Expression of the \textit{HXT1} Low Affinity Glucose Transporter Requires the Coordinated Activities of the HOG and Glucose Signalling Pathways\*  

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Expression of the \textit{HXT1} gene, which encodes a low affinity glucose transporter in \textit{Saccharomyces cerevisiae}, is regulated positively in response to glucose by the general glucose induction pathway, involving the Snf3/\textit{Rgt2} membrane glucose sensors, the SCF-Grr1 ubiquitination complex and the Rgt1 transcription factor. In this study we show that, in addition to the glucose signaling pathway, regulation of \textit{HXT1} expression also requires the HOG pathway. Deletion components in the glucose signaling pathway or in the HOG pathway results in impaired \textit{HXT1} expression. Genetic analyses showed that, whereas the glucose signaling pathway regulates \textit{HXT1} through modulation of the \textit{Rgt1} transcription factor, the HOG pathway modulates \textit{HXT1} through regulation of the Sko1-Tup1-Ssn6 complex. Coordinated regulation of the two signaling pathways is required for expression of \textit{HXT1} by glucose and in response to osmostress.

Yeast cells are able to adjust cellular metabolism, gene expression, and growth in response to environmental stimuli. For example, the presence of glucose, the most preferable carbon source, is able to elicit a complex metabolic response based in two major levels: i) allosteric modification of different enzymes and ii) regulation of gene expression. Transcriptional regulation varies from inhibition of expression (glucose repression) to activation of transcription (glucose induction) (see Refs. 1–4 for reviews). Some of the genes induced in response to glucose encode for glycolytic enzymes, ribosomal proteins, and glucose transporters. Expression of the low affinity glucose transporter \textit{HXT1} has been used as a model to study the process of transcriptional activation by glucose (3). Genetic and biochemical studies have defined several components that are involved in the regulation of \textit{HXT1} expression. Glucose availability in the surrounding media is assessed by the membrane glucose sensor proteins Snf3 and Rgt2. This signal is then transmitted to the SCF-Grr1 ubiquitination complex (5, 6), which finally modulates the activity of Rgt1, a transcription factor that belongs to the Cys6-Zinc cluster protein family, which acts as a transcriptional repressor in the absence of glucose (3, 7). Additional components of the glucose induction pathway are Std1 and Mth1, two proteins that modulate negatively \textit{HXT1} expression (3, 8); recent studies indicate that Std1 and Mth1 may interact with the C-terminal tails of the glucose sensors Rgt2 and Snf3 and with Rgt1 (9–11) and that the SCF-Grr1 complex is involved at least in the inactivation of Mth1, mediating in this way the glucose-induced dissociation of Rgt1 from \textit{HXT1} promoter and its activation (12). Moreover, data from several laboratories suggest the existence of an additional uncharacterized transcription factor, different from Rgt1, that regulates \textit{HXT1} gene expression (7).

Exposure of yeast cells to increases in extracellular osmolarity results in the activation of the Hog1 MAPK\textsuperscript{\dagger} pathway. Activation of the Hog1 MAPK induces diverse osmo-adaptive responses such as regulation of gene expression. Genome-wide transcriptional analyses showed that a great number of genes are regulated by osmotic stress in an \textit{HOG1}-dependent manner. Among these, there are genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (reviewed in Ref. 13). Several transcription factors have been reported to lie downstream of the MAPK, regulating different subsets of osmotic-stress-responsive genes by different mechanisms. The general stress response transcription factors Msn2/Msn4 and the transcriptional regulator Hot1 are important for the recruitment of the Hog1 MAPK to stress-inducible promoters (14, 15). On the other hand, modification of Smp1, a member of the MEF2 family of transcription factors, by Hog1 is important to modulate its transcriptional activity (16). Sko1, a member of the ATF-CREB family, inhibits transcription of several osmotic-stress-inducible genes through recruitment of the general co-repressor complex Tup1-Ssn6 (17–19). Sko1 is phosphorylated by the Hog1 MAPK upon stress, and this is crucial to switch Sko1-Tup1-Ssn6 from a repressor to an activator complex (20, 21).

