

Feedback Regulation of Glucose Transporter Gene Transcription in *Kluyveromyces lactis* by Glucose Uptake

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In the respirofermentative yeast *Kluyveromyces lactis*, only a single genetic locus encodes glucose transporters that can support fermentative growth. This locus is polymorphic in wild-type isolates carrying either *KHT1* and *KHT2*, two tandemly arranged *HXT*-like genes, or *RAG1*, a low-affinity transporter gene that arose by recombination between *KHT1* and *KHT2*. Here we show that *KHT1* is a glucose-induced gene encoding a low-affinity transporter very similar to *Rag1p*. *Kht2p* has a lower K_m (3.7 mM) and a more complex regulation. Transcription is high in the absence of glucose, further induced by low glucose concentrations, and repressed at higher glucose concentrations. The response of *KHT1* and *KHT2* gene regulation to high but not to low concentrations of glucose depends on glucose transport. The function of either *Kht1p* or *Kht2p* is sufficient to mediate the characteristic response to high glucose, which is impaired in a *kht1 kht2* deletion mutant. Thus, the *KHT* genes are subject to mutual feedback regulation. Moreover, glucose repression of the endogenous β -galactosidase (*LAC4*) promoter and glucose induction of pyruvate decarboxylase were abolished in the *kht1 kht2* mutant. These phenotypes could be partially restored by *HXT* gene family members from *Saccharomyces cerevisiae*. The results indicate that the specific responses to high but not to low glucose concentrations require a high rate of glucose uptake.

Most organisms have evolved sophisticated regulatory strategies to adapt their metabolism to the availability of nutrients. Substrate uptake is a first key function that is regulated. Signals that control substrate uptake depend on the nature and concentration of available nutrients and the nutritional state of the cell. Since substrate uptake feeds back on the nutritional state, a regulatory circuit exists, the components of which are only beginning to be understood even in such intensely studied pathways as glycolysis in *Saccharomyces cerevisiae* (see reference 20 for a recent review).

S. cerevisiae cells are apparently able to sense the extracellular glucose concentration and transmit the signal over the membrane into the cytoplasm (20, 32). Two types of receptors have been proposed: hexose transporter-like receptors, which are involved in controlling hexose transporter (*HXT*) gene expression (30), and a G protein-coupled receptor that is required for the activation of the protein kinase A signaling pathway by cyclic AMP (22, 32, 36).

Intracellularly, glucose-phosphorylating enzymes play an important role in glucose regulation. Whether these enzymes exert their influence on glucose regulation through their metabolic activity or whether they function as intracellular signaling molecules is still a controversial issue (13, 15, 19, 33).

A signaling function has clearly been established for the

galactose-phosphorylating enzyme of the yeast *Kluyveromyces lactis*. This galactokinase (*KIGal1p*) is required to activate transcription of lactose and galactose metabolic genes (26). Upon binding of its substrates galactose and ATP, *KIGal1p* can interact with the *KIGal80* protein, an inhibitor of the transcription activator *KIGal4p* (an ortholog of *S. cerevisiae* Gal4p) (45). *KIGal1p-KIGal80p* interaction relieves *KIGal4p* inhibition and does not require galactokinase enzymatic activity. The *KIGAL80* gene is also under control of *KIGal4p*, and *KIGAL80* induction counteracts *KIGal4p* activation (44). Thus, the dynamics of induction depends crucially on the dynamics of *KIGal80p* inactivation. This, in turn, depends on the rate of lactose and galactose uptake, since the signaling molecule is intracellular galactose.

The induction process can be impaired in the presence of glucose if the concentration of *KIGal4p* is below a critical threshold (34, 43). Transcription of the *LAC4-LAC12* genes, encoding β -galactosidase and lactose permease, respectively, and controlled by *KIGal4p* from a large bidirectional promoter (9, 17, 35), is particularly sensitive to glucose.

By screening for reduced β -galactosidase expression in glucose-lactose medium, mutations in glucose transporters were obtained that reduced the inhibitory effect of glucose (38).

By complementation of these mutants, two new *K. lactis* hexose transporter genes were isolated, *KHT1* and *KHT2* (38). These genes are closely linked and tandemly transcribed. Mutations in any of these genes slightly reduced the repression by glucose of lactose induction, whereas in the *kht1 kht2* double mutant, the glucose effect was completely abolished. Not only the inducible *LAC/GAL* regulon, but also glucose repression of lactate dehydrogenase and malate dehydrogenase, was affected in the *kht1 kht2* mutant.

