATGTTTCTACTTATCTGTTTGGCACTACCTGGGCTCCAACTCCATTTGTTTAACTC
    C F Y L F C F A T T W A P I P F U U N S
2881 TGAACCTTTCCATGAGAGTTAAGTCCAGGTATGGCTATTGCTCAAGCTTGTAACTG
    E T F P L R U K S K C M A I A Q A C N W
2941 GATCTGGGTTTCTGATTTGTTTCTTACTCATTATTTTCAAGTGTCTATGATTTCTA
    I W G F L I G F F T P F I S G A I D F Y
3001 CTACGGTTATGTTTTTATGGGCTGTTTGGTCTTTTCTTACTTACCTTCTTCTTCTG
    Y G Y U F M G C L U F S Y F Y U F F F U
3061 TCCAGAACTAAAGGTTTGAATTTAGAGAGTTTAAACCTTTATGGGAGAGGTTT
    P E T K G L T L E E U N T L W E E G U L
3121 GCCATGGAATACCTTCTTGGGTTCCACCAACAGAGAGGTAAGTACTACACGCTGA
    P W K S P S W U P P N K R G T D V N A D
3181 TGATCTAATGATGATCAACCTTTTACAGAGAGTGTTCGGAAGAAAGTAGGTCAG
    D L M N D D Q P F Y K K M F G K K *
3241 TAAATTAATATAGACTTCTTCAAGGAATAAATAATATTTGATCCTTCCAAATTT
    TTAACGCTTTTGAATATATATTTTATGATTTAATAGTTTATAGATACTTTATCAA
3301
3361 CTACAAATTTGAATATGTTTTTGTTCATAGTTTGGAGAGGTTAGGAATAGGCCGA

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FIGURE 2.—Nucleotide and predicted amino acid sequence of the *HXT4* gene. The DDSE region in the *HXT4* promoter is underlined. Predicted transmembrane domains in the *HXT4* peptide sequence are boxed. Highly conserved amino acids including the leucine zipper motif, the “PESPR” box and two “GRR” boxes are shown outlined. The *HXT4* DNA sequence has been entered in GenBank (accession no. M81960).

HXT4-LacZ fusion plasmids:		Promoter length:	β -galactosidase units ● 60 hours:	<i>snf3</i> suppression	
				by <i>lacZ</i> fusion plasmid:	by <i>HXT4</i> plasmid with equivalent promoter:
pGT2R	H DDSE P	1 kb	4300	+	+
pGT8R	X	440 bp	9700	-	+
pGT7R	X	145 bp	15200	-	+

FIGURE 3.—Comparison of different promoter lengths with respect to expression and *snf3* suppression. β -Galactosidase units are expressed as nanomoles of ONPG hydrolyzed/min/mg of protein. *snf3* suppression was determined by plating cells onto YEP 0.05% glucose, 2 μ g/ml antimycin A plates. “+++” connotes strong growth after 2 days, “++” connotes moderate growth after 2 days, “+” connotes growth after 3 days.

FIGURE 4.—Eadie-Hofstee plots showing restoration of glucose uptake to *snf3* null strains by *HXT4*-bearing multicopy plasmids. Cells were grown to mid-log and then shifted to low or high glucose for 4 h. V = nanomoles/min/mg dry weight of cells, S = glucose concentration (mM).

could also suppress *snf3* in multicopy (Figure 8). The promoter of *SNF3* could not suppress *snf3* (Figure 8). Multicopy plasmids containing the intact *HXX1*, *HXX2*, *GLK1*, *GRR1*, *ENO2*, *SUC2* and *GAL2* genes together with their upstream regulatory regions could not suppress *snf3*. DDSE contains several consensus transcription factor binding sites found in glycolytic gene promoters. These included several *MIG1* binding sites (also found in the *SUC2* promoter) and two *GCR1* binding sites (also found in the *ENO2* promoter). The finding that other promoters which contain these binding sites do not suppress *snf3* suggests that these binding domains are not sufficient to cause *snf3* suppression, and further suggests the presence of previously uncharacterized proteins specifically involved in regulating a subset of genes involved in glucose metabolism, which includes the *HXT* genes but may also include other genes as well.

The only other DDSE that had been sequenced was the one in the *HXT2* promoter. Therefore, the DDSE in

the *HXT1* promoter was sequenced as well to determine if there was a DDSE consensus sequence. After comparing the DDSEs of *HXT1*, *HXT2* and *HXT4* no strong DDSE consensus was apparent. Some similarities were found among the different promoters, most notably A- and T-rich stretches flanked by G and C (accession nos. M33270, L07080 and M81960). No ORFs of substantial length were found in the *HXT1* and *HXT2* DDSEs.

