Glucose transport in Saccharomyces cerevisiae effects on growth and metabolism
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Chapter 2

Functional expression, quantitation and cellular localization of the Hxt2 hexose transporter of Saccharomyces cerevisiae tagged with the green fluorescent protein

SUMMARY

The Hxt2 glucose transport protein of Saccharomyces cerevisiae was genetically fused at its carboxyl terminus with the green fluorescent protein (GFP). The Hxt2::GFP fusion protein is a functional hexose transporter: it restored growth on glucose to a strain bearing null mutations in the hexose transporter genes GAL2 and HXT1 through HXT7. Furthermore, the glucose transport activity in this null strain was not markedly different from the activity of that with the wildtype Hxt2 protein.

Both Hxt2 and Hxt2::GFP showed biphasic uptake kinetics with a high-affinity component ($K_m = 0.25$ mM) and a low-affinity component ($K_m = 15$ and $13$ mM). We calculated from the fluorescence level and transport kinetics that induced cells had $1.4 \times 10^5$ Hxt2::GFP molecules per cell, and that the catalytic-center activity of the Hxt2::GFP molecule in vivo is $65 \text{ s}^{-1}$ at $30 \text{ °C}$.

Expression of Hxt2::GFP was induced by growth on low glucose. Under inducing conditions the Hxt2::GFP fluorescence was localized to the plasma membrane. In a strain impaired in fusion of secretory vesicles with the plasma membrane, the fusion protein accumulated in the cytoplasm. When induced cells were treated with high glucose, the fusion protein was redistributed to the vacuole within 4 h. When endocytosis was genetically blocked, the fusion protein remained in the plasma membrane after treatment with high glucose.

INTRODUCTION

Glucose is the preferred nutrient of *S. cerevisiae* for carbon and energy. The concentration of glucose in the yeast environment also regulates metabolic and cellular activity and gene expression. Transport of glucose and other hexoses is necessary for their metabolism, and is also implicated in their regulatory effects. Glucose transport in yeast is mediated by proteins encoded by the *HXT* gene family, of which twenty members have been identified genetically or by sequence homology (123). This is a remarkably large number of genes for a biochemically simple function in a unicellular organism; mammals express only six homologous glucose transporters (76).

Experimental evidence has implicated seven members of the *HXT* family in metabolically significant hexose transport; these are *GAL2* (the galactose transporter) and *HXT1* – *HXT4*, *HXT6*, and *HXT7* (which transport glucose, fructose and mannose) (185, 186). Two other members of the family, *SNF3* and *RGT2*, encode glucose sensors (165, 166); the remaining eleven members are not well characterized (15). The transport kinetics of the seven metabolically significant Hxt proteins have been measured *in vivo* (185). Their affinities for glucose vary by two orders of magnitude, whereas the maximal uptake velocity of cells expressing these proteins individually differs by only a factor of three. Assuming that the transporters have a similar catalytic-center activity for glucose, this suggests that they be expressed to approximately the same level.

These *HXT* genes are differentially regulated at the levels of expression and inactivation in response to the growth conditions (15). However, under normal conditions the expression intervals of different *HXT* genes can overlap (46). The transport kinetics of batch-cultivated cells changes constantly during growth on glucose (235). This reflects the arrival of newly synthesized transporters at the plasma membrane via the secretory pathway and the inactivation of transporters via endocytosis and degradation (8, 149, 189, 222). Therefore, the cell should have a mechanism to selectively remove from the membrane only those transporters whose characteristics are inappropriate for the prevailing conditions (for example, low-affinity transporters in a low glucose environment) while leaving their close homologues with appropriate characteristics in place.

*HXT2* encodes a protein with high homology to other yeast sugar transporters (125). *hxt2* null mutant strains are partially defective in high-affinity glucose transport and in growth on low glucose concentrations (125, 186, 240). Expression of Hxt2 protein is stimulated by shifting batch-cultured cells from high to low glucose media (240). These observations suggest that Hxt2 is a high affinity glucose transporter. However, a strain expressing only *HXT2* displays
peculiar transport kinetics: when grown on low glucose, it displays both high- and low-
affinity kinetics, whereas when grown on high glucose, it displays transport kinetics of
intermediate affinity.

In order to investigate further the properties and regulation of Hxt2 we have tagged it with the
green fluorescent protein (GFP) of *Aequorea victoria*. GFP matures after translation to be an
intrinsically fluorescent protein (26). It has a number of characteristics that make it a useful
tool for monitoring biochemical and cellular phenomena. First, genetically constructed
chimeras between GFP and other proteins frequently display functional characteristics that are
indistinguishable from those of the native proteins. For example, a chimera between GFP and
the OSCP subunit of the yeast mitochondrial ATPase is translocated into mitochondria and
assembled into functional ATPase complex (176). Second, GFP can be a quantitative reporter
of protein abundance by use of fluorimetric and spectrophotometric techniques. The detection
threshold of GFP fluorescence in the cytoplasm has been reported to be 200 nM (172).
Assuming a cytoplasmic volume of 500 nl per $10^7$ cells (35), this corresponds to 6000
molecules per yeast cell. Third, the fluorescent signal from a GFP-tagged fusion protein can
be used to monitor the subcellular localization and dynamics of protein trafficking in living
cells. For example, Niedenthal *et al.* (160) tagged three open reading frames of unknown but
essential function in yeast chromosome XIV with GFP. The chimeric genes complemented
null mutations of each locus. One of the fusion proteins was localized to the nucleus, one was
localized to the vacuole, and one was present in the cytoplasm.

Some integral membrane proteins fused with GFP appear to be targeted properly, despite the
constraints imposed on the sequence and structure of these proteins by membrane topogenesis
and trafficking. For example, both isoforms of yeast hydroxymethyl glutaryl (Hmg)-CoA
reductase fused with GFP are correctly localized to the endoplasmic reticulum membrane, and
under conditions where regulated degradation of the Hmg2 isoform occurs, the degradation
rate is similar for the native and fusion proteins (84). Another pertinent example is the fusion
of the mammalian GLUT4 glucose transporter with GFP. GLUT4 resides in intracellular
vesicles in resting cells, and is translocated to the plasma membrane upon insulin stimulation.
Removal of insulin causes recycling of GLUT4 to an intracellular pool (118). Fusions of
GLUT4 with GFP display the same insulin-responsive translocation when observed by
confocal microscopy. Fusion proteins with GFP at the carboxyl terminus of GLUT4 also
undergo re-internalization upon insulin removal; however, fusions with GFP at the amino
terminus do not (49). This suggests that GFP is not an entirely silent tag in such fusion
proteins. Transporter function has not been reported for the GFP-tagged GLUT4.
We have constructed an Hxt2::GFP fusion protein for use as a reporter of Hxt2 expression, abundance, and localization within the yeast cell. We find that Hxt2 tagged with GFP is a functional glucose transporter, and is localized to the plasma membrane. We exploit the fluorescent properties of the fusion protein to quantitate its cellular abundance and to observe its trafficking, in both wildtype cells in various growth conditions and in cells with defects in secretion or endocytosis. Our results demonstrate that this hexose transporter moves to the plasma membrane via the secretory pathway, and is removed from the membrane via endocytosis.

