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RESEARCH ARTICLE

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Expression of the *HXT13, HXT15* and *HXT17* genes in *Saccharomyces cerevisiae* and stabilization of the *HXT1* gene transcript by sugar-induced osmotic stress

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Abstract *Saccharomyces cerevisiae* contains a family of 17 *hex*ose *t*ransporter (*HXT*) genes; only nine have assigned functions, some of which are still poorly defined. Despite extensive efforts to characterize the hexose transporters, the expression of *HXT6* and *HXT8-17* remains an enigma. In nature, *S. cerevisiae* finds itself under extreme nutritional conditions including sugars in excess of 40% (w/v), depletion of nutrients and extremes of both temperature and pH. Using *HXT* promoter–*lacZ* fusions, we have identified novel conditions under which the *HXT17* gene is expressed; *HXT17* promoter activity is up-regulated in media containing raffinose and galactose at pH 7.7 versus pH 4.7. We demonstrated that *HXT5, HXT13* and, to a lesser extent, *HXT15* were all induced in the presence of non-fermentable carbon sources. *HXT1* encodes a low-affinity transporter and in short-term osmotic shock experiments, *HXT1* promoter activity was reduced when cells were exposed to media containing 40% glucose. However, we found that the *HXT1* mRNA transcript was stabilized under conditions of osmotic stress. Furthermore, the stabilization of *HXT1* mRNA does not appear to be gene specific because 30 min after transcriptional arrest there is a fourfold more mRNA in osmotically stressed versus non-stressed yeast cells. A large portion of *S. cerevisiae* mRNA molecules may, therefore, have a decreased rate of turnover during exposure to osmotic stress indicating that post-transcriptional regulation plays an important role in the adaptation of *S. cerevisiae* to osmotic stress.

Keywords Hexose transport · Osmotic stress · Transcription · mRNA turnover

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Introduction

Glucose import into the yeast cell is facilitated by a group of membrane-spanning proteins, termed *hex*ose *t*ransporters (HXT). There are at least 20 members of the yeast hexose transporter family (*HXT1* to *HXT17*, *SNF3, RGT2* and *GAL2*), as identified by genetic studies and/or sequence homology (for reviews, see Bisson et al. 1993; Kruckeberg 1996; Boles and Hollenberg 1997; Ozcan and Johnston 1999). All the HXT gene products, with the exception of Hxt12p, are able to support growth on glucose when expressed individually in a strain deleted for all 20 transporter genes (Wieczorke et al. 1999), indicating all the HXT genes encode functional glucose transporters. *HXT1, HXT2, HXT3* and *HXT4* are the best characterized members of the HXT family. The presence of multiple hexose transporters with differing affinities for glucose is reasonable given that *Saccharomyces cerevisiae* is able to grow in an extensive range of sugar concentrations (0.1 to >40% w/v).

HXT1 encodes a low-affinity transporter that is maximally expressed in the presence of high levels of extracellular glucose (>1% w/v or 56 mM) (Ozcan and Johnston 1995). *HXT1* was originally isolated as a multicopy suppressor of a high-affinity glucose transport defect in *snf3Δ* cells (Lewis and Bisson 1991) and later as a suppressor of a potassium transport defect in *trk1Δtrk2Δ* cells (Ko et al. 1993). Early studies revealed that *HXT1* expression was maximal during lag and early-exponential phases of growth (Lewis and Bisson 1991). *HXT1* expression increased during exposure to osmotic stress caused by salt (1.0 M NaCl), sorbitol (1.5 M) (Hirayama et al. 1995) or high sugar (40% w/v) (Erasmus et al. 2003). Induction of *HXT1* by osmotic stress is dependent on the high osmolarity glycerol (HOG) pathway (Rep et al. 2000). It has been proposed that *HOG1*-dependent *HXT1* expression provides additional glucose for the synthesis of glycerol, a compatible solute that accumulates during conditions of osmotic stress (Hirayama et al. 1995).

HXT2 and *HXT4* are high-affinity transporters. Expression of *HXT2* and *HXT4* is increased approximately 5 to 20-fold in cells growing in the presence of low glucose [~0.1% (w/v) or 5.6 mM] versus cells grown either in the absence of glucose or in the presence of high glucose (Ozcan and Johnston 1995). Like *HXT1*, *HXT2* and *HXT4* were cloned as multicopy suppressors of the high-affinity glucose uptake defect in *snf3Δ* mutants (Kruckeberg and Bisson 1990). *HXT2* and *HXT4* are maximally expressed at low concentrations [~0.1% (w/v) or 5.6 mM] of glucose (Ozcan and Johnston 1995). Rgt1p represses the expression of *HXT2* and *HXT4* in the absence of glucose. However, these genes have an additional level of regulation by Mig1p and Snf1p that limits their expression to low concentrations of glucose. Indeed *HXT4* has a K_m for glucose of 6.2–9.0 mM, whereas the corresponding K_m values reported for *HXT2* are 1.5–2.9 mM (Reifenberger et al. 1997; Maier et al. 2002).

HXT3 is a low-affinity glucose transporter that was originally identified along with *HXT1* as a suppressor of a potassium transport defect in *trk1Δtrk2Δ* cells (Ko et al. 1993). *HXT3* is also a multicopy suppressor of the *snf3Δ* growth defect on raffinose (Ko et al. 1993; Theodoris et al. 1994). Raffinose is a trisaccharide composed of galactose–glucose–fructose. Raffinose serves as a low source of fermentable carbon as the glucose–fructose bond can be gradually hydrolyzed by invertase. *HXT3* promoter activity is constitutive in the presence of glucose but is independent of sugar concentration (Ozcan and Johnston 1995). In the absence of glucose, *HXT3* is repressed by Rgt1p (Ozcan and Johnston 1995). *HXT3* expression reaches maximal levels upon entry into stationary phase (Ko et al. 1993).

HXT5 encodes a functional hexose transporter with moderate affinity for glucose ($K_m = 10$ mM) that is maximally expressed under conditions that cause slow growth (Diderich et al. 2001; Verwaal et al. 2002). For example, in batch cultures increases in temperature or osmolarity, as well as growth in the presence of ethanol or glycerol or a depletion of glucose, all induce the expression of *HXT5* (Verwaal et al. 2002). Microarray data have also identified *HXT5* as inducible by increased temperature (Gasch et al. 2000) or osmolarity (Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Yale and Bohnert 2001; Erasmus et al. 2003). The induction of *HXT5* when glucose is depleted is a function of growth rate and is independent of glucose derepression because in exponentially growing *hxk2Δ* cells in the presence of glucose, *HXT2* and *HXT4* are derepressed, while *HXT5* expression is not detected.

HXT6 and *HXT7* exist in tandem on chromosome IV, separated by approximately 3.5 kb, and are 1.5 kb downstream of *HXT3*. Hxt6p and Hxt7p are highly related, differing by only two amino acids over the entire 570 amino acid sequence (Reifenberger et al. 1995). Neither of the differing amino acids appears conserved within the hexose transporter family (Boles and Hollenberg 1997). Of the characterized transporters from this family (*HXT1-7*), *HXT6* and *HXT7* have the highest affinity for glucose, with a K_m value of approximately 1.0 mM (Reifenberger et al. 1997; Maier et al. 2002). In wild-type strains, the expression of *HXT7* is repressed in the presence of high concentrations of glucose, but increases as glucose is depleted (Ye et al. 2001). Interestingly, despite their high sequence similarity, *HXT6* and *HXT7* appear to be regulated independently. The expression of *HXT7* is much higher than that of *HXT6* in wild-type strains under similar growth conditions (Reifenberger et al. 1997; Diderich et al. 1999). Furthermore, *HXT6*, in addition to being regulated by the general glucose repression pathway, responds to a novel signal transduction pathway involving Snf3p (Liang and Gaber 1996). More specifically, the maintenance of *HXT6* glucose repression is dependent on *SNF3* expression even when glucose is abundant.