KHT1 and *KHT2* map to the same chromosomal locus as the low-affinity glucose transporter gene *RAG1* described earlier

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TABLE 1. Yeast strains used in this study

| Strain | Relevant characteristics | Parent strain(s) | Source or reference |
|----------------------|--|---|---------------------|
| <i>K. lactis</i> | | | |
| JA6 | α <i>ade1-600 adeT-600 trp1-11 ura3-12</i> | SD11.U2 \times W600B | 9 |
| JA6/DL4R | <i>lac4::ura3</i> | JA6/DL4 (<i>lac4::URA3</i>) fluoroorotic acid-resistant derivative | 17 |
| JA6/CM57 | Chromosomal integration of <i>KHT1-lacZ</i> fusion | JA6 (integration of pBM3157) | This work |
| JA6/CM58 | Chromosomal integration of <i>KHT2-lacZ</i> fusion | JA6 (integration of pBM3158) | This work |
| JA6/DT12R | <i>kht1::ura3::kht2</i> | JA6/DT12 (<i>kht1::URA3::kht2</i>) fluoroorotic acid resistant derivative | 38 |
| DT12R/57 | Chromosomal insertion of <i>KHT1-lacZ</i> fusion | JA6/DT12R integration of pBM3157 | This work |
| DT12R/58 | Chromosomal insertion of <i>KHT2-lacZ</i> fusion | JA6/DT12R integration of pBM3158 | This work |
| ST105 | <i>RAG1</i> | AWJ-8c \times MG1/2 | 38 |
| ST106 | <i>rag1::URA3</i> | ST105 | 38 |
| <i>S. cerevisiae</i> | | | |
| EBY.VW4000 | Δ <i>hxt1-17 gal2 stl1 agt1 mph2-3</i> | CEN.PK2-1C | 40 |

(11). The sequence of *RAG1* is almost identical to that of *KHT1*, except for the 3' end (encoding K-A-M-L in *RAG1* and K-R-F in *KHT1*), which is identical to the 3' end of *KHT2*, indicating that *RAG1* arose by recombination between *KHT1* and *KHT2* (38).

The presence of *KHT1* and *KHT2* correlated with a higher sensitivity to glucose repression found in only a few *K. lactis* strains (7). A natural isolate that was entirely insensitive to glucose repression was shown to carry a defective *rag1* allele (18). These findings suggested that glucose repression in *K. lactis* depends on particular glucose transporters.

The *KHT1/RAG1-KHT2* gene cluster is the only genetic locus in the respirofermentative yeast *K. lactis* that contains glucose permeases relevant for fermentative metabolism.

Another hexose transporter gene, *HGT1*, encoding a high-affinity transporter has a transport capacity too low to suppress the so-called "Rag⁻" phenotype of *rag1* mutants (Rag⁺ = resistant against antimycin A on glucose) (18). A *rag1 hgt1* double mutant like the *kht1 kht2* mutant is still able to grow on glucose as a carbon source, indicating the presence of more still unidentified glucose transporters (5). Here we extend the studies on glucose transport in *K. lactis* by characterizing the Kht1p and Kht2p transport kinetics and by analyzing regulation of the *KHT1* and *KHT2* genes. We show that transporter gene regulation is influenced by transport activity and that Kht1p and Kht2p mutually control each other, depending on

glucose availability. In the absence of both transporters, the high glucose response is impaired, supporting the view that the rate of sugar transport is a crucial parameter in intracellular glucose signaling.

MATERIALS AND METHODS

Strains and culture conditions. The yeast strains used in this study are listed in Table 1. Strains JA6/CM57, JA6/CM58, DT12R/57, and DT12R/58 arose from ectopic integration of *Bam*HI-linearized plasmids pBM3157 and pBM3158, respectively, into the genomes of strains JA6/DL4R (*lac4*) and JA6/DT12R (*kht1 kht2*). JA6/CM57 and JA6/CM58 carry single integrations, as shown by Southern blot hybridization, whereas DT12R/57 and DT12R/58 carry multiple copies. Cells were grown in batch culture at 30°C in synthetic minimal medium containing (per liter) 6.7 g of yeast nitrogen base (YNB) without amino acids (Difco) supplemented with required amino acids and bases. Carbon sources were routinely added at 2% (wt/vol) for glucose and galactose and 3% (wt/vol) for glycerol. Solid media were prepared by adding Bacto agar (Difco) to a final concentration of 2% (wt/vol). The β -galactosidase activity of *K. lactis* clones was checked on solid medium containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside [40 μ g/ml]).

Yeast transformation. Competent cells of *K. lactis* were routinely prepared according to the method of Klebe et al. (21) and stored at -70°C (14). Transformation with linearized plasmid DNA for chromosomal integration was performed by the lithium acetate method (1).