In this work we show that regulation of \textit{HXT1} expression is achieved by two independent transcription factors, Rgt1 and Sko1, controlled by the glucose induction and HOG signaling pathways, respectively. Thus, induction of \textit{HXT1} gene expression in response to glucose and in response to osmotic stress...
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Experimental Procedures

Strains and Genetic Methods—Saccharomyces cerevisiae strains used in this study are listed in Table I. snf1Δ::KanMX-mutated alleles were obtained by gene disruption using a BamHI fragment from plasmid pUC-snflΔ::KanMX (11). In this study, TRP1-mutated alleles were obtained by gene disruption using plasmid pDG16 (22). rgt1Δ::TRP1-mutated alleles were obtained by gene disruption using plasmid pUC-rgt1Δ::URA3 (see below). All mutants were confirmed by PCR analysis using specific oligonucleotides. Standard methods for genetic analysis and transformation were used. Yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids, supplemented with different carbon sources.

Plasmids—Centromeric plasmid pC-HXT1-lacZ (LEU2) was described previously (23). The HXT1 expression cassette (HXT1 promoter fused to Escherichia coli lacZ gene) was subcloned into plasmids pRS313/His3, pRS314/Trp1, and pRS316/Ura3 (24). Plasmids pEG202-Rgt1 (LexA-Rgt1) and the corresponding empty vector pEG202 were described in a previous study (11). Plasmid pSH18–18 (6lexAop- lacZ) was described previously (25). To perform promoter analysis, PCR-generated DNA fragments containing several regions of the HXT1 promoter up to the ATG were cloned into Yip358R(Ura3) or Yip368R(Leu2) (26). To analyze internal promoter regions, different stretches from the 5′ upstream region were amplified by PCR and inserted into the CYC1-lacZ reporter construct pJS205 (27).

Plasmid pUC-rgt1Δ::URA3 was constructed in this way. Plasmid pUC-lacZ (11) was digested with BglII and dephosphorylated with calf intestinal phosphatase. In this way we removed a central 2806-bp region of RGT1, leaving 444 and 335 bp at 5′ and 3′ ends, respectively, as flanking regions. A BamHI fragment from plasmid YDP-U (28), containing the URA3 selection marker, was subcloned into the BglIII sites of the former plasmid to give pUC-rgt1Δ::URA3, which was digested with BamHI and Sall to obtain a linear fragment that was used in the disruption experiments.

Enzyme Assays—Cells growing exponentially in 2% raffinose plus 0.05% glucose were pulse-labeled with either 0.4 M NaCl (final concentration), 2% glucose (final concentration) or a combination of 0.4 M NaCl and 2% glucose (final concentration). At times 0 and 60 min, aliquots were taken from the cultures, and the β-galactosidase activity was assayed in permeabilized cells and expressed in Miller units as in Ref. 29. Values are means from three to four independent transformants (S.D. < 15% in all cases). TUP1-deficient strains flocculate and, thus, β-galactosidase activity was assayed in yeast extracts as in a previous study (30) and expressed in Miller units/mg of protein. Invertase activity was assayed in whole cells as described in a previous study (31).

Immunoblot Analysis—Preparation of protein extracts was essentially performed as described (30). The extraction buffer was 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM dithiothre- tose, dextrose) at 30 °C. Precipitation PCR assays were performed as described previously (14). In all ChIP experiments, yeast cultures were grown in raffinose to early log phase (∼1.0) before cells were exposed to 2% glucose or osmotic stress.

Results

The HOG Pathway Regulates HXT1 Gene Expression by Glucose and Osmostress—Expression of the HXT1 low affinity glucose transporter is regulated by glucose availability, being inhibited when glucose levels are scarce and activated in the presence of the sugar (see Ref. 3 for review). As shown in Fig. 1, cells growing exponentially in 2% raffinose showed very low levels of HXT1 expression (measured as a transcriptional fusion of the HXT1 promoter to the lacZ gene, encoding β-galacto- sidase enzyme; see “Experimental Procedures”). After a pulse of 2% glucose, expression of HXT1 was induced, in agreement with what it has been reported previously (see Ref. 3 for re- view). However, HXT1 induction rate was higher when cells...