To date, little is known about the regulation of *HXT8-16*. *HXT11* is capable of restoring glucose uptake in a *rag1Δ* strain of *K. lactis* (Nourani et al. 1997). *HXT11* and *HXT9* were also identified as targets for the transcriptional activator Pdr3p (for *p*leiotropic *d*rug *r*esistance) (Nourani et al. 1997). Deletion of *HXT11* and/or *HXT9* confers resistance to cycloheximide (protein synthesis inhibitor), sulfomethuron methyl (acetolactate synthase inhibitor) or 4-nitroquinoline-*N*-oxide (mutagen). This is interesting given that when expressed individually, Hxt9p and Hxt11p are functional glucose carriers (Wieczorke et al. 1999), and yet their expression is entirely independent of extracellular glucose. Rather, their expression is linked to a transcription activator that also regulates proteins that confer drug resistance.

HXT17 was identified by a microarray experiment as a target of a constitutively active form of the Mac1p transcription factor (Gross et al. 2000). Mac1p regulates the expression of high-affinity copper uptake genes under copper-deficient conditions (Jungmann et al. 1993). However, when cells were treated with a copper-specific chelator to mimic copper limitation, *HXT17* was not induced, indicating the effect may have been due to a property of the specific *MAC1* mutant allele.

Regulatory components of *HXT* gene expression: Rgt1p, Grr1p, Mth1p and Std1p

RGT1 encodes a DNA-binding protein that serves as both an activator and a repressor of *HXT* gene expression. In the absence of glucose, Rgt1p represses *HXT1–HXT4*; addition of glucose to the media causes inhibition of Rgt1p activity and subsequent derepression of various *HXT* genes (Ozcan and Johnston 1995). Repression of transcription by Rgt1p requires the general transcriptional repressors Ssn6p and Tup1p (Ozcan et al. 1996). Glucose-mediated inhibition of Rgt1p activity requires Grr1p. Interestingly, Rgt1p is required for both repression and activation of *HXT1* gene expression. In *rgt1Δ* cells, *HXT1* is expressed in the absence of glucose, but does not reach maximum

expression levels in the presence of high amounts of glucose (2% w/v) (Ozcan and Johnston 1995). Recently it was shown that Rgt1p becomes hyperphosphorylated in response to high concentrations of glucose (4% w/v) (Mosley et al. 2003). Hyperphosphorylation is required for converting the protein to an activator because phosphorylation was abolished in *snf3Δ, rgt2Δ* and *grr1Δ* mutants, and induction of *HXT1* expression is also lacking in these strains.

GRR1 (*g*lucose *r*epression *r*esistant) encodes an F-box protein associated with the ubiquitin proteolysis machinery (Li and Johnston 1997). *GRR1* expression is required for *HXT* gene expression, and this is due to the requirement of Grr1p for Rgt1p inactivation. The glucose repression defect of *grr1Δ* strains is therefore indirect, as cells are unable to transport any amount of glucose (Vallier et al. 1994; Ozcan and Johnston 1995). It has been proposed that Grr1p regulates Rgt1p activity by targeting the protein for degradation (Ozcan and Johnston 1999). However, a recent report suggests an intermediate, Mth1p, links these two proteins (Flick et al. 2003). *MTH1* and the closely related *STD1* are two genes that are important for the proper regulation of *HXT* gene expression (Schmidt et al. 1999; Schulte et al. 2000); *mth1Δstd1Δ* cells express *HXT1-4* even in the absence of glucose (Schmidt et al. 1999). Both Mth1p and Std1p are able to interact with the membrane-bound glucose sensors Rgt2p and Snf3p (Schmidt et al. 1999; Lafuente et al. 2000), and both proteins localize to the membrane and the nucleus, making them good candidates as transducers of the glucose signal that activates or derepresses transcription (Schmidt et al. 1999). Indeed, it was recently established that the phosphorylation and dissociation of Rgt1p from *HXT* promoters is mediated by Grr1p-dependent degradation of Mth1p (Flick et al. 2003). Three modes of transcriptional regulation of HXT transporters by different levels of glucose are summarized in Fig. 1.

In this study we examined the transcriptional regulation of yeast hexose transporter genes, with a particular emphasis on the response of *HXT* genes to conditions that may be encountered during wine fermentations rather than laboratory conditions. Our primary focus was on *HXT* transcription in the presence of 0.2–40% (w/v) glucose or fructose, as well as the effect of anaerobiosis, changing pH, osmotic pressure and glucose starvation on *HXT* mRNA levels. We identified novel conditions under which the *HXT17* gene is expressed; *HXT17* promoter activity is up-regulated in media containing raffinose and galactose at pH 7.7 versus pH 4.7. We demonstrated that *HXT5, HXT13* and, to a lesser extent, *HXT15* were all induced in the

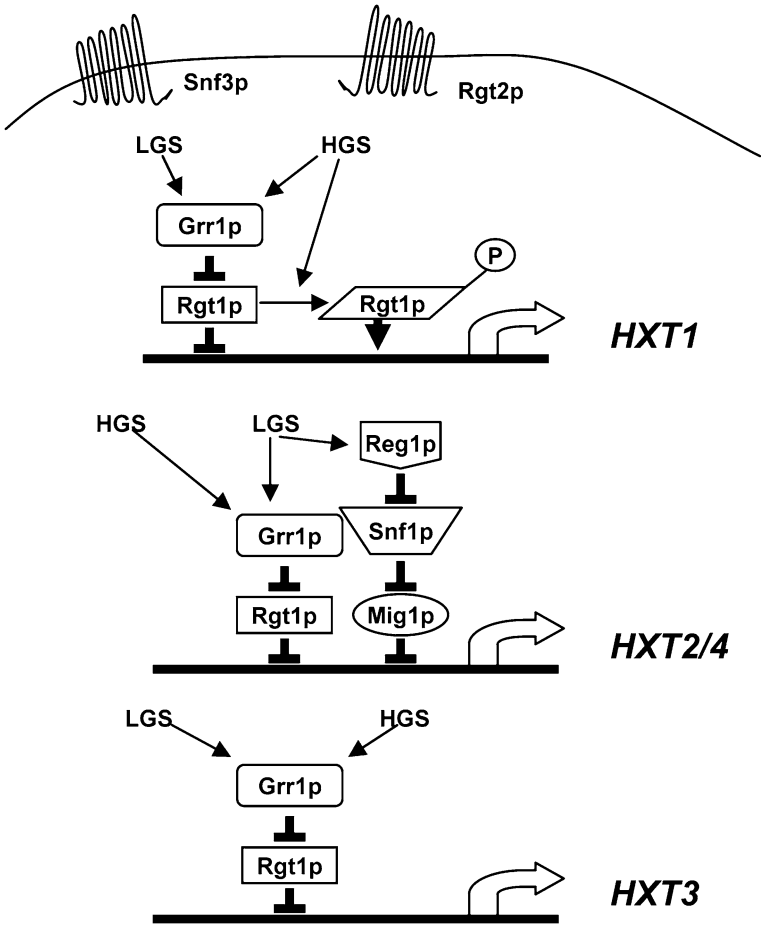

Fig. 1 Three models of transcriptional regulation of the yeast hexose transporters by different concentrations of glucose. *Arrows* represent positive regulation and *blunt-ended lines* represent negative regulation. (Adapted from Ozcan and Johnston 1999). *LGS* low glucose, *HGS* high glucose

presence of non-fermentable carbon sources. Furthermore, *HXT1* mRNA transcripts were stabilized in a 40% glucose-containing medium. Global mRNA decay was reduced in osmotically stressed yeast cells and a large portion of *S. cerevisiae* mRNA molecules has a decreased rate of turnover during exposure to osmotic stress. Yeast cells therefore seem to adapt to osmotic stress by regulating transcription as well as mRNA turnover.

