Chapter 2

HXT5 expression is determined by growth rates in *Saccharomyces cerevisiae*

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Abstract

In the yeast *Saccharomyces cerevisiae* hexose transporter (Hxt) proteins transport glucose across the plasma membrane. The Hxt proteins are encoded by a multigene family with 20 members, of which Hxt1-4p and Hxt6-7p are the major hexose transporters. The remaining Hxt proteins have other or unknown functions. In this study, expression of *HXT5* under different experimental set-ups is determined. In glucose-grown batch cultures, *HXT5* is expressed prior to glucose depletion. Independent of the carbon source used in batch cultures, *HXT5* is expressed after 24 hours of growth and during growth on ethanol or glycerol, which indicates that growth on glucose is not necessary for expression of *HXT5*. Increasing the temperature or osmolarity of the growth medium also induces expression of *HXT5*. In fed-batch cultures, expression of *HXT5* is only observed at low glucose consumption rates, independent of the extracellular glucose concentration. The only common parameter in these experiments is that an increase of *HXT5* expression is accompanied by a decrease of the growth rate of cells. To determine whether *HXT5* expression is determined by the growth rate, cells were grown in a nitrogen-limited continuous culture, which enables modulation of only the growth rate of cells. Indeed, *HXT5* is expressed only at low dilution rates. Therefore, our results indicate that expression of *HXT5* is regulated by growth rates of cells, rather than by extracellular glucose concentrations, as is the case for the major *HXTs*. A possible function for Hxt5p and factors responsible for increased expression of *HXT5* upon low growth rates will be discussed.

Introduction

The initial step in glucose metabolism is uptake of glucose, which is carried out by specific hexose transporter (Hxt) proteins that are localized in the plasma membrane (1,2,3,4). Uptake of glucose is mediated by facilitated diffusion. A multigene family of 20 genes, which encode different putative *HXTs*, namely *HXT1-HXT17, GAL2, SNF3* and *RGT2*, has been identified. *HXT1-4* and *HXT6-7* encode the major hexose transporters, because expression of each of these genes in the MC996 background strain deleted for *HXT1-7* allows growth on glucose (5). Overexpression of *HXT8-17* (except *HXT12)* individually in an *hxt1-17 gal2* deletion strain in the CEN.PK

background restored growth on glucose, indicating that Hxt8-11p and Hxt13-17p are also able to transport glucose (6), but expression is low under normal growth conditions (4). Recently, it was shown that Hxt5p also has glucose transport capacity (7). Expression of the major *HXTs* is mainly regulated by extracellular glucose concentrations (4). *SNF3* and *RGT2* encode plasma membrane proteins that are involved in sensing the amount of glucose to induce expression of specific *HXTs* (8,9).

HXTs encode highly homologous proteins with 12 putative transmembrane segments, with amino- and carboxy-terminal domains localized in the cytoplasm (2). The intracellular amino-terminal domains of Hxt proteins show little homology amongst each other in contrast to the remaining domains. The *HXT5* gene encodes a protein of 592 amino acids, which is approximately 20 amino acids larger compared to the major hexose transporters. The intracellular amino-terminal domains of Hxt1p, Hxt6p and Hxt7p contain 59 amino acids, those of Hxt2p, Hxt3p and Hxt4p 50, 56 and 65 amino acids respectively, but the amino-terminal intracellular domain of Hxt5p is 82 amino acids. Because Hxt5p has a longer amino-terminal domain, it is tempting to speculate that Hxt5p may have a specific function in addition to glucose transport in yeast. Furthermore, *HXT5* has a different expression pattern compared to the major hexose transporters, as determined by DNA micro array experiments. In glucose-grown batch culture experiments, expression of *HXT5* was highly induced upon glucose depletion (10), which was confirmed at the protein level by monitoring expression of Hxt5-GFP (7). Other studies indicated that expression of *HXT5* is induced by increasing the osmolarity of the growth medium after addition of NaCl or sorbitol (11,12,13,14), or by increasing the temperature (11). Increased expression of *HXT5* upon glucose depletion, temperature and osmotic up-shift suggests a specific role for Hxt5p in adaptation of cells to these new conditions. To test whether Hxt5p was essential for growth, *HXT5* was deleted, which did not result in a clear phenotype. Inoculation of stationary phase cells, which normally would have expressed *HXT5*, into fresh medium containing high levels of glucose, resulted in slightly slower growth of the *HXT5* deletion strain compared to wild type cells (7).