Table I

| Strain | Genotype | Reference |
|--------|----------|-----------|
| TM141 (wild type) | MATα his3 leu2 trp1 ura3 | (16) |
| TM233 | MATα hog1Δ::TRP1 derivative of TM141 | (16) |
| snf1Δ | MATα snf1Δ::KanMX hog1Δ::TRP1 of TM141 | This study |
| TM257 | MATα phe2Δ::Ura3 derivative of TM141 | This study |
| FP50 | MATα ste11Δ::His3 ste22Δ::LEU2 ste32Δ::HIS3 his3 leu2 ura3 | (42) |
| FP57 | MATα sho1Δ::TRP1 ste11Δ::His3 derivative of TM141 | (42) |
| W303–1A (wild type) | MATα ade2 his3 leu2 trp1 can1 | (43) |
| msn2Δ::msn4Δ | MATα snf1Δ::KanMX derivative of W303 | This study |
| rgt1Δ | MATα rgt1Δ::KanMX derivative of W303 | This study |
| rgt1Δ::HXT1 | MATα rgt1Δ::KanMX derivative of W303 | This study |
| tup1Δ::KanMX | MATα tup1Δ::KanMX derivative of W303 | This study |
| sho1Δ | MATα sho1Δ::KanMX derivative of W303 | (18) |
| sho1Δ::HXT1 | MATα sho1Δ::KanMX derivative of W303 | (18) |
| sho1Δ::rgt1Δ::URA3 | MATα sho1Δ::KanMX derivative of W303 | This study |
| MSY401 (wild type) | MATα his3 leu2 trp1 ura3 | (5) |
| MSY441 | MATα snf3Δ::hisG rgt2Δ::HIS3 derivative of MSY401 | (9) |
| MSY192 | MATα std1Δ::HIS3 mth1Δ2 derivative of MSY401 | (9) |
| std1Δ::mth1Δ2::HOG1 | MATα std1Δ::HIS3 mth1Δ2 hog1Δ::TRP1 derivative of MSY401 | This study |
| ENY.ura3Δ-1A (wild type) | MATα his3 leu2 trp1 ura3 | (45) |
| ENY.cat80-8b | MATα gro1Δ (cat80–24) derivative of ENY.WA-1A | (45) |
| LC99 | MATα ura3 leu2 his3 rgt1Δ::KAN Yip386R-HXT1 (URA3) | This study |
| LC91 | MATα ura3 leu2 trp1 his3 rgt1Δ::KAN Yip358R HXT1 (URA3) | This study |
HOG Pathway Regulates Yeast HXT1 Expression

Fig. 1. The Hog1 MAPK plays a major role in the induction of HXT1 expression by glucose. Wild type (TM141) and hog1Δ (TM233) cells were transformed with plasmid pC-HXT1-lacZ. Transformants were grown to mid-logarithmic phase in selective SC-2% raffinose plus 0.05% glucose medium. β-Galactosidase activity was assayed in cells 60 min after a pulse of either 2% glucose, 0.4 M NaCl, or 0.4 M NaCl plus 2% glucose. Values are mean β-galactosidase activities from four to six transformants (bars represent S.D.).

were subjected simultaneously to 2% glucose plus 0.4 M NaCl (Fig. 1). Similar results were obtained when 1 M sorbitol was used instead of NaCl (data not shown). These results were in agreement with data from microarray analyses that indicated that HXT1 expression was enhanced after treatment with 0.4 M NaCl (33), 1 M NaCl (34), or 1 M sorbitol (35) in the presence of glucose. It is worth noting that no induction of HXT1 expression was observed if cells were subjected only to osmotic stress in the absence of glucose (Fig. 1).

To determine if the HOG pathway was responsible for over-induction of HXT1 expression in response to glucose plus osmotic stress, we analyzed HXT1-lacZ expression in a hog1Δ mutant strain (Fig. 1). To our surprise, HXT1 expression was not induced even by glucose alone, indicating that the Hog1 protein kinase was required not only to overinduce HXT1 expression by glucose plus osmotic stress but also to regulate HXT1 expression by glucose. The absence of induction by glucose in a hog1Δ mutant was not due to a delay in the rate of induction, because when cells were grown overnight in 2% glucose, hog1Δ mutant cells still showed very reduced levels of HXT1 expression in comparison to wild type cells (data not shown). A recovery in the induction of HXT1 by glucose was obtained if hog1Δ mutants were transformed with a plasmid carrying a wild type Hog1 kinase, but not with a plasmid with a catalytically inactive form (Hog1IKS-KN) (data not shown), indicating that the activity of the Hog1 kinase was necessary to allow induction of HXT1 expression by glucose. In contrast to HXT1, expression of HXT2, encoding an intermediate-affinity glucose transporter that is repressed by glucose, was not affected in a hog1Δ strain (data not shown), indicating that the action of Hog1 was specific on HXT1 expression.

Snf1 protein kinase activity affects negatively HXT1 expression (11). To rule out the possibility that the absence of Hog1 kinase could stimulate the activity of the Snf1 kinase and then inhibit HXT1 expression, we studied the activity of Snf1 protein kinase in a hog1Δ mutant by analyzing the regulation of the expression of SUC2 (a glucose repressed gene) and found that it was similar to wild type (Table II). More importantly, induction of HXT1 expression by glucose in a double hog1Δsnf1Δ mutant was similar to the hog1Δ mutant (Table II). These results indicated that the defect in the induction of HXT1 by glucose in hog1Δ cells was not related to the activation of Snf1 protein kinase and that the Hog1 MAPK played a crucial role in the regulation of HXT1 induction by glucose.