Suppression of *snf3* by DDSE is dependent on the presence of *HXT2* or *HXT3*: DDSE suppression was examined in strains carrying multiple null mutations of the *HXT* genes (Figure 9). Any one of the four *HXT* genes is required for growth on low glucose and raffinose in a wild-type *SNF3* strain (Ko *et al.* 1993). Deleting all four *HXT* genes results in a growth defect on low glucose and raffinose (Ko *et al.* 1993). DDSE was unable to suppress the growth defect in strain, CY290 (*SNF3*, *hxt1*, *hxt2*, *hxt3*, *hxt4*) or strain LBY313 (*snf3*, *hxt1*, *hxt2*, *hxt3*, *hxt4*) on 0.05% glucose or 2% raffinose. DDSE was only partially able to suppress strain CY291 (*snf3*, *HXT1*,

FIGURE 5.—Eadie-Hofstee plot showing an increase in low affinity glucose uptake in the wild-type YPH500 strain by the *HXT4*-bearing multicopy plasmid pGT7. Cells were grown to mid-log and then shifted to high glucose for 4 hr. See Figure 4 legend for *V* and *S*.

FIGURE 6.—Eadie-Hofstee plot showing glucose uptake in the *snf3* null strain LBY403 with multicopy DDSE-bearing plasmids. Cells were grown to mid-log and then shifted to low glucose for 4 hr. The results shown are a compilation of three separate experiments. Two of the experiments used the DDSE bearing plasmid pGT9 and the corresponding vector YEp352 and the other one used the DDSE bearing plasmid pGT94 and the corresponding vector pRS424. Glucose uptake in the wild-type strain YPH500 is shown for comparison. Error bars depict the variation seen in the different experiments. See Figure 4 legend for *V* and *S*.

hxt2, *hxt3*, *HXT4*). Some transformants produced small and scattered colonies after a prolonged incubation (5 days) on 0.05% glucose plates while other colonies from the same transformation did not. No colonies were seen on 2% raffinose. Multicopy DDSE strongly suppressed strains CY292 (*snf3*, *hxt1*, *HXT2*, *hxt3*, *hxt4*) and CY293 (*snf3*, *hxt1*, *hxt2*, *HXT3*, *hxt4*) on low glucose and raffinose. We interpret these results to mean that *snf3* suppression by DDSE is dependent on the *HXT* genes since strain CY290 was not suppressed by DDSE. The effect of DDSE sequences on restoration of growth on low glucose concentrations to *snf3* mutants is dependent on the presence of either *HXT2* or *HXT3* since strain CY291 was suppressed very poorly while CY292 and CY293 were strongly suppressed. DDSE in multicopy could result in increased expression of *HXT2* and *HXT3* or perhaps an unknown gene or genes, such as some type of nutrient

sensor or component of a nutrient sensing pathway that results in suppression only in the presence of *HXT2* or *HXT3* and, in the absence of *SNF3*, neither *HXT2* nor *HXT3* are activated for transport. In the course of these and other studies on *snf3* mutant strains, we have found that phenotype modifying or suppressing mutations arise at a high frequency. Careful attention must be paid to avoid misinterpretation of the data.

Multicopy *HXT4*, *SNF3* and DDSEs suppress *grr1* in multicopy: We have found that in some strain backgrounds *grr1* null mutations have a growth defect on low glucose/antimycin A medium. Not all *grr1* strains examined had this growth defect. The YM *grr1* strains obtained from MARK JOHNSTON grew on low glucose/antimycin A, while MARIAN CARLSON *grr1* strains, as well as *grr1* strains constructed in our laboratory, were unable to grow on low glucose/antimycin A. Multicopy

FIGURE 7.— β -Galactosidase activity of the *HXT4* promoter-lacZ fusion. Wild-type strain YPH500 was transformed with the *HXT4-lacZ* fusion plasmid pGT2R. Samples were taken in triplicate spanning the growth curve for measurement of β -galactosidase activity, and OD₅₈₀. β -Galactosidase activity expressed in nanomoles ONPG/min/total protein is indicated by open circles and optical density at 580 nm is indicated by open squares.