Materials and methods

Strains, plasmids and media

The yeast strains used in this study are listed in Table 1. Yeast cells were grown on standard media: YPD [1% (w/v) yeast extract (Difco), 2% (w/v) Bacto peptone (Difco), 2% (w/v) dextrose] or YNB [0.67% (w/v) yeast nitrogen base without amino acids or ammonium sulfate (Difco) supplemented with the appropriate amino acids, 0.5% (w/v) ammonium sulfate and containing raffinose/galactose (1.5:0.5% w/v) or glucose or fructose (0.2–40% (w/v) as indicated]. *HXT* promoters (0.6–1.8 kb DNA fragments generated by restriction enzyme digests) were fused to *lacZ* in the vector YEp357R (Ozcan and Johnston 1995, 1999). Plasmids are shown in Table 2.

Yeast transformations and β-galactosidase assays

Individual *HXT–lacZ* plasmids (Table 2) were transformed into *S. cerevisiae* TCY1 cells using the high-efficiency lithium acetate method (Gietz and Schiestl 1995). Transformed cells were inoculated into *lacZ* medium (final density $A_{600\ nm}$ 0.05) and grown with shaking at 30°C until an OD of $A_{600\ nm}$ of 0.6–0.8 was reached. β-Galactosidase (β-gal) assays were performed as described previously (Ausubel 1995), except that 2 or 10 ml of cell culture was analyzed instead of 25 ml. Each sample was analyzed in duplicate within the experiment, and the entire experiment was performed in triplicate. β-gal activity values are reported in Miller units (Miller 1972).

RNA isolation

Total RNA was isolated using the hot phenol method (Ausubel 1995). For real-time PCR analyses, an additional step was required during isolation to remove contamination by genomic DNA. This was performed using Qiagen RNeasy kits (catalogue # 74104), with the Rnase-free DNase kit (catalogue # 79254) using the manufacturer's recommended protocols (available at http://www.qiagen.com).

Real-time PCR

cDNA synthesis from 2.0 μg of total RNA was performed using the Omniscript RT Kit (Qiagen) according to the manufacturer's suggested protocol. The reverse transcription reaction was primed using random hexamer oligonucleotides at a final concentration of 2.5 μM. The final cDNA product was dissolved in DEPC-treated H_2O to a final volume of 500 μl.

Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's standard protocol, except that the final reaction volume was reduced to 20 μl. Gene-specific oligonucleotide primers (Table 3) were used at a final concentration of 0.5 μM. PCR reactions were performed in an ABI Prism® 7000 Sequence Detector (Applied Biosystems) with the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were assayed in triplicate for each gene, and differences in cDNA synthesis efficiency were corrected for by normalizing all expression values to constitutively expressed 18S rRNA.

Determination of water activity (a_w)

Water activity measurements were carried out in triplicate, using an Aqualab Series 3 water activity meter (Decagon Devices, Pullman, WA, USA). The values reported represent the mean of three assays.

Osmotic shock assays

TCY1 cells transformed with *HXT1–lacZ* were grown in 300 ml YNB (galactose 2% w/v) to mid-log phase and then centrifuged at 9,000*g* for 10 min at room temperature. The supernatant was discarded, and the cells were resuspended in 6 ml sterile dH_2O. Flasks containing 50 ml of YNB media plus the indicated carbon or salt were inoculated with 1 ml (approximately 1.6×10^7 cells)

Table 1 Yeast strains used in this study

Strain	Genotype	Reference/source
TCY1	*MATα ura3Δ lys2Δ*	van der Merwe et al. (2001)
BY4742	*MATα his3Δ1 ura3Δ lys2Δ leu2Δ*	Brachmann et al. (1998)
BY4742 *hog1Δ*	*MATα his3Δ1 ura3Δ lys2Δ leu2Δ hog1::kanMX*	Invitrogen Life Technologies, Carlsbad, CA, USA
Y260	*MATa ura3Δ52 rpb1Δ1*	Nonet et al. (1987)

Table 2 Plasmids used in this study

Plasmid	Description	Reference
pBM2636	*HXT1–lacZ*	Ozcan and Johnston (1995, 1999)
pBM2717	*HXT2–lacZ*	
pBM2819	*HXT3–lacZ*	
pBM2800	*HXT4–lacZ*	
pBM3555	*HXT5–lacZ*	
pBM3537	*HXT10–lacZ*	
pBM3539	*HXT11–lacZ*	
pBM3538	*HXT12–lacZ*	
pBM3466	*HXT13–lacZ*	
pBM3573	*HXT14–lacZ*	
pBM3472	*HXT15–lacZ*	
pBM3574	*HXT16–lacZ*	
pBM3476	*HXT17–lacZ*	

All plasmids are derived from YEp357R

of the transformants and then incubated at 30°C. After 4 h, 10 ml of each culture was harvested in duplicate and analyzed for β-galactosidase expression. The experiment was performed at least three times using independent cultures.

RNA stability assays

For these studies the yeast strain Y260 was employed. Y260 cells bear a temperature-sensitive mutation in an essential RNA polymerase II protein, Rpb1p, and rapidly arrest mRNA transcription upon shifting to the non-permissive temperature (Nonet et al. 1987). A 1 l volume of YPD was inoculated with Y260 cells at an initial cell density of $A_{600\ nm}=0.2$ and grown at 25°C until an $A_{600\ nm}$ 1.0 was reached (~11 h). The cells were harvested at 9,000*g* for 5 min at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 4 ml sterile YPD media, pre-warmed to 37°C (the non-permissive temperature). Seven 50 ml volumes of YPD, containing either 2% glucose (w/v) or 40% glucose (w/v) as the carbon source, were warmed to 37°C and then added to Y260 cell pellets to yield a final concentration of approximately 3×10^7 cells/ml. All flasks were immediately placed in a water bath at 37°C with shaking. In addition, one flask was immediately centrifuged after inoculation, the supernatant was discarded and the cells were quickly frozen by placing the tube in ethanol at −80°C. This marked the zero-minute time point. For each time point (15, 30 and 60 min) the entire 50 ml culture was harvested by centrifugation, and the cell pellet was frozen in the same manner and stored at −80°C until RNA extractions were performed.

For quantifying mRNA as a percentage of total RNA, poly-adenylated molecules were purified from 1 mg DNase-treated total RNA using the Qiagen Oligotex mRNA Midi kit (catalogue # 70042). Total RNA and mRNA were quantified by measuring the absorbance at 260 nm. Experiments were repeated three times.

Statistical analyses

A two-way analysis of variance (ANOVA) was used to evaluate the effect of carbon source and sugar concentration on *HXT* promoter activities and for comparing mRNA levels after transcriptional arrest in low and high-sugar media. Differences in promoter activity were analyzed using a Fisher's least significant difference (LSD) test ($P=0.05$). Statistics were calculated using Minitab software (release 14, Minitab Inc., USA).