DNA manipulation, preparation of yeast RNA, and Northern blot analysis. The plasmids used are listed in Table 2. pBM3157 and pBM3158 are derivatives of plasmid YEp357R (28) carrying the promoter sequences of *KHT1* and *KHT2*, respectively, as *Bam*HI-*Eco*RI fragments fused to '*lacZ*'. Both plasmids were kindly supplied by S. Özcan (Lexington, Ky.). The isolation of total DNA from

TABLE 2. Plasmids used in this study

| Plasmid | Relevant characteristics ^a | Source or reference |
|----------|---|----------------------------|
| pBM3157 | YEp357 with <i>KHT1</i> promoter (+3 to -1110) fused to ' <i>lacZ</i> ' | S. Özcan, unpublished data |
| pBM3158 | YEp357 with <i>KHT2</i> promoter (+3 to -1642) fused to ' <i>lacZ</i> ' | S. Özcan, unpublished data |
| pKATUC4 | KICEN2-ARS1-KARS12- <i>ScTRP1-ScURA3</i> -vector | 44 |
| pJW10-7 | pUC12 with <i>RAG1</i> gene | 38 |
| pJW10-23 | PUC12 with <i>KHT1-KHT2</i> | 38 |
| pY10-7 | KATUC4 cassette in pJW10-7 | 37 |
| pY10-23 | KATUC4 cassette in pJW10-23 (<i>KHT1-KHT2</i>) | 37 |
| pVSH1 | pKATUC42 with <i>HXT1</i> | This work |
| pVGAL2 | pKATUC42 with <i>GAL2</i> | This work |
| p33-KHT1 | YCplac33 with <i>KHT1</i> | 16; this work |
| p33-KHT2 | YCplac33 with <i>KHT2</i> | 16; this work |

^a Details available on request.

K. lactis was done as described earlier (7). All DNA techniques were performed according to standard procedures (1, 25).

Total RNA was isolated from *K. lactis* cells grown to the exponential phase by a hot phenol method. For Northern blot analysis, total RNA was fractionated by electrophoresis in 1.3% agarose-formaldehyde gels. Separated RNA was transferred to nylon membranes (Qiabran; Qiagen) by capillary blotting with $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (25). Prehybridization and hybridization were performed in high-sodium dodecyl sulfate (SDS) buffer (Church buffer) containing 7% (wt/vol) SDS, 50% (vol/vol) formamide, $5\times$ SSC, 2% (wt/vol) Boehringer blocking reagent, 50 mM sodium phosphate (pH 7.0), and 0.1% (wt/vol) *N*-lauroylsarcosine. Incubation was overnight at 53°C. The following washes were performed: (i) $2\times$ SSC-0.1% SDS at room temperature (twice, 15 min per wash) and (ii) $0.5\times$ SSC-0.1% SDS at 68°C (twice, 15 min per wash). Membranes were exposed to X-ray film at -70°C . Labeling of DNA probes was done by random-primed DNA synthesis with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ by using the Hexa labeling kit (MBI Fermentas).

β -Galactosidase assays. β -Galactosidase activity (*K. lactis* Lac4p) was determined in crude extracts at 30°C as described previously (43). The activity of LacZ fusion proteins was measured in crude extracts at 37°C in LacZ buffer (27). In JA6/DT12-derived strains containing *lacZ* fusion genes, *LAC4*-derived background β -galactosidase activity was subtracted from the total activity by including parent strain DT12R in each experiment. The background never exceeded 15% of the *lacZ* activity and was not influenced by the glucose concentration. Protein concentrations were determined according to the method of Lowry (24) with bovine serum albumin as a standard.

Glucose determination. Glucose concentrations in the culture supernatants were determined by using the glucose oxidase/peroxidase assay (3).

Glucose transport measurements. Transport of $[\text{C}^{14}]\text{glucose}$ was determined in 5-s glucose-uptake assays as described previously (31).

RESULTS AND DISCUSSION

***KHT1* and *KHT2* encode functional glucose transporters with low and intermediate affinity, respectively.** The fact that *KHT1* and *KHT2* are both able to complement the *Rag1*⁻ phenotype of a *K. lactis* *rag1* mutant indicated that they encode functional glucose transporters (38). To further characterize the gene products, we first compared the kinetics of glucose uptake in a wild-type strain carrying both genes to that of a strain containing only *RAG1*. Glucose transport kinetics was determined in 5-s-uptake measurements (Fig. 1A). For strain JA6 (*KHT1 KHT2*), a biphasic curve with a high-affinity branch ($K_m \approx 4.5$ mM) was obtained. In strain ST105 (*RAG1*), the high-affinity branch was missing. Exact quantitation of K_m was difficult in this strain due to high background at the high substrate concentrations; a rough estimate of 80 mM may not be significantly different from the K_m of 20 to 50 mM described before for *RAG1* (39).