Osmostress Caused by Extracellular Glucose Results in Hog1 Activation and Induction of HXT1 Gene Expression—To analyze whether only the Hog1 MAPK or the integrity of the HOG pathway was needed for the induction of HXT1 by glucose, we followed HXT1 expression in mutants on several components of the HOG pathway. As shown in Fig. 2A, deletion of the PBS2 MAPK kinase or simultaneous deletion of the three MAPK kinase kinases of the HOG pathway, STE11, SSK2, and SSK22, abolished induction of HXT1 by glucose. Thus, the integrity of the main core of the HOG pathway is required for HXT1 induction by glucose.

Two upstream sensing mechanisms activate the core of the HOG pathway, the Snf1 “two-component” osmosensor and a second mechanism that involves the Sho1 transmembrane protein (36). Mutations in the Sho1 branch (double sho1Δste11Δ mutant) did not alter HXT1 expression (Fig. 2A). However, mutants in the Snf1 branch of the HOG pathway (ssk2Δssk22Δ or ssk1Δ data not shown) showed a clear defect in the induction of HXT1 by glucose (Fig. 2A). It is worth noting that, on the later strains, induction of HXT1 was similar to wild type only when both glucose and NaCl were added (Fig. 2A). These results indicate that induction of HXT1 expression by glucose is mediated by the Snf1 branch of the HOG pathway.

Activation of the Hog1 MAPK by phosphorylation has been described to occur in response to osmotic stress (see the introduction). To test whether glucose “per se” or the osmotic stress caused by the addition of 2% glucose to the medium was responsible for Hog1 activation, we followed Hog1 phosphorylation in response to the addition of sugar. As shown in Fig. 2B, addition of 2% (110 mM) glucose or 2% galactose to raffinose growing cells induced Hog1 phosphorylation to the same extent as treatment with 110 mM NaCl. Moreover, addition of higher concentrations of glucose or galactose led to higher levels of Hog1 phosphorylation (data not shown). As expected, phosphorylation of Hog1 by sugar occurred in wild type cells but not in pbs2Δ cells (Fig. 2B). Time course experiments showed that phosphorylation of Hog1 by glucose was transient (Fig. 2C), as it has been described for NaCl (13). Thus, Hog1 activation is caused by an increase in extracellular osmolarity caused by the addition of sugar, not necessarily restricted to glucose.

Because the presence of glucose was always necessary to stimulate HXT1 expression and because in the absence of an active HOG pathway no induction of HXT1 was observed (Figs. 1 and 2), we suggest the possibility that the addition of 2% glucose to raffinose growing cells would elicit two different signals, one that would be transmitted through the glucose induction pathway (see below) and another, where glucose would act as an osmolite that would activate the Snf1 branch of

Table II

| Strain | (HXT1-lacZ) β-galactosidase | (SUC2) invertase |
|--------|----------------------------|---------------|
| Wild type | 10.0 ± 0.4 | <1 |
| snf1Δ | 114.8 ± 0.4 | <1 |
| hog1Δ | 10.4 ± 0.4 | <1 |
| snf1Δ hog1Δ | 13.2 ± 0.4 | <1 |
the HOG pathway, more sensitive to osmotic changes in the environment (13).

**Induction of HXT1 Expression upon Glucose Plus Osmotic Stress Depends on the Integrity of the Glucose Signaling Pathway**—Induction of HXT1 by glucose depends on the glucose signaling pathway (3). Then, we wanted to test whether integrity of the glucose signaling pathway was required to allow overinduction of HXT1 in response to glucose plus osmostress. Inactivation of the membrane glucose sensors Snf3 and Rgt2, and the SCF-Grr1 ubiquitination complex abolished HXT1 expression by both glucose and osmostress (Fig. 3A). In contrast, deletion of the MTH1 and STD1 genes, known regulators of Rgt1 transcriptional repressor (8), resulted in constitutive expression of HXT1. However, in the double std1Δmth1Δ mutant, osmostress but not glucose, was able to induce HXT1 expression at even higher levels in a Hog1-dependent manner (Fig. 3A). Thus, integrity of the main core of the glucose signaling pathway (Snf3/Rgt2 and SCF-Grr1) is essential to allow overinduction of HXT1 in response to glucose plus osmostress. If repressing properties of Rgt1 are avoided (std1Δmth1Δ mutants), then the HOG pathway may overinduce HXT1 expression in response to osmostress.