Results

Effect of glucose or fructose on *HXT* promoter activity at concentrations ranging between 0.2 and 40% (w/v)

To advance our understanding of the regulation of yeast hexose transporter gene expression, we tested whether increased sugar concentrations (up to 40% w/v) or specific carbon sources (fructose vs. glucose) affected *HXT* expression. As an initial screen, we prepared X-gal plates containing either glucose or fructose from 0.2 to 40% (w/v) glucose or fructose as carbon source. We incubated TCY1 cells transformed with one of *HXT1-5* and *HXT10-17* promoter–*lacZ* fusions on these plates for several days but observed no significant difference between glucose and fructose-grown cells. In addition, the only *HXT* promoters that exhibited activity under these conditions were *HXT1-5* and *HXT13* (data not shown). To examine the promoter activity of these genes more closely, we assayed liquid-grown cultures of TCY1 transformed with *HXT1-5, HXT13–lacZ* for β-gal activity. *HXT1–lacZ* expression increased almost linearly with increasing sugar concentrations up to 30% (w/v) ($R^2=0.8662$, Fig. 2a). There was, however, no statistical difference in *HXT1* promoter activity in cells grown in 30 versus 40% glucose or fructose. *HXT2* promoter activity was increased in cells exposed to sugar concentrations at or below 2% (w/v). Interestingly, induction of *HXT2* was twofold higher with 2% fructose

Table 3 Primers used in this study

Gene name	Forward primer	Reverse primer
GPD1	5'-CCAGAAGTTTTCGCTCCAATAGTA-3'	5'-AGCAACCAAATTGTCGGGTAGA-3'
HXT1	5'-CCCGATCTAATATCTCCTCAGAAATCC-3'	5'-CCACCGAAAGCAACCATAACAC-3'
IPP1	5'-ACAGCAAGGGTATTGATTTGACCA-3'	5'-AAGCTGGTGGGATGGCATCA-3'
18S rRNA	5'-GGTGAAATTCTTGGATTTATTGAAGAC-3'	5'-TTGATTTCTCGTAAGGTGCCGAGT-3'

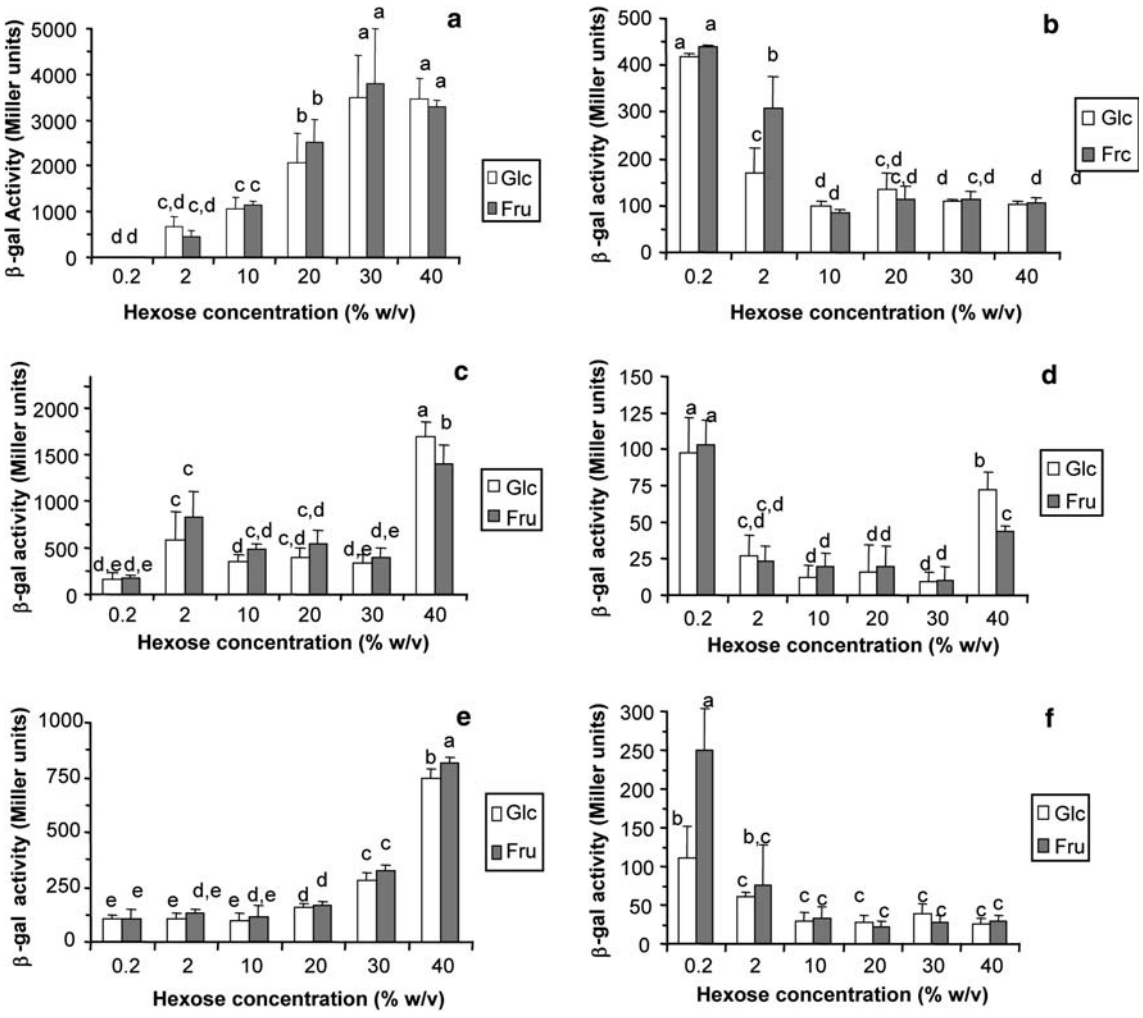

Fig. 2 Promoter activity of *HXT1* (**a**), *HXT2* (**b**), *HXT3* (**c**), *HXT4* (**d**), *HXT5* (**e**) and *HXT13* (**f**) in response to various concentrations of extracellular glucose or fructose. Strain TCY1 was grown in YNB media with the carbon source as indicated. At mid-log phase, cells were harvested and assayed for *β*-gal activity. Results shown represent the mean of three experiments. *Error bars* represent one standard deviation. Means with the same letter are not significantly different (Fisher's LSD **a** = 871.8, **b** = 57.5, **c** = 267.7, **d** = 22.4, **e** = 55.2, **f** = 43.9, $P = 0.05$)

compared to 2% glucose (Fig. 2b). *HXT3* was expressed across all sugar concentrations (Fig. 2c), while *HXT4* activity was found to be highest at 0.2% (w/v) glucose and fructose (Fig. 2d), in agreement with previous observations (Ozcan and Johnston 1995). Surprisingly both *HXT3* and *HXT4* had a spike of promoter activity in cells grown in 40% glucose or fructose (w/v). *HXT5* promoter activity remained relatively unchanged in both glucose and fructose-grown cultures until the sugar concentration exceeded 20% (w/v). Increasing the sugar concentration from 20 to 40% induced the *HXT5* promoter approximately fivefold (Fig. 2e). *HXT13–lacZ* was moderately induced in response to low (< 2%) glucose and fructose (Fig. 2f). Similar to *HXT2*, there appeared to be a differential induction of *HXT13* between glucose and fructose at very low sugar concentrations (0.2%). It is important to note that the cells grown in the 0.2% glucose or fructose media had likely reached carbon starvation conditions at the time of harvest. Indeed these cells entered stationary phase at a lower cell density (not shown).