LB403(*snf3*) multicopy plasmids containing the following DNA sequences

FIGURE 8.—Suppression of the *snf3* growth defect by multicopy plasmids bearing fragments from *HXT* promoters: *HXT4* promoter (pGT94), *HXT2* promoter (pGT1C3), *HXT1* (pDL502). Plasmid pAK15 which contains the *SNF3* promoter was unable to suppress. Plasmid pGT166 which contains the *HXT3* promoter was able to suppress (data not shown). Suppression was tested by streaking cells onto YEP 0.05% glucose, 2 μ g/ml antimycin A and incubating for 3 days at 30°.

HXT4, *SNF3* and DDSEs were able to suppress this growth defect in multicopy. Although *SNF3* was able to suppress *grr1* in multicopy, *GRR1* was unable to suppress *snf3* in multicopy. These plasmids were unable to suppress the abnormal cellular morphologies found in *grr1*. Multicopy DDSE was unable to restore glucose repression to *HXT2* and *HXT4* protein- β -galactosidase fusions (data not shown). Multicopy DDSE, *HXT4* and *SNF3* did not appear to restore glucose repression to a *GAL1* promoter. Strain YM2957, a *grr1* strain containing a *GAL1::LacZ* fusion appears blue on X-gal, 2% glucose plates, while wild-type *GRR1* strains containing this fusion appear white on X-gal/2% glucose plates. YM2957 appears blue on X-gal/2% glucose plates even when transformed with multicopy plasmids containing DDSE, *HXT4* or *SNF3* (data not shown). Thus, although these plasmids suppress the growth defect of *grr1* strains

on low glucose they do not suppress the glucose repression defect.

When streaked onto X-gal/2% glucose plates, the *HXT4* and *HXT2-lacZ* fusion plasmids, pGT2R and pAK12, appear white in *GRR1* strains versus blue on X-gal/0.05% glucose plates, indicating that glucose repression is operating on these genes. The pleiotropic loss of glucose repression characteristic of *grr1* strains is apparent with *HXT2* and *HXT4* as well. When streaked onto X-gal/2% glucose plates, *grr1* null strains transformed with pGT2R and pAK12 appeared blue (data not shown). Preliminary evidence indicates that in *grr1* strains, the level of Hxt2p is very low. Therefore, although *HXT2* message may be transcribed at a high level, the level of Hxt2p is diminished in *grr1* (G. THEODORIS, D. L. WENDELL and L. F. BISSEON, unpublished results).

DISCUSSION

HXT4 is the newest member of the hexose transporter family in *Saccharomyces*. *HXT4* is the first multicopy suppressor characterized which appears to increase both high and low affinity glucose transport, suggesting that high and low affinity glucose transport in *Saccharomyces* may be mediated in part by the same transporters. Alternatively, increasing low affinity transport with *HXT4* may simultaneously and indirectly activate high affinity transport. PRIOR *et al.* (1993) found that *HXT4* but not *HXT1* was able to suppress *rag1*, a mutation in the related yeast *Kluyveromyces fragilis* that is defective in low affinity transport. *HXT4* was able to suppress *rag1* on multicopy and single copy plasmids (PRIOR *et al.* 1993).

The need for a family of glucose transporters in *Saccharomyces* has been unclear. Like yeast, mammals also have multiple glucose transporters (*GLUT1-7*) (BALDWIN 1993). Expression of *GLUT* genes in mammals is tissue-specific, *i.e.*, *GLUT1* is found in brain, *GLUT2* is found in the pancreas (GOULD and BELL 1990). Ex-

Strain:	<i>SNF3</i>	Genotype:				multicopy suppression by DDSE
		<i>HXT1</i>	<i>HXT2</i>	<i>HXT3</i>	<i>HXT4</i>	
LBY313	Δ	Δ	Δ	Δ	Δ	—
LBY403	Δ	+	+	+	+	+
CY290	+	Δ	Δ	Δ	Δ	—
LBY203	Δ	Δ	+	+	+	+
LBY416	Δ	+	Δ	+	+	+
LBY901	Δ	+	+	+	Δ	+
CY291	Δ	+	Δ	Δ	+	— — / +*
CY292	Δ	Δ	+	Δ	Δ	+
CY293	Δ	Δ	Δ	+	Δ	+

FIGURE 9.—DDSE-mediated suppression in multiple *hxt* and *snf3* null strains. "Δ" indicates gene deletion, "+" indicates the presence of the wild-type gene. Suppression was assayed by streaking yeast onto YEP plates containing 0.05% glucose or 2% raffinose plates containing 2 μg/ml antimycin A and incubating for 3 days. "+" indicates growth after 3 days. Strain CY291 was only very weakly suppressed by multicopy DDSE. Small and scattered colonies formed after an incubation of 5 days on 0.05% glucose and no colonies formed on raffinose plates.