Deletion of RGT1 repressor resulted in a mild deregulation of HXT1 expression in absence of glucose and no further induction by glucose (Ref. 3 and Fig. 3B). In contrast, a clear induction of HXT1 expression was observed by NaCl alone or by glucose plus NaCl (Fig. 3B). These effects were dependent on the presence of Hog1 kinase, because in the double rgt1Δhog1Δ mutant no induction of HXT1 was observed under any condition (Fig. 3B). Thus, in the absence of the Rgt1 transcriptional repressor, activation of the HOG pathway by osmostress leads to full HXT1 induction, even in the absence of glucose.

An alternative explanation for the results presented so far was that the function of Rgt1 could be regulated directly by the Hog1 kinase. However, this was unlikely, because when we tested the transcriptional properties of a LexA-Rgt1 fusion, these were similar in both wild type and hog1Δ mutant (Table III).

**Regulation of HXT1 Expression by the HOG and Glucose Signaling Pathways Is Exerted at Different Sites on the HXT1 Promoter**—As shown above, induction of HXT1 by glucose and osmotic stress requires the activation of two independent signaling pathways, glucose induction and HOG pathways. To identify the upstream control region of HXT1 that are important for regulation by any of these pathways, we investigated the expression of a set of segments of the HXT1 pro-
moter fused to the lacZ gene, in cells grown in raffinose and then pulsed with glucose, NaCl, or glucose plus NaCl, as above (Fig. 4). Insertion of a fragment of ~200 bp (from ~223 to ATG) to the Yip358R reporter vector gave high levels of β-galactosidase activity in any of the conditions tested, whereas insertion of larger fragments (~1200 to ATG or ~821 to ATG) resulted in strong repression under basal conditions (raffinose-growing cells) and strong induction in response to glucose or to glucose plus NaCl (Fig. 4B). These results indicated that regulation of HXT1 expression consists mainly of a derepression process. Because we observed a similar derepression pattern when we assayed a fragment containing from ~821 to ATG in comparison to full-length HXT1 promoter (from ~1200 to ATG), we suggest that the fragment comprised between ~821 to ~223 contained the main regulatory elements of HXT1.

Further deletion analysis showed that a fragment containing from ~521 to ATG was not induced by glucose, indicating that in the ~821 to ~521 region there must be sequences related to the induction of HXT1 by glucose. We also observed that this ~521 to ATG fragment was not induced by NaCl alone, but it was fully induced by glucose plus NaCl (Fig. 4B). Interestingly, deletion of RGT1 allowed full induction of this ~521 to ~223 contained the main regulatory elements of HXT1.

Further deletion analysis showed that a fragment containing from ~521 to ATG was not induced by glucose, indicating that in the ~821 to ~521 region there must be sequences related to the induction of HXT1 by glucose. We also observed that this ~521 to ~223 contained the main regulatory elements of HXT1.

TABLE III
Transactivating properties of Rgt1 are not affected in a hog1Δ mutant
Wild type (TM141) and hog1Δ (TM233) cells were transformed with plasmid pSH18-18 (containing 6LexAop-lacZ) and either plasmid pEG202 (LexA) or pEG202-Rgt1 (LexA-Rgt1). Transformants were grown to mid-logarithmic phase in selective SC-4% glucose medium. Values are mean β-galactosidase activities from four to six transformants (S.D. < 15% in all cases).

|                         | LexA | LexA-Rgt1 |
|-------------------------|------|----------|
| Wild type               | <1   | 1940     |
| hog1Δ                   | <1   | 2153     |
located between −521 and −426 and that the HOG pathway affected another putative repressor that interacted with a promoter region located between −426 and −223.

**Sko1 Transcription Factor Regulates HXT1 Expression under the Control of the HOG Pathway**—As just mentioned, analysis of the HXT1 promoter suggested the presence of an uncharacterized transcription factor regulated by HOG pathway that repressed HXT1 expression. Inspection of the HXT1 promoter did not yield any sequence known to be regulated by specific transcription factors other than stress response elements. STRE elements are known to be binding sites for Msn2 and Msn4 transcription factors (37). However, when we tested HXT1 expression in yeast cells deficient in both MSN2 and MSN4 genes, we observed a similar pattern of HXT1 expression, compared with the wild type strain (data not shown).