MATERIALS AND METHODS

Strains, media and growth

The E. coli strains used in this work were DH5α (F',φ80dpirΔZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17 (rk-,mk+), supE44, λ-, thi-1, gyrA96, relA1) and DM1 (F', dam'13::Tn9 (CmR), dcm'mcrB, hsdRM', galI, gal2, ara-, lac' thr', leu', tonR, tsxR, Su6) from Life Technologies and BL21(DE3) (F',ompT, hsdSB, (rB'mB'), dcm, gal, (DE3)) from Promega. They were grown in LB medium, and transformed by the calcium chloride method as described (143). Transformants were grown in the presence of 60 μg ml⁻¹ ampicillin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>KY73</td>
<td>MATα hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::hxt6 hxt7::HIS3 gal2Δ::DRk ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</td>
<td>this work</td>
</tr>
<tr>
<td>RE102</td>
<td>MATα hxt2Δ::HIS3 ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</td>
<td>(186)</td>
</tr>
<tr>
<td>RE700</td>
<td>MATα hxt1Δ::HIS3::Δhxt4 hxt5::LEU2 hxt2Δ::HIS3 hxt3Δ::LEU2::hxt6 hxt7::HIS3 ura3-52 his3-11,15 leu2-3,112 MAL2 SUC2 GAL MEL</td>
<td>(186)</td>
</tr>
<tr>
<td>NY17</td>
<td>MATα sec6-4 ura3-52</td>
<td>(156)</td>
</tr>
<tr>
<td>RH1800</td>
<td>MATα his4 leu2 ura3 bar1-1</td>
<td>(184)</td>
</tr>
<tr>
<td>RH1623</td>
<td>MATα his4 leu2 ura3 bar1-1 end3-1</td>
<td>(184)</td>
</tr>
<tr>
<td>RH1597</td>
<td>MATα his4 leu2 ura3 bar1-1 end4-1</td>
<td>(184)</td>
</tr>
</tbody>
</table>

*Direct repeat.*

The yeast strains used in this study are listed in Table 2.1. Strain KY73 was constructed as follows: a 3.5-kb EcoRI/SmaI fragment containing the GAL2 gene was cloned from plasmid pS25 (158) into pUC18 to create plasmid pAK82. The EcoRV fragment of this plasmid (containing the GAL2 open reading frame) was replaced with the Klenow-treated
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HindIII/Smal fragment of pJR-URA3 (190). This fragment contains the URA3 gene flanked by short direct repeats. The resulting plasmid, pAK83, was digested with EcoRI and Smal and transformed into strain RE700 (186). Transformants were selected for uracil prototrophy. Ura^+ isolates were plated on medium containing 5-fluoroorotic acid (FOA), and FOA-resistant isolates were recovered. These were screened for uracil auxotrophy and impaired galactose growth. Southern blotting with probes to the URA3 and GAL2 genes showed that the Ura^+ Gal^+ isolates had a deletion of the GAL2 gene and had lost the URA3 marker from the gaI2Δ locus (data not shown). Strain KY73 shall subsequently be referred to as the hxt null strain.

The liquid medium used in these experiments consisted of 1.6 g l^-1 Yeast Nitrogen Base (Difco 0335-15-9), 5 g l^-1 ammonium sulfate, 1 g l^-1 casamino acids (Difco), and 20 mg l^-1 tryptophan. It was supplemented with a low concentration of glucose (0.1%), with a high concentration of glucose (5%), or with maltose (2%). Solid media for strain propagation, yeast transformation, and FOA selection were prepared as described (204).

The induction experiments described below involved transfer of exponentially growing cells from non-inducing medium to low- and high-glucose medium, after centrifugation and washing in pre-warmed medium. Strain KY73 was grown on maltose medium prior to induction; other strains were grown on solid medium containing 2% glucose or in liquid medium containing high (5%) glucose unless otherwise indicated. Strains with temperature sensitive alleles (NY17, RH1597, RH1623) were grown at 24 °C as the permissive temperature and 37 °C as the restrictive temperature. Other strains were grown at 30 °C unless otherwise indicated.

**HXT2::GFP plasmid construction**

The plasmid pAK1a, containing the entire HXT2 gene in YEp352 (95), has been described (125). An Ascl restriction site was introduced at the 3' end of the HXT2 open reading frame via overlap-extension PCR (100) as follows: pAK1a was amplified with Taq DNA polymerase in two separate reactions. Reaction 1 included oligonucleotides AK8 and AK9, and reaction 2 included oligonucleotides AK10 and AK11 (Table 2.2). The products of these reactions were gel-purified, combined with oligonucleotides AK8 and AK11, and re-amplified. The product of this secondary reaction was digested with BclI. pAK1a was purified from strain DM1, digested with BclI, and ligated with the BclI-digested PCR product. Three plasmids in which the HXT2::Ascl cassette was in the correct orientation were identified by DNA sequencing. Each of them had the sequence GGCGCGCGCCG between the last codon and the stop codon of HXT2. This sequence contains the recognition sequence of Ascl (shown
in bold) and encodes glycine, alanine and proline. The remainder of the sequence was unaltered from that previously reported (125). The \textit{HXT2::Asc\textsubscript{i}} plasmid used below was named pAK121.

### Table 2.2 Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>AK8</td>
<td>ACCCTGTTTTTTCTTATCAG</td>
</tr>
<tr>
<td>AK9</td>
<td>CTCTTACGGCCGGCTTCTCGGAAACTCTTTTTC</td>
</tr>
<tr>
<td>AK10</td>
<td>GGAAGGC6C6CGCGAAGAGATTATACCTAAGAC</td>
</tr>
<tr>
<td>AK11</td>
<td>TTAACGTCGAGTCCGTAAG</td>
</tr>
<tr>
<td>AK12</td>
<td>CGCGATGGCTTCTATGACCGGTGGTCAACAAATGGGTGG</td>
</tr>
<tr>
<td>AK13</td>
<td>CGCGCCACCCATTTGTTGACCACCGTCATAGAAGCCAT</td>
</tr>
<tr>
<td>AK22</td>
<td>TTGGCGCGCCGATGAGTAAAGGAGAAGAAC</td>
</tr>
<tr>
<td>AK23</td>
<td>TTGGCGCGCCGCTTTGTATAGTTCATCCG</td>
</tr>
</tbody>
</table>

An F64L/S65T allele of GFP in plasmid pRSET\textsubscript{B} was amplified by PCR with the primers AK22 and AK23 (Table 2.2); the product was cloned into the \textit{Asc\textsubscript{i}} site of pNEB193. The resulting plasmid, pGFP, was digested with \textit{Asc\textsubscript{i}} and the GFP cassette was gel-purified. pAK121 was digested with \textit{Asc\textsubscript{i}}, ligated with the GFP cassette, and \textit{HXT2::GFP} chimeras were identified by restriction analysis of the resulting recombinant plasmids. The amplification of GFP and subsequent cloning steps were performed in triplicate and products were functionally tested for GFP fluorescence to ensure that the polymerase chain reaction did not introduce any deleterious mutations in the DNA encoding GFP. The resulting plasmid containing the chimeric \textit{HXT2::GFP} gene was named pAG1-5.