pression of the *HXT* genes appears to growth cycle specific and is influenced by environmental factors: *HXT1* expression appears to be dependent upon the nitrogen composition of the medium and is not under glucose repressional control (N. M. FONG and L. F. BISSEON, unpublished observations). *HXT2* is under glucose repression control and highly expressed upon a shift to low glucose, but not expressed when grown to low glucose (WENDELL and BISSEON 1993). *HXT3* is expressed in stationary phase (KO *et al.* 1993), as is *HXT4*. These genes may be adapted for functioning under different environmental conditions, in much the same way the *GLUT* genes may be adapted for functioning in different tissues or cell types. The characterization of *HXT4* led to the discovery of a new class of mechanistically unique multicopy suppressors of glucose transporter defects: regulatory elements in the promoters of the *HXT* genes. We have designated these suppressors DDSEs (DNA sequence dependent suppressing elements). Although a small ORF exists in the *HXT4* promoter, extensive analysis showed that it was not required for the multicopy suppression. ORFs of substantial length were not found in the *HXT1* and *HXT2* DDSEs. The suppression of *snf3* and *grr1* by these sequences likely results from the titration of a repressing transcription factor(s) by the DNA sequence. Preliminary evidence indicates that the expression of *HXT2*, and *HXT4* was slightly increased in *SNF3* strains containing multicopy DDSE (G. THEODORIS, D. L. WENDELL and L. F. BISSEON, unpublished observations). The suppression of *snf3* by DDSE was found to be dependent on either *HXT2* or *HXT3*. Increasing transcription of these genes may be necessary for *snf3* suppression. However, we believe it is possible that increasing transcription of non-*HXT* genes may also be involved in suppression. Researchers using multicopy suppressor screens should be aware that this mechanism of suppression exists.

Several lines of evidence suggest that chromosomal gene expression can be modulated by multicopy plasmid-borne regulatory DNA sequences. GOZALBO (1992) has shown that multiple copies of *SUC4* regulatory regions may cause partial de-repression of invertase synthesis. In a screen for multicopy suppressors of lethality due to overexpression of cAMP-dependent protein kinase driven by a *GAL1* promoter, NEHLIN *et al.* (1989) isolated plasmids containing promoter sequences with *GAL4* binding sites (RONNE *et al.* 1991). They suggest that the suppression is mediated by competition for the *GAL4*-activating protein by the promoter sequences. The minimal DDSE suppressing region appears to be rather large and no striking DDSE consensus is apparent. Thus, a simple model involving titration of a single factor by a specific consensus binding site appears unlikely, and multiple factors may be involved. Titration of a limiting factor or factors may increase transcription of chromosomal gene(s) leading to *snf3* suppression. Possible candidates for titrated factors include: (1) *RGT1* (restoration of glucose transport); *rgt1* is a loss of function mutation isolated as a second site suppressor of *snf3* (MARSHALL-CARLSON *et al.* 1991). The *RGT1* gene product functions by either repressing expression of genes involved in transport or by inhibiting the function of the transporters themselves (MARSHALL-CARLSON *et al.* 1991). (2) *HTR1* (hexose transport regulation); *HTR* is a dominant mutation which is defective in glucose transport and transcription of *HXT* genes (OZCAN *et al.* 1993). OZCAN *et al.* (1993) found that the *HXT4* and *HXT3* promoters can suppress this mutation in multicopy. (3) *SSN6* (suppressor of *snf*) *SSN6* is a repressing transcription factor in yeast. *SSN6* does not contain a DNA binding motif. Instead it is believed to be brought to DNA sequences by other transcription factors (KELEHER *et al.* 1992). *SSN6* is a second site suppressor of *snf3* (NEIGEBORN and CARLSON

1984). Expression of *HXT2* has been found to be greatly increased in *ssn6* strains (WENDELL and BISSON 1993).

In conclusion, both *HXT* genes and their promoter regions are able to independently suppress *snf3*. The *HXT* genes appear to encode high affinity glucose transporters, the overexpression of which explains suppression. We have shown that overexpression of *HXT4* in high glucose media leads to an increase in low affinity transport thus providing the first link between the *HXT* genes and low affinity transport in *Saccharomyces*. The mechanism of suppression by DDSEs appears to be complex, involving the titration of negative regulatory factors by the DDSE DNA sequences leading to increased transcription of chromosomal genes. *SNF3* itself does not appear to be a bona fide glucose transporter, but rather a protein required for the functional expression of the *HXT* proteins which appear to be responsible for sugar uptake.

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