Effect of pH on *HXT17* promoter activity

During growth on X-gal plates containing raffinose and galactose as the carbon source (1.5:0.5% w/v), there appeared to be an increase in *HXT17* promoter activity when compared to cells grown on 2% glucose (w/v) (data not shown). Initial attempts to quantify this induction using *β*-gal assays in liquid-grown cultures were unsuccessful. However, one key difference between plate and liquid *β*-gal assays is that the X-gal plates are buffered to have a neutral pH, whereas liquid YNB media has a pH of 4.5–4.7. After adjusting the pH of the YNB media with KOH, we observed that *HXT17* pro-

moter activity in cultures grown in pH 7.7 versus 4.7 (using raffinose and galactose as the carbon source) was approximately 12-fold higher (Fig. 3).

HXT5, *HXT13* and *HXT15* are expressed during growth on ethanol or glycerol and ethanol together

After prolonged incubation of X-gal plates containing low amounts of glucose or fructose we observed slight induction of *HXT5, HXT13* and *HXT15–lacZ* (data not shown). We hypothesized that these genes may be induced in response to glucose starvation and hence tested the expression level of these genes in cells grown on the non-fermentable carbon source ethanol alone (2% v/v) or in combination with glycerol (2% v/v each). As seen in Fig. 4, *HXT5*, *HXT13* and to a lesser extent *HXT15* are up-regulated during growth on ethanol and glycerol versus 2% glucose (ethanol data is similar to that of the ethanol/glycerol combination and is therefore not shown). We also tested glycerol alone, but TCY1 cells were unable to grow under this condition. Because *HXT15* was expressed much lower than the other two transporters, the physiological significance of this observation is uncertain.

Reduction of *HXT1* promoter activity by high glucose (40% w/v) and 1.4 M NaCl

HXT1 promoter activity is up-regulated by osmotic shock in media containing NaCl (0.7–1.0 M) or sorbitol (0.95–1.5 M) as osmolyte (Hirayama et al. 1995; Rep et al. 2000). The water activity of media containing 1.4 M NaCl is approximately 0.952 (Erasmus et al. 2003). This is similar to the water activity of 40% glucose (w/v) which we found to be approximately 0.939. To compare the effects of NaCl and 40% glucose (w/v)

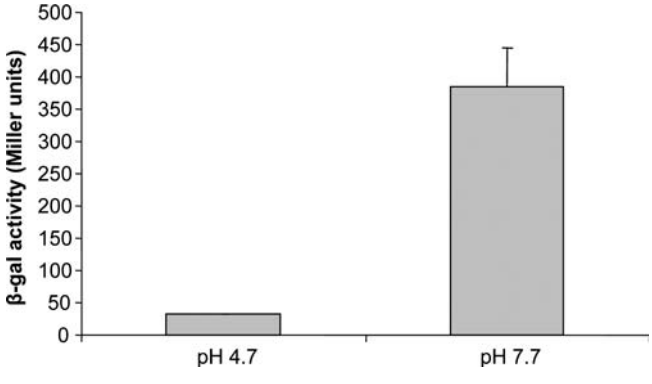

Fig. 3 *HXT17* is expressed in cells grown with raffinose and galactose (1.5:0.5% w/v) as the carbon source at pH 7.7 versus 4.7. Strain TCY1 was grown in YNB media containing raffinose and galactose with the pH adjusted to 4.7 or 7.7. At mid-log phase, cells were harvested and assayed for *β*-gal activity. Results shown are the mean of three experiments. *Error bars* represent one standard deviation

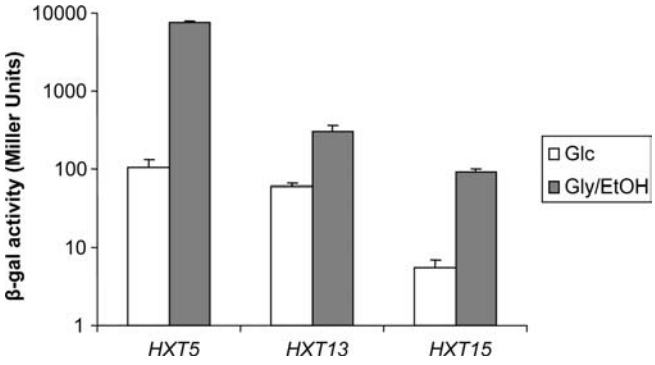

Fig. 4 *HXT5*, *HXT13* and *HXT15* are expressed in cells grown in YNB media containing glucose (2% w/v) or ethanol and glycerol (2% v/v each). TCY1 cells were grown in YNB media containing the indicated carbon source (ethanol *EtOH*, glycerol *Gly*). At mid-log phase, cells were harvested and assayed for *β*-gal activity. Results shown are the mean of three experiments. Error bars represent one standard deviation

on *HXT1* promoter activity, we designed an experiment to test the effect of a short-term osmotic shock on *HXT1–lacZ* induction. Short-term stress exposure reflects the immediate response, rather than long-term adaptation to a stressor, and is therefore distinct from experiments like those shown in Fig. 2. As seen in Fig. 5a, *HXT1* was highly induced by 2% glucose (3,500 Miller units); 1.4 M NaCl (8% w/v) in the presence of glucose activated the *HXT1* promoter to the same extent as 40% glucose (250 Miller units). Unexpectedly, the level of promoter activation was ~14-fold higher in 2% glucose compared to 40% glucose (Fig. 5a). These data indicated that osmotic shock actually reduces the *HXT1* promoter activity. Galactose (2% w/v) and 2% galactose plus 1.4 M NaCl failed to activate the *HXT1* promoter (Fig. 5b).

Effect of high glucose concentrations on *HXT1* mRNA stability

Previous studies (northern blotting and/or DNA microarrays) have indicated that *HXT1* mRNA levels are increased in response to NaCl or sorbitol (Hirayama et al. 1995; Rep et al. 2000). However, our results indicated that the *HXT1* promoter activity was reduced by osmotic shock. To reconcile these observations, we hypothesized that for *HXT1* mRNA to accumulate under osmotic stress despite the promoter being down-regulated, the turnover of the molecule must be decreased. To address this possibility, the yeast strain Y260, bearing the temperature-sensitive *rpb1-1* allele was used. This conditional RNA polymerase mutant rapidly ceases mRNA synthesis at the non-permissive temperature (Nonet et al. 1987). *S. cerevisiae* Y260 was shifted to the non-permissive temperature in rich media containing either 2% glucose or 40% glucose (w/v) as the carbon source. Total RNA was harvested at various time points following cessation of transcription, and the