\textit{HXT2} was tagged with DNA encoding an epitope tag from the bacteriophage T7 gene 10 protein by digestion of pAK121 with \textit{Asc\textsubscript{i}} and ligation with annealed oligonucleotides AK12 and AK13 (Table 2.2). Sequence analysis showed that plasmid pAK125 had the tag sequence in the proper orientation. The sequence encodes the epitope MASMTGGQQMG using the preferred codons of \textit{S. cerevisiae} (M. Cherry, personal communication) except for the threonine codon (ACC); use of this codon (the third most abundant threonine codon) introduces an \textit{Agl} restriction site into the DNA sequence.

Single-copy vectors bearing \textit{HXT2} and \textit{HXT2::GFP} were constructed by digesting pAK1a and pAG1-5 with \textit{Sac\textsubscript{i}} and \textit{BamHI} and ligating the fragments of interest into \textit{Sac\textsubscript{i}/BamHI}-digested centromeric vector YCplac33 (74). They were named pAK145 and pAK146, respectively.

The plasmids used in this study are listed in Table 2.3.
### Table 2.3 Plasmids used in this research

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Vector</th>
<th>Source or reference</th>
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<tr>
<td>pRS426</td>
<td>negative control</td>
<td>2 µ URA3</td>
<td>(208)</td>
</tr>
<tr>
<td>YCplac33</td>
<td>negative control</td>
<td>CEN URA3</td>
<td>(74)</td>
</tr>
<tr>
<td>pAK1la</td>
<td>HXT2</td>
<td>2 µ URA3</td>
<td>this work</td>
</tr>
<tr>
<td>pAGl-5</td>
<td>HXT2::GFP</td>
<td>2 µ URA3</td>
<td>this work</td>
</tr>
<tr>
<td>pAK125</td>
<td>HXT2::T7</td>
<td>2 µ URA3</td>
<td>this work</td>
</tr>
<tr>
<td>pAK145</td>
<td>HXT2</td>
<td>CEN URA3</td>
<td>this work</td>
</tr>
<tr>
<td>pAK146</td>
<td>HXT2::GFP</td>
<td>CEN URA3</td>
<td>this work</td>
</tr>
</tbody>
</table>

### Glucose transport assay

Transport of glucose was measured on the basis of zero-trans D-U-[14C]-glucose uptake assay at 30 °C as described (235). A detailed protocol is available from the author of this thesis on request. The suspension density was approximately 7.5% wet weight : volume. Twenty-four hours later counting was performed by Wallac Win Spectral 1414 Liquid Scintillation Counter. Kinetic parameters were determined using ENZFITTER software (Elsevier-Biosoft). Total cell protein was measured following the Lowry assay by COBAS FARA (Roche) after digestion of cells overnight in 1N NaOH, with bovine serum albumin as a standard, and cell number was determined by counting at least 1000 cells with a haemocytometer.

### Microscopy

Living cells were examined with a Leitz Aristoplan epifluorescence microscope using filter cube 1001 HQ-FITC (Chroma) for GFP excitation, filter cube N2.1 (Leica) for conA-Texas Red excitation and filter A for CMAC-Arg. Micrographs were recorded using an UltraPix 12-bit CCD camera and processed for display using Adobe Photoshop.

### Fluorimetry

A Hitachi RF-5001PC fluorimeter was used to scan the excitation and emission spectra of whole cell suspensions in 0.1 M potassium phosphate buffer pH 6.5. Spectra of cells expressing HXT2::GFP were normalized to cell density, and were corrected for background by subtraction of spectra determined for cells expressing HXT2 cultured under identical conditions. Emission spectra were collected between 500 and 550 nm, with excitation at 489 nm and excitation and emission slit widths of 3 nm. Preliminary experiments showed that the fluorescence signal at the emission peak for GFP F64L/S65T (509 nm; ref. 172) was essentially linear for cell suspensions between optical densities (A600) of 0.1 and 2. For the experiments on intracellular quenching of GFP fluorescence, spectra were recorded using an
Aminco DW2000 spectrophotometer and an SLM-Aminco Bowman series 2 luminescence spectrometer.

**Immunofluorescence**

Concentrated formaldehyde solution was directly added to the culture dropwise to a concentration of 3.7% (w/v) as described (ref. 177; Jos Grimbergen, personal communication). After 30 min at room temperature, the cells were recovered by centrifugation and resuspended in phosphate-buffered formaldehyde solution (100 mM potassium phosphate, pH 6.5, containing 0.5 mM MgCl$_2$ and 3.7% formaldehyde). After 2 h at room temperature, the fixed cells were washed twice in phosphate-buffered saline (PBS, 0.14 M NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, and 1.5 mM KH$_2$PO$_4$, pH 7.3) and once with solution A (100 mM KH$_2$PO$_4$ and K$_2$HPO$_4$, pH 7.5, 1.2 M sorbitol). Cells were resuspended in solution A containing 0.2% β-mercaptoethanol and 20 μg/ml zymolyase, and incubated 30 min at 37 °C for permeabilization. After digestion the cells were spun down at 6000 rpm for 20 seconds and washed 3 times with solution A. The pellets were resuspended in solution A at a suitable density before proceeding.

10 μl polylysine (1 mg ml$^{-1}$) was added to each well of a multiwell slide (Polysciences, Inc), aspirated off after 10 s and air-dried. The slide was rinsed in distilled water for 10 min and thoroughly air-dried.

10 μl permeabilized cells were placed in each well. After 10 s the liquid was aspirated off and air-dried. The attached cells were treated with 20 μl 0.5% Triton X-100–PBS in each well for 15 min at room temperature. The cells were then washed 3 times in PBS, 3 times with solution A and finally air-dried. The cells were incubated at room temperature in a moist environment (a Petri dish with a wet paper in it) for 45 min with 8-10 μl anti-GFP (rabbit serum) which was diluted 1:5000 with PBS containing 1 mg ml$^{-1}$ BSA (PBS-BSA). The anti-GFP antibody had undergone affinity purification on nitrocellulose according to Pringle (177) and was pretreated with denatured whole-cell extracts of RE102 before use to improve the specificity of binding.

The primary antibody was aspirated off, and the cells were washed 10 times with PBS-BSA containing 0.1% Tween-20. During these procedures the wells were not allowed to dry out.

Then 5 μl fluorophore-conjugated secondary antibody (donkey anti-rabbit serum/Cy3) diluted 1:2000 in PBS-BSA was immediately added to each well. The slide was incubated at room temperature in a moist environment avoiding light for 30 min. The wells were washed 10 times with PBS-BSA containing 0.1% Tween-20.
For Hxt2::T7 detection the cells were incubated with 1:1000 anti-T7 antibody and 1:2000 donkey anti-mouse/TRITC secondary antibody respectively.

After removing the wash solution, 5 µl mounting medium (1 mg/ml p-phenylenediamine dissolved in 1× PBS, pH 9, 90% (v/v) glycerol, and 22.5 ng ml⁻¹ DAPI) was immediately added to each well. The coverslip was sealed around the edges with nail polish. The slides can be stored at -20 °C in dark for more than one year without gross deterioration of immunofluorescence images.