To identify the additional repressing factor that regulates
HXT1 expression, we conducted a mutant screening on the basis of the assumption that simultaneous inactivation of RGT1 and the unknown transcriptional repressor would render HXT1 expression constitutively activated. Briefly, rgt1Δ cells growing on raffinose and containing an integrated HXT1-lacZ reporter construct were mutagenized with MNNG, and positive clones were selected by their ability to induce HXT1 expression and, therefore, to produce β-galactosidase on X-gal-containing plates (described under “Experimental Procedures”). In this way, 30 positive clones were identified from 55,000 colonies. Recessive mutants were selected and classified into a number of complementation groups. Three of the largest complementation groups were identified as ssn6Δ, tup1Δ, and sko1Δ mutants by complementation cloning.

We then tested the effect of the deletion of SKO1 in cells carrying the centromeric HXT1-lacZ reporter construct. As shown in Fig. 5A, deletion of SKO1 resulted in cells able to induce HXT1 expression in response to glucose, but no further induction of HXT1 expression was observed by the combined action of glucose plus osmotic stress. Moreover, a double sko1Δ hog1Δ mutant strain showed the same pattern of expression as the sko1Δ strain, indicating that the lack of expression of HXT1 in a hog1Δ in response to glucose (Fig. 1) was caused by the inability of this strain to release Sko1 repression. Therefore, Sko1 mediates Hog1 regulation of HXT1 expression.

Apart from sko1Δ mutants, we identified in our screening mutations in TUP1 and SNN6 genes. It is known that the Tup1-Ssn6 general co-repressor complex interacts with Sko1 to repress transcription of osmostress-regulated genes (17, 18). In addition, it is also known that the Tup1-Ssn6 complex interacts with Rgt1 to repress transcription of HXT1 in low glucose conditions (11, 38). Consistent with these observations, mutations in TUP1 or SNN6 resulted in constitutive expression of HXT1 that was not significantly enhanced by addition of glucose or NaCl (Fig. 5B; data not shown for the ssn6Δ mutant). As expected, deletion of HOG1 in a tup1Δ strain did not affect HXT1 expression. Therefore, our data suggest that two transcriptional repressors, Sko1 and Rgt1, are controlling HXT1 gene expression by their binding to the Tup1-Ssn6 complex.

Sko1 Controls HXT1 Transcription by Direct Binding to the Promoter—Chromatin immunoprecipitation (ChIP) analyses have shown that the Hog1 MAPK is actively recruited to os-

**Fig. 5.** The Sko1-Tup1-Ssn6 complex controls HXT1 expression under the control of the Hog1 MAPK. A, Hog1 regulates HXT1 expression through the Sko1 transcriptional factor. Wild type (W303–1A), sko1Δ, hog1Δ, and sko1Δhog1Δ strains (A), and wild type (W303–1A), tup1Δ and tup1Δhog1Δ cells (B) were transformed with the appropriate centromeric pHXT1-lacZ plasmids. Transformants were treated and analyzed as in Fig. 1. In A: values are mean β-galactosidase activities from four to six transformants expressed in Miller units. In B: β-galactosidase activity (units/mg) was measured in cell extracts; values are mean β-galactosidase activities from four to six transformants (bars represent S.D.).
most stress responsive promoters (14, 21). Consistently, our ChIP analyses showed that Hog1 was also recruited to the HXT1 promoter in response to osmostress (data not shown). To test whether Sko1 was also present at the HXT1 promoter in cells growing in raffinose, samples for ChIP analyses were taken before (Raffinose) or after 15 min of a pulse of 2% glucose (Gluc), 0.4 M NaCl (NaCl), or 2% glucose plus 0.4 M NaCl (Gluc + NaCl). Immunoprecipitations were performed by using mouse anti-HA monoclonal antibodies. PCR was performed with primers spanning the promoter region of HXT1, GRE2, and control oligonucleotides spanning the GAL1 gene region. The exact primer sequences are available upon request. Control lanes show DNA amplified from extracts from cells without tagged protein (Control), or prior to immunoprecipitation (WCE). Data represents -fold increase over control without tag. Quantification was performed using Quantity One software from Bio-Rad.

ChIP analyses from several groups have described the presence of Rgt1 at the HXT1 promoter in the absence of glucose and its release in response to a pulse of glucose (7, 12, 39). Consistent with these results, we found Rgt1 present at the HXT1 promoter in cells growing in raffinose, but its binding was not affected by osmostress (Fig. 6B). As expected, binding of Rgt1 to the HXT1 promoter was diminished in the presence of glucose plus NaCl (Fig. 6B). Taking all these results together, we suggest that in cells growing in low glucose conditions, Sko1 and Rgt1 are present at the HXT1 promoter and co-repress gene transcription.