In this study we determined the expression of the hexose transporter *HXT5* to obtain insight in the regulation of expression and to obtain clues about a possible function for Hxt5p in addition to glucose transport. *HXT5* expression was determined under different conditions, including batch growth, fed-batch growth and well-defined growth conditions in continuous cultures. It was shown that expression of *HXT5* mRNA

and Hxt5p is not regulated by extracellular glucose concentrations, as is the case for major Hxt proteins, but merely by the growth rates of cells. Based on the unique expression profile of *HXT5* and the presence of an extended amino-terminal domain, a possible function for Hxt5p will be discussed.

Materials and Methods

Strains, media and growth conditions

The *Saccharomyces cerevisiae* strains used in this study include CEN.PK 113-7D (MATa, *SUC2, MAL2-8^c*) and was kindly provided by P. Kötter (Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany), KY98 (MATa, *SUC2, MAL2-8^c, HXT5::GFP*) by A. Kruckeberg (E. C. Slater Institute, University of Amsterdam, Amsterdam, The Netherlands) and strain JBY20 (MATa, *SUC2, MAL2-8^c, ura3, HXT5::HA*) by J. Becker (Institut für Mikrobiologie, Heinrich-Heine-Universität, Düsseldorf, Germany). During batch culture experiments yeast cells were grown on 0.67% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) of the carbon source as indicated in the text. Cells were grown at 30ºC at 180 rpm in a shaking incubator (New Brunswick Scientific). For temperature up-shift experiments cells were grown to an OD_{600} of 1.2 (± 0.1). The culture was subdivided into 15 ml falcon tubes and incubated in a water bath of 42ºC, while shaking the tubes every 10 minutes. For the osmotic up-shift experiments the cells were also grown to an OD_{600} of 1.2 (± 0.1) and concentrated 5 M NaCl was added to a final concentration of 0.7 M. Samples were directly frozen in liquid nitrogen and stored at –80ºC.

Fed-batch growth conditions

Experiments with synchronous cultures in fed-batch experiments were performed as described earlier, with some modifications (15). Cells were grown in YNB medium without amino acids with glucose as carbon source at a cell density of $1.2x10^7$ cells.ml^{-1}, and an initial extracellular glucose concentration of 0.05 mM. Glucose dissolved in YNB medium was continuously added at rates of 10 fmol and 50 fmol cell^{-1}.h^{-1} respectively. The number of cells and the external glucose concentration was monitored throughout the experiment.

Cell synchronization

Centrifugal elutriation was performed as described previously, with some modifications (16). Cells were grown until the exponential phase in YNB medium containing 1% galactose at 30ºC. $2x10^{10}$ cells were loaded in a 40 ml chamber of a Beckman J-6MI centrifuge with a JE-5.0 rotor at 30ºC and 2000 rpm. Cells were grown in the elutriation chamber on YNB 1% galactose medium. Newborn daughter cells were collected on ice, centrifuged and kept overnight on ice in YNB 1% galactose medium. The cell size was monitored with a Coulter Multizer II, and the flow rate of the elutriation was adapted to maintain a constant cell size.

Continuous culturing

Cells were grown in a 2 L BiofloIII chemostat (New Brunswick Scientific) connected to a computer controller unit with Advanced Fermentation Software (New Brunswick Scientific). Cells were inoculated in the EGLI culture medium as described previously (17) and continuous feed was connected after overnight batch growth. During the different dilution rates, the NH_4^+ concentration was adapted to 1.5 $g.l^{-1}$ to maintain nitrogen limitation, the glucose concentration in the feed was 200 mM. Steady state samples were taken as described (17).

Glucose concentration

Extracellular glucose concentrations were determined as described (18). Samples were mixed with hexokinase/glucose-6-phosphate dehydrogenase (Boehringer Mannheim), $NADP^+$ (Roche) in a 100 mM imidazole, 10 mM $MgCl_2$ buffer pH 7.0 and the conversion of $NADP^+$ into NADPH was determined using a spectrophotometer (Pharmacia Biotech).