**Immunoblotting**

Cells were harvested by centrifugation, washed once in 1% KCl, and extracted by abrasion with glass beads in buffer B (50 mM Tris-Cl pH 8, 10 mM EDTA, 5% glycerol plus protease inhibitors (1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin A, 0.2 mM AEBSF in DMSO)). The lysates were cleared by centrifugation at 1300 g for 3 min, and total membrane proteins were harvested by centrifugation at 100,000 g for 1 h at 4 °C. The membrane pellets were resuspended in buffer B, the protein concentration was determined, and the samples were diluted to 1 µg/µl in buffer B. An equal volume of loading buffer was added, then the samples were heated to 40 °C for 15 min. 5 µg protein was loaded in each lane and electrophoresed in a 10% SDS-PAGE minigel. The proteins were transferred to PVDF membrane using a mini trans-blot electrophoretic transfer cell (Bio-Rad) with buffer C (48 mM Tris, 39 mM glycine, 5% methanol, 0.05% SDS) at 200 V for 1 h at 4 °C. Membranes were incubated with gentle agitation in PBS + 0.2% Tween-20 as follows: (1) blocking for at least 1 h in 5% nonfat milk; (2) incubation with primary antibodies for 16 h at 4 °C in 1% nonfat milk, followed by 4×10 min washes; (3) incubation with horseradish peroxidase-conjugated secondary antibody for 1 h in 1% nonfat milk, followed by 3×5 min washes. The final wash was done without Tween-20 in the buffer. Immunodetection was carried out by chemiluminescence as described (201). The antibodies were diluted as follows: anti-Hxt2 and anti-GFP, 1/40,000; anti-T7, 1/10,000; and goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates, 1/10,000. Primary antibodies were routinely pretreated with denatured whole-cell extracts of RE102 before use.

**GFP purification**

Plasmid pRSETB::GFP-F64L/S65T was transformed into strain BL21(DE3). Fresh colonies were inoculated into LB-ampicillin medium and grown until the culture reached an OD₆₀₀ of 2.5. Cells were lysed by sonication and hexahistidine-tagged GFP was purified by nickel-chelate affinity chromatography as described (172). The purified protein was dialyzed against 0.1 M phosphate buffer pH 6.5. The molar extinction coefficient of GFP F64L/S65T at 489
nm is 55,000 M$^{-1}$ cm$^{-1}$ ± 5000 (172). Using a Hewlett Packard 8452A diode array spectrophotometer, the concentration of the GFP stock solution was determined to be 2.5 μM.

**Chemicals and reagents**

The anti-Hxt2 antibody has been described (239, 240). Anti-GFP antibody, Texas Red-conjugated concanavalin A (conA-Texas Red), and CMAC-Arg were from Molecular Probes. The anti-T7 antibody was from Novagen. Fluorophore-conjugated donkey anti-rabbit serum/Cy3 and donkey anti-mouse serum/TRITC were from Jackson. Electrophoresis and immunoblotting reagents were from Bio-Rad except for prestained protein molecular weight markers, which were from Fluka. Restriction enzyme Ascl and plasmid pNEB193 were from New England Biolabs. Maltose (M-5885, containing less than 0.3% glucose) was from Sigma. D-U-[14C] glucose was from Amersham. Custom oligonucleotides were synthesized by Life Technologies and by Pharmacia. Ni-NTA-agarose was from QIAgen. Bovine serum albumin (A 4503, prepared from Fraction V albumin, ≥96% albumin) was from Sigma. All other chemicals were of reagent grade.

**RESULTS**

**Hxt2 fused with GFP is a functional hexose transporter**

The hxt null strain KY73 was transformed with either single-copy or multi-copy plasmids containing the HXT2 gene or the chimeric HXT2::GFP or HXT2::T7 genes, or with empty vectors. The transformants were restreaked onto glucose medium and growth was assessed after 3 d. As shown in Figure 2.1 the chimeric genes restored growth on glucose to the hxt null strain to the same extent as the native HXT2 gene. Cells containing the empty vector were unable to form visible colonies.

Cells of the null strain carrying single-copy plasmids with HXT2, HXT2::GFP or the empty vector were grown overnight in maltose medium to mid-log phase and then shifted to low glucose medium. After 4 h the cells were harvested and zero-trans glucose transport was assayed. Data from a representative experiment are shown as an Eadie-Hofstee plot in Figure 2.2. When the results were fitted to a single-component transport system obeying Michaelis-Menten kinetics, a large deviation resulted. Fitting the results to a two-component system increased the statistical significance of the calculated transport parameters. The $K_m$ and $V_{max}$ values for a two-component system are presented in Figure 2.2. The kinetics of glucose transport conferred by HXT2 and HXT2::GFP were not markedly different, although the HXT2::GFP gene reproducibly resulted in cells with a slightly lower $K_m$ of the low-affinity component and higher $V_{max}$ values than those of the native HXT2 gene product.
Figure 2.1 *HXT2::GFP restores growth on glucose to the hxt null strain* Strain KY73 was transformed with (A) multi-copy plasmids pAG1-5 (*HXT2::GFP*), pAK125 (*HXT2::T7*), pAK1a (*HXT2*), or pRS426 (multi-copy vector), or (B) single-copy plasmids pAK145 (*HXT2*), pAK146 (*HXT2::GFP*), or YCplac33 (vector). Transformants were restreaked onto YNB medium containing casamino acids, tryptophan, and 2% glucose and photographed after 3 d.

Catalytic-center activity of the Hxt2::GFP glucose transporter

We used the fluorescent signal from Hxt2::GFP to determine the cellular concentration and catalytic-center activity of the fusion protein, using purified GFP as a fluorescent standard. We first examined the effect of intracellular components on GFP fluorescence. Light absorption and fluorescence emission spectra were recorded from suspensions of cells expressing *HXT2* or *HXT2::GFP*. The suspensions were then French-pressed in 50 mM Tris-HCl pH 8 (GFP fluorescence is maximal at pH 8; ref. 172) and spectra were recorded from the lysates. The fluorescence intensity measured in the lysate, when corrected for the decrease in light extinction at the excitation and emission wavelengths, corresponded with 102% of the emission from whole cells. Therefore we conclude that GFP fluorescence is not quenched by intracellular components.

In order to quantify the cellular abundance of the Hxt2::GFP molecules, the Hxt2 and Hxt2::GFP cell suspensions that were used for the glucose transport assays were diluted 1:10 in phosphate buffer to the same optical density, and fluorescence spectra were recorded. Then fluorescence spectra of the Hxt2 cell suspension were recorded at intervals during titration with purified GFP protein. The concentration of pure protein required to match the fluorescent signal of cells expressing Hxt2::GFP was 19.4 nM (n=3). The density of the cell suspension in the GFP titration was $8.5 \times 10^7$ cells ml$^{-1}$. Therefore each cell contained $1.4 \times 10^5$ Hxt2::GFP molecules.
The cellular abundance of the Hxt2::GFP chimera and the glucose transport activity mediated by that protein were used to calculate the catalytic-center activity for glucose of the transporter. The Hxt2::GFP cell suspension contained 0.61 mg (total cell protein) ml$^{-1}$, giving a relationship of 32 pmol Hxt2::GFP mg (total cell protein)$^{-1}$. The $V_{\text{max}}$ for glucose transport of these cells, 125 nmoles min$^{-1}$ mg (total cell protein)$^{-1}$ (Figure 2.2) was divided by this value, resulting in an estimate of 65 s$^{-1}$ for the catalytic-center activity of Hxt2::GFP. All of the Hxt2::GFP in the cells appeared to be at the plasma membrane when examined by fluorescence microscopy (not shown).