Regulation of HXT1 Expression Is Mediated by the Coordinated Regulation of Rgt1 and Sko1 Transcriptional Activities—Analysis of the HXT1 promoter (see above) showed that a small region between −521 to −223 contained possible Rgt1 and Sko1 regulatory elements that could be critical to understand the relationship between the HOG and glucose signaling pathways in the regulation of HXT1 expression. To analyze this relationship at the promoter level, we investigated a promoter fragment of HXT1 containing from −521 to −220 in a CYC1-lacZ reporter vector under the same growth conditions as above. As shown in Fig. 7, this 301-bp fragment was able to repress transcription of the CYC1-lacZ system in low glucose medium and derepressed transcription in response to glucose or to glucose plus NaCl, similarly to what we observed when we used the full-length promoter in a wild type strain (Fig. 1). Rgt1 was still able to play a negative role in the regulation of this fragment in low glucose, because deletion of RGT1 increased expression under this condition. Interestingly, osmostress, but not glucose, fully induced expression of the reporter in an rgt1Δ strain, indicating that, when there is no Rgt1, the release of Sko1 by the activation of the HOG pathway results in full expression of the reporter. Consistently, the lack of Sko1 results in defective derepression by NaCl and no overinduction of the reporter by the combined action of glucose plus osmostress. In addition, the simultaneous deletion of RGT1 and SKO1 led to constitutive expression of the reporter construct under any condition. Therefore, Rgt1 and Sko1 acted independently but coordinately to regulate expression of HXT1 in response to glucose and osmostress. Our results also suggest that full HXT1 expression requires the activity of both glucose induction and HOG signaling pathways to eliminate both repressing activities, Rgt1 and Sko1.

**DISCUSSION**

Yeast cells are able to adjust cellular metabolism, gene expression, and growth in response to environmental stimuli. In this sense, *S. cerevisiae* can deal with an extremely broad range of sugar concentrations and can metabolize glucose, its most preferable carbon source, from higher than 1.5 M (as in drying fruits) down to micromolar concentrations. To be adapted to any environmental sugar condition, yeast have developed an unusual diversity of glucose transporter proteins (17 different Hxt’s) with specific individual properties and kinetics. *S. cerevisiae* has from low affinity glucose transporters such as HXT1 and HXT3 (Km from 50 to 100 mM), that function when there is a good supply of sugar, to intermediate affinity transporters such as HXT2 and HXT4 (Km around 10 mM), and high affinity transporters such as HXT6 and HXT7 (Km around 1 mM), that function when the amount of the sugar is becoming scarce. Expression of all these transporter genes is tightly regulated at
the transcriptional level by the amount of substrate in the environment. Thus, the expression of \textit{HXT1}, a low affinity glucose transporter, is induced in the presence of glucose, whereas the expression of \textit{HXT2} (intermediate affinity) and \textit{HXT6} (low affinity) glucose transporters is repressed by the presence of the sugar (see Refs. 3, 40, 41 for review).

In this report, we show that full induction of \textit{HXT1} expression requires the coordinated action of two independent signaling pathways, the glucose signaling and HOG signaling pathways. A plausible interpretation of this result could be that by increasing the expression of \textit{HXT1} by hyperosmotic conditions, yeast could provide more substrate (glucose) for the synthesis of the osmoprotectant glycerol (see Ref. 13 for review on glycerol biosynthesis) to cope with the osmostress conditions. Activation of the glucose signaling pathway is mediated by the transmembrane glucose sensors Snf3 and Rgt2. On the other hand, activation of the HOG pathway can be mediated by two independent sensing systems: the two-component sensor that involves the Sln1 histidine kinase and the Sho1 sensing system (36). It has been shown that both systems are capable of leading to Hog1 activation in response to changes in the extracellular osmolarity, however, they seem to react slightly different. The Sln1 sensor is able to sense small changes in the environment and induce progressive Hog1 activation, whereas the Sho1 sensing system induces full response but only once a threshold level of osmotic stress in the environment is reached (22). The different sensitivity of the two osmosensing systems was already studied under laboratory conditions, but the physiological meaning of this different sensitivity has not been completely understood. Here, we show that small changes in extracellular sugar concentration, which result in small changes in extracellular osmolarity, are sufficient to induce Sln1-mediated Hog1 activation, whereas these changes are not high enough to induce the Sho1-sensing system (Fig. 2). This different sensitivity of the two signaling systems might have a significant physiological role, because if under specific conditions only a partial activation of Hog1 MAPK is required, a fine tuning mechanism would avoid full induction of adaptive responses that might be too energy-consuming for the cell.