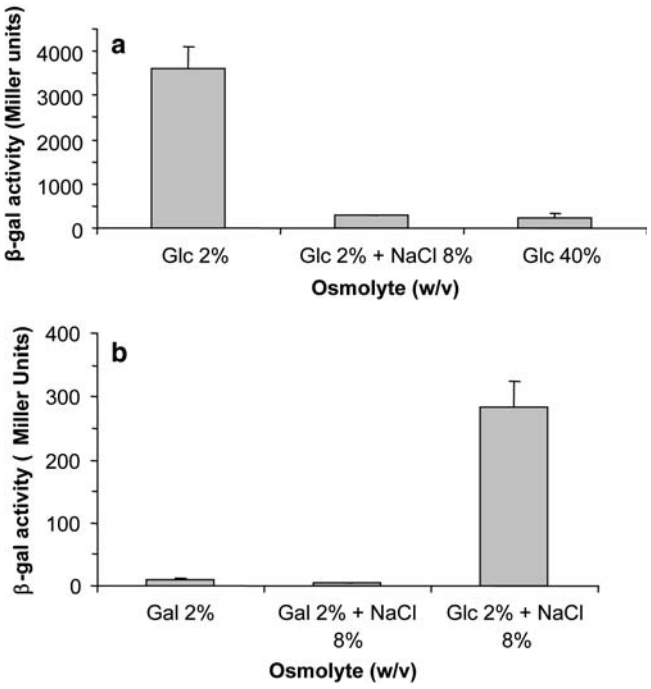

Fig. 5 Short-term osmotic shock-induced *HXT1* expression requires glucose but decreases *HXT1* promoter activity relative to non-stressed cells. **a** Osmotic shock reduced the *HXT1* promoter activity. TCY1 cells were transformed with an *HXT1–lacZ* construct and grown in YNB media containing 2% galactose (w/v) as the carbon source. At mid-log phase cells were harvested and inoculated into media containing either 2% glucose (with or without 8% NaCl) or 40% glucose (w/v). After 4 h cells were harvested and analyzed for β-gal activity. **b** Glucose is required for *HXT1* promoter activation in response to osmotic shock. TCY1 cells were prepared as described in **a**, but inoculated into YNB media containing glucose (2% w/v) or galactose (2% w/v), in the presence or absence of 8% NaCl (w/v). The results shown are the mean of three experiments. *Error bars* represent one standard deviation

level of *HXT1* mRNA was assayed by real-time PCR. As confirmation of the functionality of the *rpb1-1* allele, the cells that were shifted to 37°C failed to double and did not appear to arrest at any particular stage of the cell cycle (data not shown), both of which are characteristics of the Y260 strain (Nonet et al. 1987). As expected, in 2% glucose (w/v) the level of *HXT1* mRNA declined immediately upon shifting to the non-permissive temperature and had diminished approximately 16-fold after 1 h at 37°C (Fig. 6a). However, in the presence of 40% (w/v) glucose there was only a threefold decline in *HXT1* mRNA over the same time period. If transcription occurred at the non-permissive temperature, one would expect to see an increase in *GPD1* mRNA levels since *GPD1* is up-regulated within 10 min of exposure to osmotic stress (Rep et al. 1999a, b), and *GPD1* expression increases greater than 30-fold in response to salt-induced osmotic stress (Rep et al. 2000). We included *GPD1* as a positive control and did not observe a significant increase in *GPD1* mRNA above the initial levels. Moreover, the level of *GPD1* mRNA was maintained at a higher level in cells exposed to 40% glucose versus 2% glucose (Fig. 6b), indicating that this transcript also seems to be stabilized by osmotic stress. Finally, *IPP1*, which encodes the inorganic pyrophosphatase enzyme and is not transcriptionally regulated by osmotic stress (Rep et al. 1999a), was included as negative control; its mRNA decayed at a similar rate in both 2 and 40% glucose-treated cells as was expected (Fig. 6c).

Osmotic stress enhances retention of mRNA molecules after termination of transcription in yeast cells

Due to the observation that *HXT1* and *GPD1* are both stabilized by osmotic stress, we investigated whether other transcripts may be stabilized by low water activity stress. As a reflection of mRNA levels, we purified polyadenylated molecules from total RNA derived from Y260 cells after 30 min at the non-permissive temperature in either low (2% w/v) or high (40% w/v) glucose. As seen in Fig. 7, there was a fourfold reduction in cellular mRNA (as a percentage of total RNA) from cells grown at the permissive temperature to cells in 2% (w/v) glucose for 30 min at the non-permissive temperature. Remarkably, the decrease in mRNA was negligible for osmotically stressed cells at the non-permissive temperature.

Discussion

Effect of glucose or fructose on *HXT* promoter activity at concentrations of 0.2–40% (w/v)

For the *HXT* genes that have been characterized thus far the primary determinant of expression level is the extracellular glucose concentration. Each transporter protein has a unique affinity for glucose (and fructose), with higher affinity transporters being expressed at the lowest concentrations of glucose and vice versa. Despite the fact that *S. cerevisiae* can readily metabolize glucose at extracellular concentrations of less than 0.1% to greater than 40% (w/v), the transcriptional regulation of yeast *HXT* genes has only been tested up to 8% glucose (w/v). We hypothesized that the *HXT* genes with previously unknown function may be transcribed only at high (>10% w/v) sugar concentrations since *S. cerevisiae* is regularly exposed to such conditions in nature or during industrial fermentations. Furthermore previous studies have focused primarily on glucose-mediated regulation of *HXT* genes. Therefore, using *HXT* promoter–*lacZ* fusion constructs, we tested the promoter activity of *HXT1-5* and *HXT10-17* in glucose and fructose concentrations ranging from 0.2 to 40% (w/v). In agreement with previous observations (Ozcan and Johnston 1995), we showed that *HXT2* and *HXT4* are expressed when extracellular sugar concentrations are <2% (Fig. 2). Furthermore, *HXT3* is expressed constitutively, and *HXT1* is induced in the presence of