![Figure 2.2](image)

**Figure 2.2** HXT2::GFP confers high affinity glucose uptake on the hxt null strain
Strain KY73, transformed with pAK145, pAK146, or YCplac33 was grown overnight in maltose medium, and then shifted to low glucose medium. After 4 h the cells were harvested and assayed for $^{14}$C-glucose uptake. The results are shown as Eadie-Hofstee plots. ●, pAK146 (HXT2::GFP); ■, pAK145 (HXT2); ●, YCplac33 (vector only).

**Hxt2::GFP expression is glucose-repressed**

We initially observed that cells carrying the HXT2::GFP chimera were fluorescent, and that the fluorescence level was glucose dependent. The time course of expression of fluorescence reached a maximum 4 - 8 h after shifting cells to low glucose; 8 - 12 h after shifting cells to high glucose (data not shown). In subsequent experiments cells were analyzed 4 h after the medium shift unless otherwise indicated.

The level of Hxt2::GFP expression was assessed using fluorimetry and immunoblotting. Comparisons were made between uninduced and induced cultures of the hxt null strain and
the hxt2Δ strain RE102, each carrying single-copy or multi-copy plasmids. In the null strain (Figure 2.3A) the fluorescence due to Hxt2::GFP was significantly increased by growth in low glucose medium. This was seen for cells bearing HXT2::GFP on both single-copy and multi-copy plasmids. The absolute level of fluorescence was higher when the HXT2::GFP chimera was borne on the multi-copy vector. Similarly, the expression of fluorescence by HXT2::GFP in the hxt2Δ strain was higher when the cells were cultured in low glucose medium than in high glucose medium (Figure 2.3B). The expression level was also higher when the chimeric gene was present on a multi-copy plasmid. However, the absolute level of fluorescence was in all cases lower in the hxt2Δ strain than in the hxt null strain (compare Figure 2.3B with Figure 2.3A).

![Fluorescence emission spectra](image)

Figure 2.3 Fluorescence emission spectra of cells expressing Hxt2::GFP protein (A). Strain KY73 (hxt null). (B). Strain RE102 (hxt2Δ). The spectral traces are labeled as follows: MC, multi-copy plasmid (pAG1-5); SC, single-copy plasmid (pAK146); L, low glucose (0.1%); and H, high glucose (5%). Spectra were recorded as described in Materials and Methods.
In a separate experiment, the amount of Hxt2::GFP protein was monitored by immunoblotting (Figure 2.4A). Significantly more protein was expressed by cultivation in low glucose than in high glucose media. This was observed for the hxt null strain with multi-copy and single-copy HXT2::GFP. A similar expression pattern was observed with the hxt2Δ strain carrying multi-copy and single-copy HXT2::GFP plasmids. The relative expression levels were estimated for the hxt2Δ strain with single-copy HXT2::GFP and for the hxt null strain with multi-copy HXT2::GFP using quantitative immunoblotting (Figure 2.5A). The Hxt2::GFP level is more than 16-fold higher in induced cells than in uninduced cells of the hxt2Δ strain, and is approximately 16-fold higher in the hxt null strain.

![Figure 2.4 Western analysis of Hxt2::GFP and Hxt2 protein levels (A). Detection with anti-GFP antibody. Lanes: 1, vector pRS426 in RE102; 2, pAK1a (HXT2) in KY73; 3, pAK125 (HXT2::T7) in KY73; 4, molecular mass markers: 116, 84, 58, 48.5, 36.5, and 26.6 kDa; 5-12, HXT2::GFP; 5-8, KY73; 9-12, RE102; 5, 6, 9, 10, multi-copy vector; 7, 8, 11, 12, single-copy vector; 5, 7, 9, 11, high glucose; 6, 8, 10, 12, low glucose; 13, purified GFP protein. (B). Detection with anti-Hxt2 antibody. Lanes: 1, vector pRS426 in RE102; 2, pAG1-5 (HXT2::GFP) in KY73; 3, pAK125 (HXT2::T7) in KY73; 4, molecular mass markers as in (A); 5-12, HXT2; 5-8, KY73; 9-12, RE102; 5, 6, 9, 10, multi-copy vector; 7, 8, 11, 12, single-copy vector; 5, 7, 9, 11, high glucose; 6, 8, 10, 12, low glucose. 5 μg membrane protein was loaded in each lane.]
Cellular Hxt2 protein levels from the same culture conditions were assessed by immunoblotting (Figure 2.4B). As with Hxt2::GFP levels, the Hxt2 protein was considerably more abundant in low glucose cells than in high glucose; indeed, in the exposure shown, the Hxt2 protein could not be detected in the high glucose samples. Moreover, the Hxt2 protein was over-expressed in cells containing multi-copy $HXT2$. However, the levels of Hxt2 protein were similar in $hxt2A$ and $hxt$ null strains in each condition. The level of Hxt2 induction after shifting cells to low glucose was assessed by quantitative immunoblotting (Figure 2.5B). For the $hxt2A$ strain with single-copy $HXT2$ the amount of Hxt2 protein in induced cells was more than 16-fold higher than in repressed cells. For the null strain with multi-copy $HXT2$ the protein level was induced approximately 16-fold by glucose. Hxt2 protein was approximately two-fold more abundant in the null strain with multi-copy plasmid than in RE102 with the single-copy plasmid.

![Figure 2.5](image-url)

**Figure 2.5** Quantitative immunoblotting of Hxt2::GFP and Hxt2 (A). Detection of Hxt2::GFP with anti-GFP antibody. (B). Detection of Hxt2 with anti-Hxt2 antibody. 5 μg membrane protein from cells grown in high or low glucose was added in lanes H and L, respectively, and a two-fold dilution series of the low glucose extract was loaded in the subsequent four lanes. Molecular mass markers as in Figure 2.4A.
Maltose medium was used for preculture of the hxt null strain carrying HXT2 or HXT2::GFP plasmids, since it does not grow in high glucose medium. But both the GFP fluorescence emission levels and the immunoblot results showed that expression of Hxt2 and Hxt2::GFP was consistently higher in the hxt null strain than in the hxt2Δ strain (precultured in high glucose) after shifting them to high or low glucose media. We examined the possibility that the HXT2 and HXT2::GFP genes were up-regulated in the hxt null strain as a compensatory response to the absence of other functional HXT genes. The degree of repression of HXT2::GFP in maltose and high glucose media was compared in the hxt2Δ strain. Overnight pre-culture in maltose resulted in a six-fold higher basal level of fluorescence than pre-culture in high glucose (Figure 2.6A). After a shift to low glucose for 4 h, twofold more fluorescence was expressed by the maltose-pregrown cells than by the glucose-pregrown cells. Furthermore, maltose-pregrown cells had 50% more fluorescence when they were shifted to fresh maltose medium for 4 h than when they were shifted to high glucose medium (Figure 2.6B).