Activation of the glucose signaling pathway by the presence of glucose leads to regulation of the Rgt1 transcriptional repressor. However, regulation of Rgt1 is not sufficient to induce gene expression by glucose without simultaneous activation of the HOG pathway. We also present strong evidence that the action of the HOG pathway is conducted via the Hog1 MAPK and the Sko1 transcriptional repressor. Our results also suggest that both repressors, Rgt1 and Sko1, interact with different regions of the \textit{HXT1} promoter. We suggest that Rgt1 interacts, at least, with a promoter region located between −521 and −426. In fact this region is included in the fragment that was used to demonstrate a direct interaction of Rgt1 with \textit{HXT1} promoter by either DNA binding (38) or ChIP (7) analyses (fragment from −648 to −361). This region contains an spaced CGG pair sequence (−460CGG−450CGG) that fulfils the requirements of the consensus sequence identified to be necessary for Rgt1 binding (39). Moreover, additional sites for Rgt1 binding must exist, because a promoter fragment containing only from −521 to ATG was not able to be properly induced by glucose. Because we have demonstrated that a promoter region from −821 to ATG contains all the regulatory regions of \textit{HXT1}, we suggest that additional Rgt1 binding sites must be located in this −821 to −521 region. In fact we identified several spaced CGG pairs in this region (−455CGG−772CGG; −766CGG−736CGG). Thus, the −821 to ATG fragment would contain at least three spaced CGG pairs, in agreement with the described requirements for proper Rgt1 binding (39).

We also suggest that Sko1 interacts with a promoter region located between −426 and −223. However, we did not find any consensus Sko1-CRE site (TGACGTCA) in this region. Because the \textit{HAL1} promoter contains a degenerated CRE site (TTACG-...
The interaction of Sko1 with the HXT1 promoter in low glucose conditions. The addition of NaCl decreases the binding of Sko1 to the HXT1 promoter and improves the binding of the Hog1 MAPK, similarly to what it has been described for other osmostress-inducible genes (20, 21). Rgt1 also binds to HXT1 promoter in low glucose conditions, but addition of NaCl does not affect its binding. Because Rgt1 binding is only decreased by glucose (7, 12, 39), we suggest that the addition of glucose to raffinose growing cells would have a dual effect. On one hand, it would release Rgt1 from the promoter and, on the other hand, acting glucose as an osmolite, it would activate the Hog pathway and would release Sko1 from the promoter, allowing in this way the derepression of HXT1. Consistent with this suggestion, we have found that the addition of higher concentrations of glucose (4%) or the combined action of 2% glucose plus 0.4 M NaCl improved HXT1 expression.

It has been described that Sko1 inhibits transcription of several osmostress-inducible genes through recruitment of the general co-repressor complex Tup1-Ssn6 (17–19). Sko1 is phosphorylated by the Hog1 MAPK upon stress, and this is crucial to switch Sko1-Tup1-Ssn6 from a repressor to an activator complex (20, 21). At the same time, it is known that the Tup1-Ssn6 complex interacts with Rgt1 and plays a major role in repression expression of HXT1 under low glucose conditions (11, 38). Therefore, the Tup1-Ssn6 co-repressor complex seems to play a dual role in the regulation of HXT1 expression. On one hand, it helps Sko1 to repress transcription under non-osmotic stress conditions and, on the other hand, it helps Rgt1 to repress transcription in the absence of glucose. Consistent with these suggestions, mutations in TUP1 or SSN6 resulted in constitutive expression of HXT1 that was not significantly enhanced by the addition of either glucose, NaCl, or both.

Taking all the results together, we propose the following model for HXT1 gene regulation (Fig. 8). Under normal conditions (low glucose and no osmostress), HXT1 promoter would be repressed by two independent repressors, Rgt1 and Sko1. In response to glucose addition, two different pathways would activate HXT1 gene expression. Glucose would directly activate the glucose signaling pathway, which would mediate regulation of the Rgt1 repressor, and the osmostress caused by the addition of glucose would result in activation of the Hog1 MAPK that would result in regulation of the Sko1 repressor by the MAPK. Thus, the activity of two independent signaling pathways would converge in the regulation of HXT1 expression by glucose and osmostress.

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