Northern blot analysis

To isolate total RNA, yeast cells were broken with 0.45 mm glass beads in a Bead Beater (Biospec Products Inc.) in phenol and RNA extraction buffer (1 mM EDTA, 100 mM LiCl, 100 mM Tris-HCl pH 7.5, 0.5% (w/v) lithium dodecylsulfide). A phenol/chloroform extraction was performed, and total RNA was precipitated by adding 3 M NaAc pH 5.6. Samples were washed with ethanol, air-dried and suspended in DMPC-treated water. Equal amounts of total RNA (10 µg) were loaded on a 1% denaturing formamide/formaldehyde gel and RNA was separated by electrophoresis.

RNA was transferred to Hybond-N membrane (Amersham Pharmacia Biotech) and cross-linked using UV light in a UV stratalinker (Stratagene). 15 pmol of *HXT5*-specific oligonucleotides (5'-TCCCAAGGGGCCTTGATGAGCGTT-3') was labelled with T4 polynucleotide kinase (USB) and 50 μCi γ-^{32}P-ATP (Amersham Pharmacia Biotech), and purified using the QIAquick nucleotide removal kit (Qiagen). The blots were washed once in 2x SSC at room temperature, incubated for prehybridisation in hybridisation mixture (1 mM EDTA, 7% SDS, 0.5 M $NaPO_4$ pH 7.5) for at least one hour at 45ºC in a micro-4 hybridization oven (Biozym). Labelled oligonucleotides were added and hybridised overnight at 45ºC. After hybridisation the blots were washed for two minutes in 2x SSC at room temperature, twice for 20 minutes in 2x SSC, 0.1% SDS, 0.1% NaPPi at 45ºC, and once for 20 minutes with 0.5x SSC, 0.1% SDS, 0.1% NaPPi at 45ºC. After a final wash for 10 minutes in 2x SSC at room temperature, the membrane was wrapped in Saran (Dow Chemicals), and autoradiograms were developed using hyperfilm MP (Amersham). To control whether equal amounts of RNA was loaded, the gels were checked for ethidium bromide staining by UV light and the membranes were probed with an oligonucleotide against *ACT1* (5'-TGTCTTGGTCTACCGACGATAGATGGGAAG-3').

Western blot analysis

JBY20 cells were collected in Falcon tubes, frozen immediately in liquid nitrogen and stored at –80ºC. The cells were thawed on ice, collected by centrifugation, washed in ice-cold water, and resuspended in PBS containing Complete protease inhibitors (Boehringer). Equal amounts of cells were lysed by shaking vigorously with 0.45 mm glass beads in a Bead Beater. Equal amounts of protein were loaded on a 10% SDS-PAGE gel, and transferred to PVDF membrane (Roche) after electrophoresis. The membranes were blocked in 5% Protifar (Nutricia) in TBST buffer (50 mM Tris-HCl pH 7.4, 10 mM NaCl and 0.1% Tween20) for 1 hour at room temperature. The membranes were incubated with 12CA5 antibody (Roche) with TBST/0.5% Protifar for one hour at room temperature. The primary antibody was detected using peroxide-conjugated rabbit anti-mouse (Jackson Immunoresearch). Proteins were visualized by Enhanced Chemoluminescence (Renaissance).

Results

Expression of *HXT5* mRNA and Hxt5 protein in glucose-grown batch cultures

To determine whether *HXT5* expression was related to the extracellular glucose concentration, overnight-grown JBY20 cells were inoculated into fresh medium containing 2% glucose and grown in batch cultures. In JBY20 cells, *HXT5* mRNA expression was induced 9 hours after inoculation (Figure 1a). Hxt5-HA protein was expressed after 9 hours after inoculation (Figure 1b). Yeast cells had different growth rates during growth on glucose in batch cultures (Figure 1c).