Figure 2.6 Fluorescence emission spectra of RE102 cells expressing multi-copy Hxt2::GFP (A). Precultures grown overnight on maltose or on high glucose. (B). Cultures grown for 4 h after shift: maltose preculture to low glucose, M→L; high glucose preculture to low glucose, H→L; maltose preculture to maltose, M→M; maltose preculture to high glucose, M→H; high glucose preculture to high glucose, H→H.
These results suggest that HXT2 is partially derepressed in cells grown in maltose medium. However, they do not discount the possibility that repression of HXT2 is also affected by the HXT genotype (and glucose transport capacity) of the host strain. In chapter 3, we describe a similar glucose-derepression phenomenon at low transport capacity in a strain expressing only Hxt7::GFP (based on strain RE607B). In chapter 5, we show that HXT7 is derepressed on high glucose in strains with reduced glucose transport capacity.

The anti-Hxt2 antibody was raised against a peptide corresponding to the carboxyl-terminal 13 residues and the terminal carboxylate group of Hxt2 (124, 240). We found that this antibody does not recognize the Hxt2::GFP fusion protein (Figure 2.4B, lane 2). This may be due to the absence of the native carboxylate group, since the anti-Hxt2 antibody also does not recognize an Hxt2::T7 fusion protein (Figure 2.4B, lane 3), even though this fusion protein is readily detected by the anti-T7 antibody (Figure 2.7).

![Figure 2.7 Western analysis of Hxt2::T7 and Hxt2 protein levels](image)

Figure 2.7 Western analysis of Hxt2::T7 and Hxt2 protein levels Detection with anti-T7 antibody. 5 μg membrane protein was added respectively in lane 1: Vector YCplac33; lane 2: pAK125 (HXT2::T7), high glucose; lane 3: pAK125 (HXT2::T7), low glucose; lane 4: pAK1a (HXT2), high glucose; lane 5: pAK1a (HXT2), low glucose; lane 6: pAG1-5 (HXT2::GFP), high glucose; lane 7: pAG1-5 (HXT2::GFP), low glucose; lane 8: pAK146 (HXT2::GFP), high glucose; lane 9: pAK146 (HXT2::GFP), low glucose. All of the plasmids were transformed into hxt null strain KY73. Molecular mass markers: 116, 84, 58, 48.5, 36.5, and 26.6 kDa.

Hxt2::GFP is localized to the plasma membrane

The location of Hxt2::GFP fusion protein within cells was visualized by immunofluorescence. pAG1-5 (Hxt2::GFP) in KY73 and pAK125 (Hxt2::T7) in KY73 were pre-cultured in maltose medium. After shifting to a low concentration of glucose for 4-5 h, the cells were harvested and prepared for immunofluorescence microscopy as described in Methods and Materials. Using fluorescence microscopy, Hxt2::GFP in hxt null strain was observed to give a very
strong immunofluorescent signal though the slide had been kept at -20°C for over one year (Figure 2.8A). We interpret this location to be the plasma membrane. The immunofluorescent signal was much stronger than the autofluorescence signal (Figure 2.8B). Hxt2::T7 was also detected by immunofluorescence in the plasma membrane (Figure 2.8C). As controls, Hxt2::GFP cells were stained with T7 antibody and Hxt2::T7 cells with GFP antibody respectively. In this case Hxt2::GFP cells showed Hxt2::GFP autofluorescence without an immunofluorescence signal. Neither autofluorescence nor an immunofluorescence signal was detected in Hxt2::T7 cells (data not shown).

Figure 2.8 Immunofluorescence of Hxt2::GFP (A). Immunofluorescence of pAG1-5 (HXT2::GFP) in KY73. Strain KY73, transformed with pAG1-5, was grown overnight in maltose medium and then shifted to low glucose medium. After 5 h the cells were harvested and assayed by immunofluorescence with 1:5000 anti-GFP and 1:2000 fluorochrome-conjugated donkey anti-rabbit serum/Cy3. (B). Autofluorescence of the cells used in A (GFP fluorescence). (C). Immunofluorescence of pAK125 (HXT2::T7) in KY73. Detection with 1:1000 anti-T7 antibody and 1:2000 donkey anti-mouse/TRITC second antibody.

Figure 2.9 Subcellular localization of Hxt2::GFP Cells of the hxt null strain (KY73), transformed with multi-copy HXT2::GFP (pAG1-5), were induced by growth for 4 h on low glucose. (A). fluorescence of Hxt2::GFP; (B). fluorescence of conA-Texas Red decorating the cell wall.
The distribution of the Hxt2::GFP fusion protein in living cells was examined in more detail by fluorescence microscopy. Strong cellular fluorescence was observed after induction; it was predominantly localized to the cell periphery, coincident with conA-Texas Red staining of the cell wall (Figure 2.9A, B). The signal in uninduced cells was very weak, and did not appear to be localized to the plasma membrane; the autofluorescence of cells expressing Hxt2 was nil (not shown). Cells containing multi-copy HXT2::GFP plasmids were more fluorescent than cells with single-copy plasmids; however, no differences in the distribution of the fluorescence were apparent as a result of over-expression of the protein (not shown).

Induced cells were treated with 5% glucose and examined after continued incubation. The fluorescent signal was gradually lost from the plasma membrane. Early in the incubation it appeared in punctate structures close to the plasma membrane (Figure 2.10A). With longer incubation it accumulated in single large globular structures (Figure 2.10B). To characterize this localization, glucose-treated cells were incubated with CMAC-Arg, a coumarin-based compound that upon hydrolysis by vacuolar peptidases becomes fluorescent and unable to permeate the vacuolar membrane. The GFP fluorescence in cells incubated for long periods with high glucose clearly colocalized with vacuoles stained by CMAC-Arg (Figure 2.10C), and was distinct from DAPI-stained mitochondria and nuclei (not shown).

The fates of Hxt2 and Hxt2::GFP protein pools after high glucose treatment were monitored over time by immunoblotting (Figure 2.11). The levels of the proteins were low (Hxt2::GFP)
to undetectable (Hxt2) in uninduced cells, and were significantly elevated after 4 h of induction. The level of Hxt2::GFP had returned to uninduced levels between 4 and 6 h after high glucose treatment, and the level of Hxt2 was below the detection limit after 30 min. No breakdown products specific for the high-glucose treated cells could be detected. Under these culture conditions the doubling time of the cells was approximately 2 h.

![Figure 2.11 Time course of Hxt2 inactivation](image)

Cultures of RE102 containing single-copy HXT2::GFP (panel A) or HXT2 (panel B), grown on maltose medium, were shifted to low glucose (time -4), and after 4 h (time zero) were supplemented with glucose to a final concentration of 5%. Samples were harvested at the times indicated (h). 10 μg membrane protein was loaded in each lane, and the Hxt2::GFP or Hxt2 proteins detected by immunoblotting. Molecular mass markers: 116, 84, 58, 48.5, and 36.5 kDa. No low molecular weight GFP was detected during breakdown.