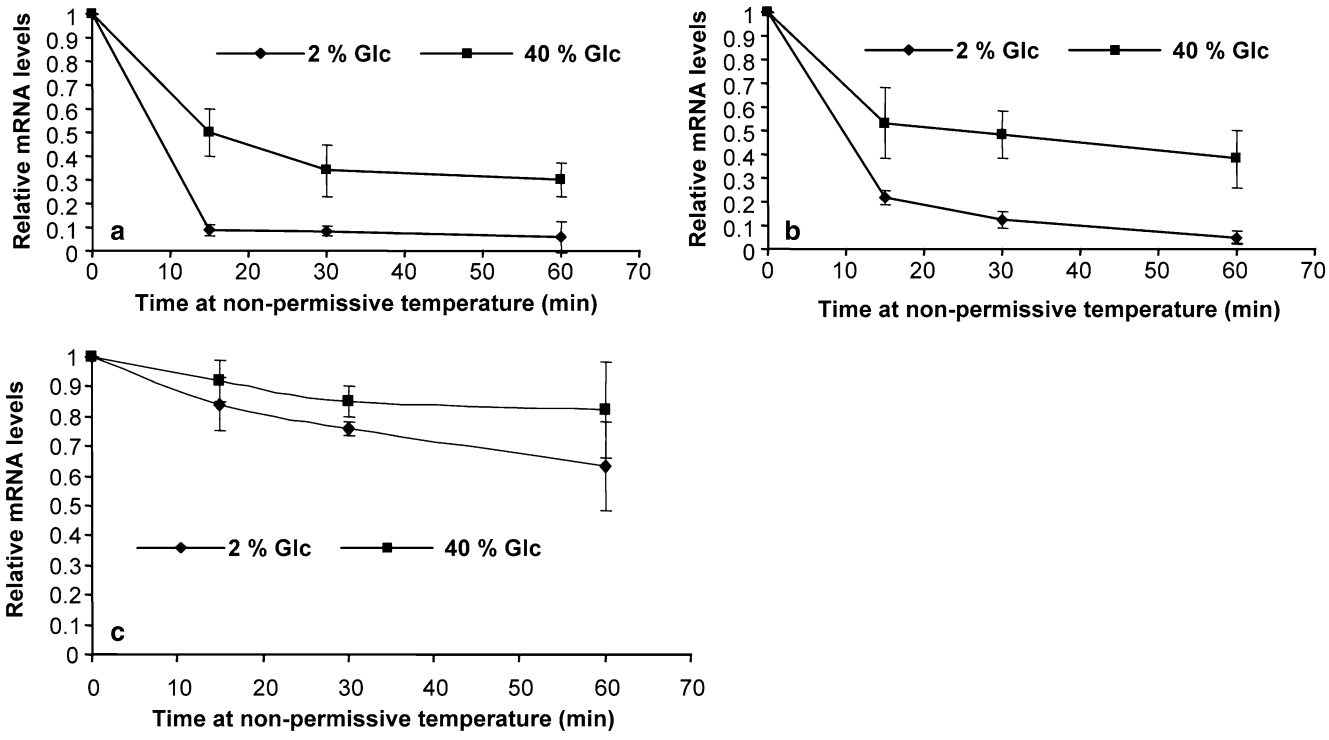

Fig. 6 Several distinct transcripts are stabilized by osmotic stress. Y260 cells were grown to mid-log phase and then shifted to the non-permissive temperature to inactivate the *rpb1-1* allele. At the time intervals indicated after the temperature shift, the cells were harvested and mRNA levels were quantified by real-time PCR. **a** *HXT1*, **b** *GPD1* and **c** *IPP1*. *Error bars* represent one standard deviation from three experiments

> 1% glucose or fructose. With the exception of *HXT2*, no differences in promoter activation were observed between glucose-grown and fructose-grown cells.

It has been reported that *hxt2Δ* strains of *S. cerevisiae* are prone to sluggish fermentations (cited in Bisson 1999). Because stuck fermentations typically arrest with 1.5–2.0% (w/v) fructose and only 0.1–0.5% glucose, it was interesting that the transcriptional activation of *HXT2* at these levels was approximately twofold higher in fructose-grown cells than glucose-grown cells. However, two observations indicate that mis-regulation of *HXT2* alone is unlikely to cause stuck fermentations. First, only one functional transporter is required to support growth on glucose (Wieczorke et al. 1999). Second, by deleting individual *HXT* genes in an industrial strain of *S. cerevisiae*, it was shown that *HXT2* is involved in growth initiation during wine fermentations when the extracellular sugar concentration was 20% (Luyten et al. 2002). This observation implies that Hxt2p is not required at the end of fermentation when glucose and fructose concentrations are limited or depleted, and therefore mis-regulation of *HXT2* is likely not associated with the residual fructose remaining in stuck fermentations.

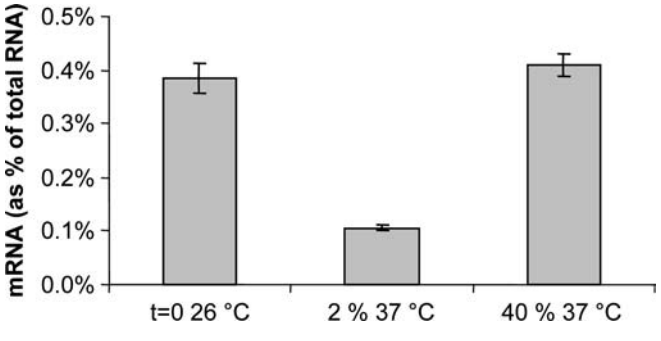

Fig. 7 Global mRNA decay is reduced in osmotically stressed yeast cells. Poly-adenylated mRNA molecules were purified from Dnase-treated total RNA derived from Y260 cells in mid-log phase at the permissive temperature or after 30 min at the non-permissive temperature with or without glucose-induced osmotic stress. mRNA levels are expressed as a percentage of total cellular RNA. The results represent the mean of three experiments and the *error bars* represent one standard deviation

Taken together, our results suggest that *HXT1, HXT3* and *HXT5* appear to be the major transporters at high sugar concentrations, making them most relevant during early stages of wine fermentations. Conversely, *HXT2, HXT4* and *HXT13* are expressed at low concentrations of extracellular glucose or fructose, suggesting these transporters could be important at the end of wine fermentations when only trace amounts of sugars remain. These observations do not entirely agree with the report of Luyten et al. (2002), which describes the role of individual transporters during the course of a wine fermentation, and further emphasizes the complexity of gene regulation of the *HXT* gene family. It could be that combinations of factors, including nitro-

gen availability, and/or ethanol concentration affect the in vivo performance of these transporter proteins. Alternatively, there may be protein–protein interactions among different transporters in vivo that are necessary for proper function and these interactions may affect the activity of these proteins when they are studied in isolation (Diderich et al. 1999; Ozcan and Johnston 1999).

Effect of pH on *HXT17* promoter activity

S. cerevisiae is able to proliferate in a wide range of external pHs. Growth is optimal at pH 4–5; the typical pH during wine fermentations ranges from 3 to 4. Growth in more alkaline conditions elicit a stress response in *S. cerevisiae*, and the transcriptional response to alkaline pH is at least partially mediated by Rim101p (Lamb et al. 2001). We observed an induction of *HXT17* promoter activity in response to a shift from pH 4.7 to 7.7 in a low-glucose environment (Fig. 3). The transcriptional response of *S. cerevisiae* to alkaline conditions has not been widely studied, although DNA microarray data have revealed that as many as 500 genes have altered expression in response to increasing extracellular pH (Causton et al. 2001; Lamb et al. 2001; Serrano et al. 2002). *HXT4* was induced within 5 min of shifting from pH 6.4 to pH 7.6 in rich media containing 2% glucose (Serrano et al. 2002). Additionally the expression levels of *HXT8, HXT9*, *HXT11* and *HXT12* were repressed at least threefold by these conditions. The significance of pH-dependent *HXT* expression is unclear; however, it is possible that individual transporters have altered affinities for glucose depending on the local proton concentration. The predicted isoelectric point for the alkaline-induced genes *HXT4* and *HXT17* is 6.37 and 6.95, respectively, indicating a net-negative charge at pH 7.6 or 7.7. Conversely, the isoelectric points for alkaline-repressed *HXT9*, *HXT11* and *HXT12* are 8.17, 8.54, and 8.48, respectively, indicating these transporters would have a net-positive charge. However, *HXT8*, which was reported as alkaline repressed with a p*I* of 5.32 (the lowest of all *HXT* genes), fails to support the pattern. Still, considering that it is only the extracellular portions of these transporter proteins that are exposed to changing pH, an examination of the net charge of these domains may prove interesting.

Based on recent reports, there appears to be a link between alkaline pH tolerance and iron and copper uptake. More specifically, several mutants that displayed a severe sensitivity to alkali were shown to be defective in gene products that are required for efficient copper and iron uptake (Serrano et al. 2004). Interestingly, the expression of high-affinity copper uptake genes is regulated by Mac1p (Jungmann et al. 1993), and a constitutively active allele of this protein $MAC1^{up1}$ was shown to increase the expression of *HXT17* more than twofold (Gross et al. 2000). However, it should be pointed out that when the same group used a copper chelator to mimic copper starvation (when *MAC1*-dependent genes are transcriptionally active), *HXT17* was only marginally induced (1.1–1.4 fold increased). Whether copper (or iron) uptake is involved in the transcriptional activation of *HXT17* remains to be seen. An interesting hypothesis would be that *HXT17* serves as a symporter for particular minerals (presumably copper and iron) along with glucose. Given that *HXT9* and *HXT11* were identified in a screen for multidrug resistance (Nourani et al. 1997) and *HXT1* and *HXT3* are suppressors of a potassium transport defect (Ko et al. 1993), it is conceivable that many of the *HXT* genes have additional (non-hexose) substrates.