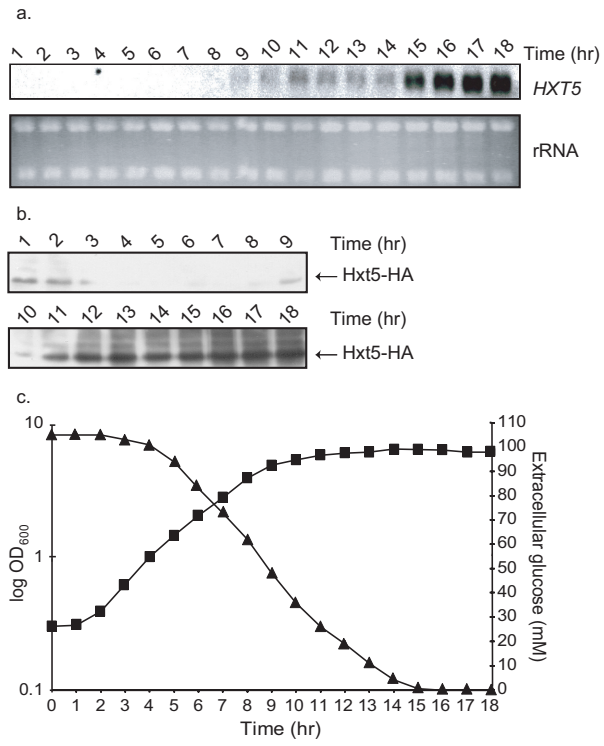

Figure 1: Expression of *HXT5* during growth on glucose in batch cultures. Strain JBY20 was grown on YNB 2% glucose in batch cultures. At the indicated time points after inoculation cells were harvested, RNA and proteins were extracted and the extracellular glucose concentration was measured. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. (c) Optical density at 600 nm (■) and extracellular glucose concentrations (▲). Results shown are representative of at least three different experiments.

JBY20 cells exhibited fast growth during the exponential growth phase (μ=0.36 h^{-1}) and slow growth upon entry in the stationary phase approximately 9 hours after inoculation (μ=0.028 h^{-1}). No *HXT5* mRNA or Hxt5-HA was expressed during the

exponential growth phase. Expression of Hxt5-HA was observed in slowly growing cells until 2 hours after inoculation. This was probably a remainder of the Hxt5-HA that was expressed in the overnight-grown cells used for inoculation, and probably not the result of *de novo* synthesis, because *HXT5* mRNA was not expressed at these time points. Furthermore, JBY20 cells grown exponentially overnight did not show expression of Hxt5-HA at the same optical densities (data not shown).

To establish whether the increased expression was related to the extracellular glucose concentration, extracellular glucose levels in the medium were measured. Extracellular glucose levels decreased, starting from 105 mM at inoculation and decreasing to 0.55 mM after 15 hours of growth, and to 0.03 mM after 18 hours of growth (Figure 1c). *HXT5* expression was already initiated 9 hours after inoculation when still 48 mM glucose was present. However, when glucose was depleted (<1 mM) 15 hours after inoculation, *HXT5* expression was maximally induced. However, Hxt5-HA expression remained constant from approximately 12 hours after inoculation. Therefore, regulation of *HXT5* expression by glucose alone seems unlikely, but a parameter that correlated with increased expression of *HXT5* after 9 hours of growth was a decrease in growth rate of the cells.

Expression of *HXT5* on different carbon sources

To investigate whether *HXT5* expression was dependent on growth on glucose, CEN.PK 113-7D cells were grown in batch cultures on YNB media containing different carbon sources. On easily fermentable carbon sources, including glucose, galactose, fructose, mannose, sucrose and maltose, cells exhibited high growth rates during the exponential phase of cell growth. Cells growing on non-easily fermentable carbon sources, i.e. ethanol and glycerol, exhibited low growth rates during the exponential phase of cell growth (Table 1).

Carbon source	μ (h^{-1}) exponential growth
Glucose	0.35
Galactose	0.19
Fructose	0.34
Mannose	0.30
Ethanol	0.10
Sucrose	0.33
Maltose	0.26
Glycerol	0.01

Table 1: Growth rates of CEN.PK 113-7D cells growing exponentially on YNB medium with 2% of different carbon sources in batch cultures.

Independently of the carbon source that was used, *HXT5* was expressed after 24 hours of growth when cells entered the stationary phase and exhibited low growth rates (Figure 2). Cells growing exponentially 6 hours after inoculation on medium containing the carbon sources glucose, galactose, fructose, mannose, sucrose and maltose did not express *HXT5*. Expression of *HXT5* was observed during the exponential phase of cell growth, when cells were grown on ethanol or glycerol (Figure 2). These results indicate that glucose is not a determining factor in the regulation of *HXT5* expression, but a decrease in growth due to the carbon source was accompanied by induction of *HXT5* expression.