It was occasionally observed that a proportion of cells in a culture (up to 5%) did not fluoresce, and that the fluorescence intensity varied from cell to cell. We do not know whether this is due to plasmid loss or to an impaired ability of some cells to respond to induction. Heterogeneity of GFP expression in yeast has been observed by others as well (160).

**Hxt2::GFP is transported via the secretory pathway**

The role of the secretory pathway in traffic of Hxt2::GFP to the plasma membrane was assessed by examining the distribution of the fusion protein in a strain that has a temperature-sensitive allele of the SEC6 gene. This gene is required for fusion of post-Golgi secretory vesicles with the plasma membrane. At the restrictive temperature in a sec6-4 strain the
secretory vesicles and their integral and lumenal proteins accumulate in the cytoplasm (162, 221). When Hxt2::GFP expression was induced in this strain grown at the permissive temperature the plasma membrane was clearly labeled by GFP (Figure 2.12A), although more fluorescence was seen in the cytoplasm of this strain than in SEC6 wildtype strains. When the fusion protein was expressed in cells maintained at the restrictive temperature of 37 °C the fluorescence developed to a similar level, but it was completely excluded from the plasma membrane. Instead it accumulated in globular bodies within the cell (Figure 2.12B).

The sec6-4 phenotype has been reported to be reversible; when cells are restored to the permissive temperature, a proportion of the invertase accumulated in the vesicles is secreted to the periplasm (237). After sec6-4 cells expressing Hxt2::GFP had been shifted from the restrictive temperature to the permissive temperature for 2 h, their plasma membranes were distinctly labeled by fluorescence (Figure 2.12C). This phenomenon was observed both in mother cells and in buds. Moreover, bright spots of GFP fluorescence were often seen in the vicinity of the bud neck. The labeling occurred even in the presence of the protein synthesis inhibitor cycloheximide. However, the fluorescent signal that had accumulated within the cells was only partially redistributed to the plasma membrane, even after 8 h of incubation at the permissive temperature and supplementation of the medium with 0.1% glucose (data not shown). The incorporation of Hxt2::GFP into the plasma membrane from secretory vesicles also occurred in the presence of high glucose: if cultures were treated with 5% glucose upon being shifted to the permissive temperature, a proportion of the GFP signal could be detected in the plasma membrane (data not shown).

Figure 2.12 Hxt2::GFP localization in a secretory mutant Cells of NY17 (sec6-1) containing pAG1-5 (multi-copy HXT2::GFP) were induced by growth in 0.1% glucose for 4 h, at the permissive temperature (panel A) or at the restrictive temperature (panel B). Cells induced at the restrictive temperature were treated with 1 µg ml⁻¹ cycloheximide and incubated at 24 °C for 2 h (panel C).
Endocytosis is involved in removal of Hxt2::GFP from the plasma membrane

Strains with the end3-l or end4-l alleles display temperature-sensitive defects in endocytosis (184). HXT2::GFP expression was induced for 4 h in strains RH1623 (end3-l) and RH1597 (end4-l) and in the wildtype strain RH1800 (END3 END4) at the permissive temperature. At this time all strains had strong GFP staining in the plasma membrane. Then the cultures were divided into four aliquots. Two were maintained at the permissive temperature and two were shifted to the restrictive temperature of 37 °C. One of the aliquots at each temperature was supplemented with 5% glucose. The cultures were examined periodically by fluorescence microscopy. After 2 h the end3 and end4 strains maintained at the permissive temperature in low glucose had strong fluorescent staining of the plasma membrane, but in high glucose had accumulated a large proportion of their fluorescence in internal structures (not shown). At the restrictive temperature both the low- (not shown) and high-glucose treated mutant cells (Figure 2.13A, B) had strong fluorescent staining of the plasma membrane. In the wildtype strain high glucose stimulated internalization of the fluorescence from the plasma membrane to the vacuole (Figure 2.13C); this internalization was more rapid at 37 °C (not shown). The retention of Hxt2::GFP in the plasma membrane of end3 and end4 cells persisted with prolonged incubation at the restrictive temperature, at which time all of the fluorescence in wildtype cells had disappeared from the plasma membrane (data not shown).

![Figure 2.13](image_url)

Figure 2.13 Hxt2::GFP is retained in the plasma membrane of cells blocked in endocytosis Cells induced by low glucose treatment at the permissive temperature were treated with 5% glucose and incubated at the restrictive temperature for 4 h. (A), RH1800 (END3 END4); (B), RH1623 (end3-l); (C), RH1597 (end4-l).

The fate of the HXT2::GFP fusion protein in single living cells was monitored over time. Cells of HXT2::GFP in hxt2Δ strain RE102 from repressing medium were resuspended in low
glucose medium plus low-melt agarose, and a single cell was monitored by epifluorescence microscopy (Figure 2.14A). In these inducing conditions Hxt2::GFP was expressed and targeted to the plasma membrane. Strong cellular fluorescence was observed after inducing for 60 min especially in the vicinity of the bud neck (Figure 2.14A). In a separate experiment, cells expressing Hxt2::GFP in the plasma membrane were resuspended in 5% glucose medium plus low-melt agarose, and a single cell was monitored. Under these conditions, HXT2::GFP expression ceased and the fusion protein was removed from the plasma membrane during 5-30 min. The fluorescent signal was redistributed to the vacuole and gradually declined during 90 min. By 21 h, the most of fluorescent signal had disappeared (Figure 2.14B).

Figure 2.14 Time course of Hxt2::GFP expression in living cells Single cells of strain RE102 (hxt2Δ) transformed with ppAK146 (HXT2::GFP) were pre-cultured in repressing or inducing media. (A). Cells from repressing medium were resuspended in low-glucose medium plus low-melt agarose and photographed periodically as indicated; a representative cell is shown. (B). Cells from inducing medium were resuspended in high-glucose medium plus low-melt agarose and photographed periodically as indicated; a representative cell is shown.
DISCUSSION

Previous studies have concluded that the kinetic parameters of Hxt2 can be modulated in response to the growth conditions. An HXT2-only strain grown on 2% glucose into the early stationary phase showed monophasic glucose transport kinetics with a $K_m$ of about 15 mM (210). Moreover, Reifenberger et al. (185) reported that the $K_m$ of Hxt2 was about 10 mM after cell growth on high glucose concentrations but appeared to be biphasic with a high-affinity component ($K_m = 1.5$ mM) and a low-affinity component ($K_m = 60$ mM) after growth on low concentrations of glucose. Our results, fitting a two-component system, demonstrate that Hxt2 in an HXT2-only strain does indeed display special transport kinetics.

We have shown that a chimera between the Hxt2 glucose transporter of *S. cerevisiae* and the green fluorescent protein is a functional transport protein with a glucose transport capacity similar to the wildtype protein. As a solute transporter, Hxt2 is inferred to be targeted to the plasma membrane, and indeed it has been identified in the plasma membrane fraction of cell lysates (240). Our results are in agreement: the Hxt2::GFP protein in induced cells was strongly localized at the plasma membrane.