HXT5, *HXT13* and *HXT15* are expressed during growth on ethanol or glycerol and ethanol together

The expression of *HXT5* is linked to growth rate in *S. cerevisiae* and previous studies have shown that cells growing on glycerol or ethanol exhibit increased *HXT5* expression relative to glucose-grown cells (Diderich et al. 2001; Verwaal et al. 2002). We observed an induction of *HXT5* when yeast cells were grown on non-fermentable carbon sources and also saw increased *HXT13* and *HXT15* expressions under the same conditions (Fig. 4). *HXT5* does not appear to be regulated by the glucose sensors Snf3p/Rgt2p (Verwaal et al. 2002) which is expected, given that many of the conditions that induce *HXT5* (low nitrogen, osmotic stress, heat stress) act independently of the extracellular glucose concentration. Our observations suggest that *HXT13* may be regulated in a similar manner. Although *HXT15* appeared to be induced on non-fermentable carbon sources, the low level of expression suggests this transporter may not be physiologically relevant under these conditions. Hxt5p has a moderate affinity for glucose ($K_m = 10$ mM) but no such data are available for Hxt13p. Our data show that *HXT13* is induced by 0.2% glucose (w/v) indicating it may be a high-affinity transporter, similar to *HXT2*, *HXT4* and *HXT6/7*. If this is the case, then Hxt5p and Hxt13p could serve as low- and high-affinity transporters, respectively, for the cell under conditions of glucose starvation. Further analysis of the promoters of *HXT5* and *HXT13* as well as a detailed biochemical study of the physical properties of these transporter proteins should help address this point. For example, a study of Hxt5p and Hxt13p protein turnover rates would be interesting because highly stable proteins would be ideally expressed under starvation conditions when transcriptional and translational activities are likely to be minimal.

HXT1 and *GPD1* mRNA is stabilized by glucose-induced osmotic stress

Others and we have observed that *HXT1* is up-regulated in response to osmotic stress (Hirayama et al. 1995; Rep et al. 1999b; Erasmus et al. 2003). Our observation that

HXT1 transcription is increased more than threefold in cells grown in 40% glucose versus 10% glucose (Fig. 5) is counter-intuitive given that the growth rate of yeast is reduced by the osmotic stress of 40% glucose. Because the glycolytic flux decreases concomitantly with the growth rate, there should already be sufficient glucose to meet cellular requirements at the level of 10%. To better understand *HXT1* promoter regulation, we compared the effect of osmotic shock caused by 40% glucose or 2% glucose with 8% NaCl. Both these media have similar water activity and therefore should inflict comparable osmotic stress upon yeast cells. The original intention was to delineate the extent of *HXT1* induction that was attributable to glucose and to osmotic stress. Using the same *HXT1–lacZ* construct, Ozcan and Johnston (1995) reported that *HXT1* reached maximal expression in cells grown in 4% glucose and remained constant up to 8%. We therefore suspected that the stress-dependent component of *HXT1* expression would require at least 10% glucose. First we observed that salt-induced osmotic stress could only activate the *HXT1* promoter in the presence of glucose (Fig. 5b). Rgt1p is a transcriptional repressor that restricts *HXT1* expression to glucose-containing environments (Ozcan et al. 1996). Since Hog1p is required for *HXT1* mRNA accumulation during osmotic stress (Rep et al. 1999b), our observation suggests that the HOG pathway is unable to overcome repression by Rgt1p. Furthermore, this result also indicates that the HOG pathway does not act directly on the *HXT1* promoter. Alternatively, osmotic stress activates a second activator or repressor on the *HXT1* promoter that acts in concert with Rgt1p (analogous to the dual repression of *HXT2* and *HXT4* by Rgt1p and Mig1p that restricts expression to low-glucose conditions). A recent report has confirmed this hypothesis, as the transcriptional repressor Sko1p was shown to bind and repress the *HXT1* promoter in the absence of Hog1p kinase activity (Tomas-Cobos et al. 2004). As little as 4% glucose or 2% glucose plus NaCl (0.4 M) was able to induce Sko1p derepression. This explains why other groups observed maximal *HXT1* promoter induction in cells grown in 4% glucose (Ozcan and Johnston 1995). Comparison of the *HXT1* promoter activity during short-term osmotic shock in 2% glucose versus 40% glucose revealed that *HXT1* was down-regulated by osmotic stress (Fig. 5a). These data are distinct from data shown in Fig. 2, since it reveals the immediate response of the promoter to the stress, rather than long-term adaptation of cells in the presence of 40% glucose as shown in Fig. 2. In order for mRNA molecules to accumulate despite lower promoter activity, the rate of turnover must be decreased. Indeed, when we tested the rate of decay of *HXT1* mRNA we found that the transcript persisted at higher levels after transcription shut-off during osmotic stress by 40% glucose compared to 2% glucose (Fig. 6).

Previous studies have demonstrated both derepression and activation mechanisms lead to *HXT1* expression (Ozcan and Johnston 1995; Tomas-Cobos et al. 2004). Here we show that there is a third component to the regulation—mRNA stabilization by osmotic stress. Given that *HXT1* was not induced by osmotic stress with galactose as the carbon source, our data and data provided by others suggest two factors contributing to the high level of *HXT1* mRNA accumulation during osmotic stress: (1) glucose-dependent promoter activation (Ozcan and Johnston 1999) and (2) osmotic stress-dependent mRNA stabilization. At this time we cannot predict what signal leads to mRNA stabilization under osmotic stress, but if there is a signal transduction pathway it does not require de novo transcription of a stabilizing factor, as *HXT1* mRNA was stabilized in the absence of ongoing transcription.

The paradigm for the transcriptional response to osmotic stress is increased *GPD1* expression (Rep et al. 1999a). In our study we found *GPD1* mRNA to be stabilized by sugar-induced osmotic stress to a similar extent as *HXT1* (Fig. 6b). An intriguing explanation for these observations is that the osmotic stress-mediated stabilization of *HXT1* and *GPD1* could occur to ensure an adequate supply of carbon for the production of glycerol, a compatible solute that is rapidly accumulated in osmotically stressed yeast cells (Blomberg and Adler 1992; Hohmann 2002).

As a reflection of cellular mRNA levels we isolated poly-adenylated molecules from yeast cells that had been without active transcription for 30 min in the presence of either 2 or 40% glucose. As a percentage of total cellular RNA, we observed a fourfold greater retention of poly-adenylated molecules in osmotically stressed yeast cells compared to non-stressed cells after transcription shut-off (Fig. 7). This implies that a large portion of *S. cerevisiae* mRNA molecules may have a decreased rate of turnover during exposure to osmotic stress. A similar observation has been made in glucose-starved yeast cells (Jona et al. 2000), indicating that it may be part of a general stress response. The same group also noticed that mRNA molecules were stabilized upon entry into stationary phase—and entry into stationary phase may be induced by stress exposure (Werner-Washburne et al. 1993; Rossignol et al. 2003). If this is true, then other stresses, to which yeast is exposed such as ethanol, should reduce mRNA turnover. Although this has not been demonstrated for *S. cerevisiae*, ethanol can stabilize TNFα mRNA in a p38-dependent manner in rat liver cells (Kishore et al. 2001, 2004).

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