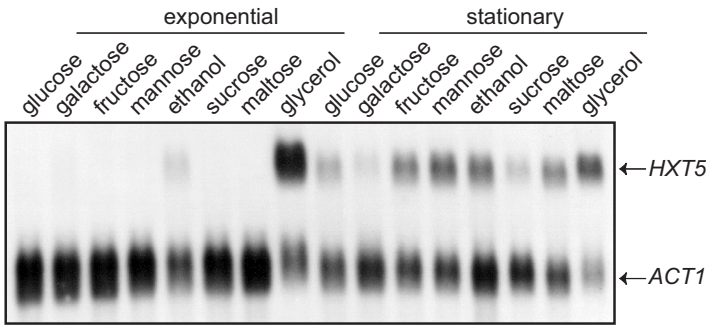

Figure 2: Expression of *HXT5* mRNA during the exponential and stationary phase of growth on different carbon sources. Strain CEN.PK 113-7D was grown on YNB medium containing 2% of each different carbon source indicated. During the exponential and stationary phase of batch growth cells were harvested and mRNA was extracted. Subsequently, expression of *HXT5* mRNA was determined by Northern blot analysis. Results shown are representative of at least three different experiments.

Expression of *HXT5* during environmental changes leading to low growth rates

Changing growth conditions by increasing the temperature or osmolarity creates conditions that influence the growth rate of cells. When the temperature of the medium of cells growing exponentially on YNB 2% glucose was changed from 30ºC to 42ºC over a 90-minute-period, growth rates decreased from μ=0.36 h^{-1} to μ=0.14 h^{-1}. Cells growing exponentially at 30ºC did not express *HXT5*. Increasing the temperature to 42ºC induced expression of *HXT5* (Figure 3a). To determine whether expression of

Hxt5 protein followed the expression pattern of *HXT5* mRNA, expression of Hxt5-HA during temperature up-shift was determined. Hxt5-HA was expressed 20 minutes after temperature up-shift and was present throughout the treatment (Figure 3b). The extracellular glucose concentration remained higher than 60 mM, indicating that increased expression of *HXT5* was not a result of glucose depletion.

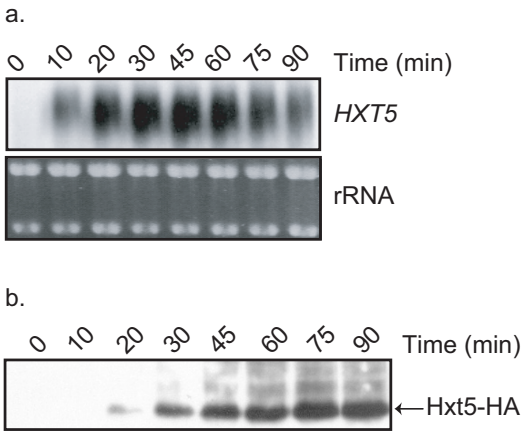

Figure 3: Expression of *HXT5* upon temperature up-shift to 42ºC. CEN.PK 113-7D cells and JBY20 cells were grown at 30ºC on YNB 2% glucose in batch cultures until the exponential phase. Then, the temperature of the medium was increased to 42ºC and at the indicated time points culture samples were harvested. mRNA was extracted from CEN.PK 113-7D cells and proteins were extracted from JBY20 cells. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. Experiments were performed at least three time and similar results were obtained: representative blots are shown.

Stress and hence a lower growth rate was also introduced by adding NaCl to a final concentration of 0.7 M to cells growing exponentially on YNB 2% glucose (μ=0.35 h^{-1} for the non-treated and μ=0.25 h^{-1} for the treated cells). This switch in external conditions was accompanied by a transient expression of *HXT5* (Figure 4a). This transient induction is not due to glucose limitation, as the glucose concentration remained higher than 60 mM throughout the experiment. During the switch of external conditions the pattern on the Hxt5-HA protein level was different from that of *HXT5* mRNA, as the protein was present throughout the experiment, and *HXT5* mRNA was

expressed transiently (Figure 4b). Again, the results of these experiments indicate that a decrease in the growth rate of cells correlates with increased expression of *HXT5*.