By quantifying the emission of GFP in the fusion protein we were able to determine a value for the catalytic-center activity of Hxt2::GFP *in vivo*. We estimate that under inducing conditions each hxt null cell transformed with single-copy HXT2::GFP has $1.4 \times 10^5$ Hxt2 molecules in the plasma membrane. Based on the $V_{\text{max}}$ determined from a single-component transport system, the catalytic-center activity of the transporter at 30 °C is 53 s$^{-1}$ (127). According to the calculated transport parameters for a two-component system, the catalytic-center activity of the transporter at 30 °C is 65 s$^{-1}$. To our knowledge this is the first empirical estimate of a catalytic-center activity for a yeast hexose transport protein. It assumes that all Hxt2::GFP molecules were actively transporting glucose, and is therefore a minimum estimate. Catalytic-center activities for other solute transporters have been estimated *in vivo* using ligand binding and transport velocity measurements. For example, the catalytic-center activities of the GLUT1 and GLUT3 human glucose transporters at 37 °C have been calculated to be 123 and 853 s$^{-1}$, respectively (142). The catalytic-center activity of the yeast purine-cytosine permease is approximately 1 s$^{-1}$ (31). The phosphate/triose phosphate translocator of the chloroplast envelope has a catalytic-center activity *in vivo* of 83 s$^{-1}$ at 20 °C (64), the phosphate/H$^+$ cotransporter of heart mitochondria has a catalytic-center activity of 350-1000 s$^{-1}$ at 25 °C (252), and the ADP/ATP translocase of heart mitochondria has a catalytic-center activity of 8-10 s$^{-1}$ at 18 °C (117). Serrano (203) suggested that the number of hexose transporters in an actively fermenting wildtype yeast cell is $10^5$-$10^6$, and from the measured fermentative capacity of yeast he calculated the catalytic-center activity of the
hexose transporters to be 10-100 s⁻¹. Using a fluorescent non-transported glucose analog, N-dansyl-D-glucosamine, Kotyk and co-workers estimated that there are up to 10⁶ glucose transporters per yeast cell (209). Thus the values that we have determined for Hxt2 are in general agreement with those in the literature for similar proteins. It will be interesting to perform comparable measurements and calculations with other yeast sugar transporters such as the physiologically important Hxt7 protein.

We found that expression of Hxt2 and Hxt2::GFP was consistently higher in the hxt null strain (KY73) than in the hxt²Δ strain (RE102). Various factors may contribute to this phenomenon: 1) In RE102 many other HXT genes can be expressed besides the HXT2 gene, while in the hxt null strain transformed with HXT2 or HXT2::GFP, the (chimeric) Hxt2 protein is the only glucose carrier available. The presence of other HXT genes may have some repressive effects on the expression of the HXT2 genes in RE102/HXT2::GFP and RE102/HXT2 strains. 2) Maltose causes some induction of HXT2 and HXT2::GFP expression when the strain is pre-cultured in maltose medium, and this increased expression may have some effect on the level of Hxt2 also after removal of the maltose. 3) Snf3 is a low glucose sensor. But what is the glucose sensor in a strain, which expresses only a single HXT gene at high glucose concentrations? Possibly Snf3 senses internal glucose or another intermediate of glycolysis. The transport capacity of the hxt null strain expressing Hxt2::GFP is lower than that in wild-type strain. Snf3 may trigger more HXT2 or HXT2::GFP expression in KY73 than in RE102 due to reduced levels of internal glucose or glycolytic intermediates. In chapter 3 and chapter 5 this hypothesis will be discussed further.

The canonical secretory pathway carries secreted proteins such as invertase and α-factor to the periplasm and other proteins such as the Pma1 ATPase and solute transporters to the plasma membrane; the SEC1 and SEC6 genes act at a late step in this pathway (97, 162, 221). It has previously been shown that Gal2 (222) and high-affinity glucose transport activity (8) reach the plasma membrane via this pathway and require SEC1 for functional expression at the plasma membrane. We show that SEC6 is involved in delivering Hxt2::GFP to the membrane. We found that Hxt2::GFP that had accumulated in secretory vesicles at the restrictive temperature in a sec6-4 strain was able to reach the plasma membrane when the cells were shifted to the permissive temperature. Plasma membrane targeting at the permissive temperature occurred even in the presence of a high concentration of glucose, demonstrating that HXT2 gene expression, but not outward trafficking of the Hxt2 protein, is under glucose control. These observations agree with those of Bisson (8) who found that high glucose did not prevent the expression of high-affinity glucose transport activity after it had been blocked in a sec1-1 mutant at the restrictive temperature.
Under high glucose conditions the hexose transporters Hxt6/7 are rapidly internalized via endocytosis and transported to the vacuole by vesicular carriers (122). Our results are similar: following treatment with high glucose, Hxt2::GFP expression ceases and the protein is removed from the plasma membrane by endocytosis. The END3 and END4 gene products are required for internalization, and the destination of the protein is the vacuole. It has been recognized that this pathway acts to internalize and inactivate a number of plasma membrane proteins, such as transporters of galactose (99), maltose (188), inositol (129), amino acids (89), nucleobases (230), drugs (52) and mating pheromones (7, 120), as well as receptors for the mating pheromones (33). We have been able to discern Hxt2::GFP-containing structures that are formed at an early stage of this process; these might be endocytic vesicles. We have also observed that these structures disappear once the fluorescence becomes localized in the vacuole.

In cells that are competent for endocytosis, Hxt2::GFP fluorescence declines markedly after prolonged growth of low-glucose cells, or after treatment with high glucose. When growth is arrested due to glucose limitation or temperature sensitivity, it is clear that this decline is not due to dilution of the GFP chromophore into new biomass but to degradation. This is in contrast with reports that describe the GFP chromophore as protease resistant (26). Sensitivity of the GFP chromophore to vacuolar proteases is advantageous for the use of GFP to tag membrane proteins, because it allows the fluorescent signal to be “chased” by vacuolar targeting of the fusion protein.

The trafficking of hexose transport proteins in the yeast cell is sketched in Figure 2.15. Under inducing conditions hexose transporters are transcribed in the nucleus, are translated at the endoplasmic reticulum, and are delivered via the Golgi apparatus and secretory vesicles to the plasma membrane. Then, the hexose transport proteins are removed via endocytosis and degraded in the vacuole. Transcription of HXT genes encoding hexose transporters is tightly controlled by extracellular glucose concentrations. The expressed Hxt transporters in the plasma membrane transport extracellular glucose into the cells. The transport activity is regulated by metabolic signals such as the intracellular glucose concentration (216). As for hexose transport protein sorting, we presume that under given conditions the hexose transporter HxtA is expressed and targeted to the plasma membrane, while HxtB is being endocytosed and degraded in the vacuole; HxtC may be retained in an intracellular pool for rapid recruitment upon a change of conditions. The formation of (hetero)dimers is also possible.

Fusions of GFP to yeast hexose transporters will be useful in addressing some of the outstanding questions about this family of proteins. By taking advantage of alleles of GFP
Hexose transporters are transcribed in the nucleus, translated at the endoplasmic reticulum, and delivered via the Golgi to the plasma membrane. Then they are internalized via endocytosis and transported to the vacuole by vesicular carriers for degradation. It is presumed that the hexose transporter HxtA is synthesized and targeted to the plasma membrane, while HxtB is being endocytosed and degraded in the vacuole; HxtC is retained in an intracellular pool for rapid recruitment upon a change of conditions.

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