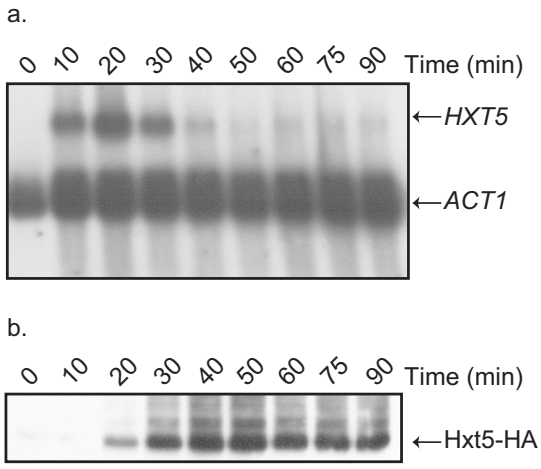

Figure 4: Expression of *HXT5* after increasing the osmolarity to 0.7 M NaCl. CEN.PK 113-7D cells and JBY20 cells were grown on YNB 2% glucose in batch cultures until the exponential phase. The osmolarity of the medium was increased to 0.7 M NaCl by adding a concentrated solution of NaCl and at the indicated time points culture samples were harvested. mRNA was extracted from CEN.PK 113-7D cells and proteins were extracted from JBY20 cells. (a) Northern blot analysis of *HXT5* mRNA. (b) Western blot analysis of Hxt5-HA. Experiments were performed at least three time and similar results were obtained: representative blots are shown.

Expression of *HXT5* during low growth rates in fed-batch cultures

To study the effect of G_1 phase elongation, and hence low growth rates, on expression of *HXT5*, Hxt5-GFP tagged cells that were synchronized early in the G_1 phase, were grown in fed-batch cultures. Low growth rates were induced by growing the cells on 10 fmol glucose $cell^{-1}.h^{-1}$, while 50 fmol glucose $cell^{-1}.h^{-1}$ was used to induce high growth rates (15). At a consumption rate of 10 fmol glucose $cell^{-1}.h^{-1}$, *HXT5* mRNA was detected. No *HXT5* mRNA was detected at a consumption rate of 50 fmol glucose $cell^{-1}.h^{-1}$ (Figure 5a). Fluorescence studies confirmed that at the consumption rate of 10 fmol glucose $cell^{-1}.h^{-1}$, Hxt5-GFP was incorporated in the plasma membrane within two hours, whereas at the consumption rate of 50 fmol glucose $cell^{-1}.h^{-1}$ even after eight hours of growth no fluorescence was observed (data not shown).

Even under these conditions the external glucose concentration did not have a regulatory function in *HXT5* expression. During the experiment extracellular glucose levels of the culture growing on 10 fmol glucose cell^{-1}.h^{-1} remained at a concentration of approximately 0.1 mM (Figure 5b). The fast growing cells with a consumption rate of 50 fmol glucose cell^{-1}.h^{-1} initially have higher extracellular glucose levels, varying between 0.2 and 0.4 mM. After 5 hours of growth, the extracellular glucose concentration diminishes to between 0.1 and 0.15 mM. These concentrations were also found in the culture growing at a consumption rate of 10 fmol glucose cell^{-1}.h^{-1} (Figure 5b). The cells growing at higher growth rates did not express *HXT5* and Hxt5-GFP after 5 hours, even when the extracellular glucose concentration is comparable to concentrations of the slowly growing culture. During slow growth in fed-batch cultures the parameter that correlated with increased expression of *HXT5* was again a decrease of the growth rate, and the growth rate *per se* might therefore regulate the expression of *HXT5*.

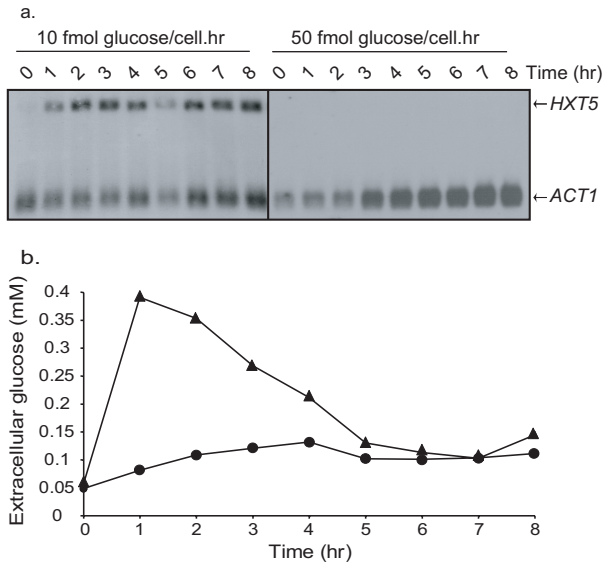

Figure 5. Expression of *HXT5* in synchronized cells in fed-batch cultures. KY98 cells were synchronized by elutriation and grown at either 10 or 50 fmol glucose cell^{-1}.h^{-1} in fed-batch cultures. At the indicated time points samples were taken and examined for *HXT5* expression and residual glucose concentration. (a) Northern blot analysis of *HXT5* mRNA. (b) Extracellular glucose concentrations of cells growing on either 10 (●) or 50 fmol (▲) glucose cell^{-1}.h^{-1} in fed-batch cultures. Results shown are representative of at least three different experiments.

Expression of *HXT5* in a nitrogen-limited continuous culture

To determine whether the growth rate was the only parameter that determines expression of *HXT5*, CEN.PK 113-7D cells were grown in a nitrogen-limited continuous culture. This experimental set-up allowed modulation of only the growth rate of yeast, by changing the dilution rate of the culture (17). Other parameters like temperature, agitation, pH and oxygen availability were constant. Also the concentration of intracellular metabolites including glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate and ATP were constant at different dilution rates (data not shown). The concentration of the limiting compound, in this case nitrogen, was also constant, namely virtually 0 mM at all dilution rates (19,20). *HXT5* was only expressed at dilution rates lower than 0.10 h^{-1}, whereas no *HXT5* expression was observed at dilution rates higher than 0.13 h^{-1} (Figure 6). At the dilution rate of 0.10 h^{-1} *HXT5* was expressed to a lower extent compared expression at dilution rates of 0.068 h^{-1} and 0.071 h^{-1}. The dilution rate is the only parameter that is changed in the continuous culture experiments. Low dilution rates, and hence low growth rates, result in increased expression of *HXT5*. Therefore, these results clearly indicate that the growth rate determines expression of *HXT5* in *Saccharomyces cerevisiae*.

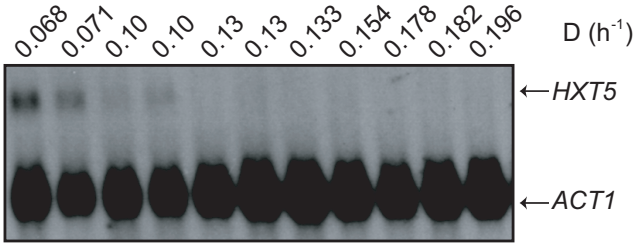

Figure 6: Expression of *HXT5* in a nitrogen-limited continuous culture. CEN.PK 113-7D cells were cultivated in a nitrogen-limited continuous culture. At the different dilution rates indicated, cells were harvested and mRNA was extracted. Expression of *HXT5* mRNA was determined by Northern blot analysis.

Discussion

To obtain clues about the function of Hxt5, and to find mechanisms that are involved in expression of *HXT5*, its expression was determined in different experimental set-ups. Batch culture experiments revealed that *HXT5* is expressed both at the mRNA and protein level when still ample glucose is available in the medium, and remained present after glucose depletion. These results are largely in agreement with earlier observations (10), however *HXT5* is also expressed prior to glucose depletion in our experiments. This suggests that Hxt5p might contribute to glucose transport, which is supported by the observation that Hxt5p is indeed able to transport glucose across the plasma membrane (7).

Independent of the carbon source in which cells are inoculated, *HXT5* is expressed after 24 hours of growth, and cells grown in ethanol or glycerol already expressed *HXT5* in the exponential phase of batch growth. These results are in agreement with earlier observations, where expression of Hxt5-GFP in cells growing on different carbon sources in batch cultures was studied (7). Increasing the temperature or osmolarity of the growth medium of exponentially growing cells resulted in increased expression of *HXT5* at both the mRNA and the protein level. These results confirm the results of various DNA micro-array experiments, which show increased expression of *HXT5* after increasing the temperature (11) or osmolarity (11,12,13,14). In fed-batch cultures *HXT5* was expressed when cells were grown at 10 fmol glucose $cell^{-1}.h^{-1}$, whereas no *HXT5* expression was observed at 50 fmol glucose $cell^{-1}.h^{-1}$. Taken together, our results indicate that during all experiments one parameter that results in induction of *HXT5* expression is in common, being a decrease in the growth rate. These results were confirmed by continuous culture experiments, which were used for modulation of only the growth rate of cells under well-defined growth conditions. *HXT5* is expressed only at dilution rates lower than 0.10 h^{-1}, and expression of *HXT5* is increased even more when the growth rate is further diminished to dilution rates of 0.068 h^{-1}. The concentration of the growth limiting substrate, in this case nitrogen, is extremely low at virtually all growth rates and is the only substrate that determines the growth rate (19,20).

Our results suggest that expression of *HXT5* is not regulated by glucose and not subjected to glucose repression. Furthermore, in a hexokinase II deletion mutant, a protein known to be involved in the regulation of glucose repression, *HXT5* expression

is not derepressed at high extracellular glucose concentrations in batch cultures (21). This result indicates that *HXT5* expression is not regulated by glucose repression. Interestingly, expression of *HXT7* is derepressed at high extracellular glucose concentrations, showing that expression of certain *HXTs* can be repressed by glucose (21). Also Snf3 and Rgt2 are not involved in regulation of *HXT5* expression, as cells deleted for *snf3* or *rgt2* still expressed *HXT5* in glucose-grown batch cultures (data not shown). The Snf3/Rgt2 pathway does regulate expression of the major *HXTs* (4). Because glucose repression and the Snf3/Rgt2 pathway are not involved in regulation of *HXT5* expression, another mechanism probably regulates expression of *HXT5*. Indeed, our results indicate that expression of *HXT5* is induced upon a decrease in growth rates of cells.

To obtain further insight how *HXT5* expression is regulated, the promoter region of the *HXT5* gene was analysed to reveal elements that might be involved in regulation of expression (22). The *HXT5* promoter contains two stress responsive elements (STREs; -472 bp and -304 bp relative to the translation initiation site respectively), two HAP2/3/4/5 binding sites (-854 bp and -785 bp respectively) and one PDS element (-544 bp) (23,24,25). Surprisingly, the promoter of *HXT5* appears to be homologous with the promoter of *GSY2*, encoding glycogen synthase 2, which is involved in glycogen synthesis (26). The promoter of *GSY2* also contains two STREs, two putative HAP2/3/4/5 complex binding sites and one PDS element. Furthermore, expression of *GSY2* exhibits a similar expression pattern as *HXT5* (27), indicating that *HXT5* and *GSY2* expression could be regulated in a similar matter. The involvement of low growth rates in *HXT5* expression does not exclude the involvement of these elements in the promoter of *HXT5* in determining expression of *HXT5* during low growth rates. Furthermore, the transcriptional elements may even be activated under conditions that cause low growth rates to induce expression of *HXT5*.

Cell cycle duration is a well-organized process of which environmental conditions are the main regulators. It was shown that cell cycle duration could be greatly elongated by growing cells in fed-batch cultures on low amounts of carbon source. Concomitantly, trehalose and glycogen were accumulated in these cells (15). Similar results were obtained from cells that were grown in continuous cultures, where also an increase in cell cycle duration is accompanied with elevated trehalose and glycogen levels (28). Surprisingly, we observed that *HXT5* was expressed whenever trehalose was accumulated during growth in batch cultures, fed-batch cultures and

continuous cultures (see Chapter 4). Furthermore, genome-wide analysis of stressful conditions including temperature up-shift, adding chemical compounds that are hazardous for cells and increased osmolarity, revealed that expression of *HXT5* is induced concomitantly with genes involved in reserve carbohydrate metabolism (11). None of the other hexose transporter has this specific expression pattern. Furthermore, Hxt5p is structurally different compared to the major Hxt proteins, because it contains a larger intracellular amino-terminal domain. Taken together, these observations suggest a specific role for Hxt5p in the accumulation or metabolism of reserve carbohydrates. The precursor for trehalose is glucose and Hxt5p may specifically regulate uptake of glucose that is designated for production of trehalose during conditions that induce low growth rates. Furthermore, it was postulated that Tps1p, a protein involved in trehalose synthesis, might function as a direct regulator of glucose transport, probably by interacting with a hexose transporter (29). In our opinion, Hxt5p seems a good candidate to interact with Tps1p, thereby regulating accumulation of trehalose.

In conclusion, our results indicate that expression of *HXT5* is determined by growth rates of cells, and not dependent on the extracellular glucose concentration. The promoter of *HXT5* contains putative regulatory elements, which may contribute to expression of *HXT5* during low growth rates. The extended amino-terminal domain of Hxt5p and the unique expression pattern of *HXT5* during various kinds of conditions leading to low growth rates that are concomitant to accumulation of trehalose, suggest a role for Hxt5p in accumulation of this reserve carbohydrate besides glucose transport.

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