Deletion of *RAG1* in the latter strain further reduced glucose uptake, but the mutant was still able to grow on glucose at about the same rate (data not shown). Therefore, to determine the kinetic parameters of *KHT1*- and *KHT2*-mediated glucose uptake in the absence of any interfering transporters, we transferred these genes individually into the glucose-negative *S. cerevisiae* strain EB.Y.VW4000. In this strain, all 17 *HXT* genes, the galactose permease gene *GAL2*, and three members of the maltose permease family had been deleted. EB.Y.VW4000 is unable to grow on glucose medium, and no glucose consumption is detectable (40). Transformation with centromeric vectors carrying the *K. lactis* gene *KHT1* or *KHT2* restored growth on glucose, confirming that both *KHT* gene products mediate glucose uptake. Apparently, no *K. lactis*-specific factors are required for functional expression of these genes in *S. cerevisiae*.

Uptake measurements gave linear Eadie-Hofstee plots (Fig.

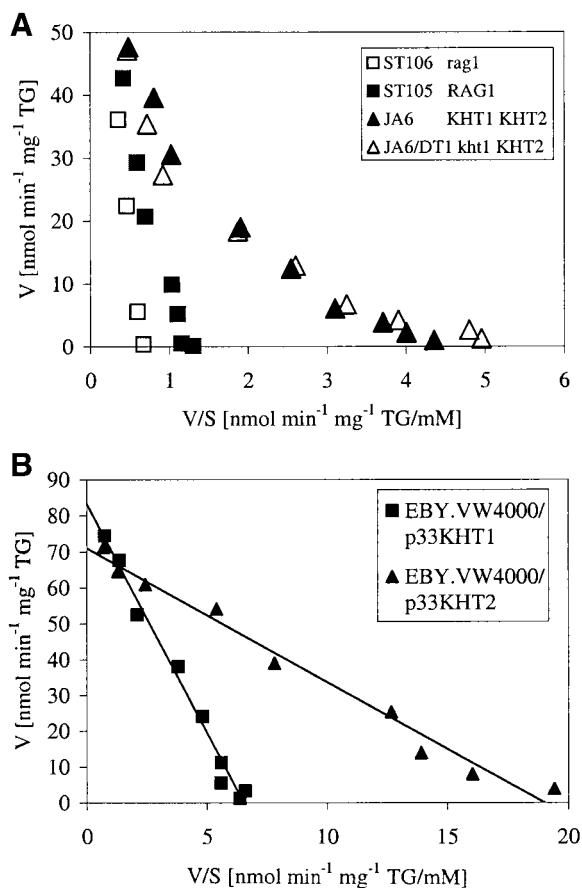


FIG. 1. (A) Transport of $[\text{C}^{14}]\text{glucose}$ in *K. lactis* strains. Glucose transport rates in different *K. lactis* strains were determined by 5-s-uptake measurements of $[\text{C}^{14}]\text{glucose}$ (31). Results are displayed in Eadie-Hofstee plots as uptake rates per milligram of dry weight (TG). The strains differed in the genetic background (squares versus triangles) and the presence (solid symbols) or absence (open symbols) of the low-affinity transporter gene *KHT1* or *RAG1*. Solid triangles, JA6 (*KHT1 KHT2*); open triangles, JA6 (*kht1 KHT2*); solid squares, ST105 (*RAG1*); open squares, ST106 (*rag1*) (38). (B) Transport of $[\text{C}^{14}]\text{glucose}$ mediated by *Kht1p* and *Kht2p* in the glucose-uptake-deficient *S. cerevisiae* mutant EB.Y.VW4000. The strain was transformed with centromeric vectors carrying *KHT1* (squares) and *KHT2* (triangles), respectively.

1B) with a K_m for *Kht1p* of 13 mM and a K_m for *Kht2p* of 3.7 mM. The K_m of *Kht1p*, as determined in *S. cerevisiae*, was somewhat lower than that of *Rag1p* (39); however, given the general difficulties in determining low-affinity uptake, we doubt that this difference reflects significant differences between *Rag1p* and *Kht1p* (38). *Kht2p* has an affinity for the glucose intermediate between the low-affinity transporter *Rag1p* and the high-affinity transporter *Hgt1p* ($K_m = 1$ mM) described before (5, 39).

Expression of *KHT1* and *KHT2* is differentially regulated by glucose. To study the regulation of the *KHT1* and *KHT2* genes, *KHT1* and *KHT2* promoter-driven *lacZ* reporter gene expression was analyzed. The fusion genes were integrated into the chromosome of *K. lactis* strain JA6/DL4R (*KHT1 KHT2 lac4*) mutated for the endogenous β -galactosidase gene, and reporter gene expression was measured in cells shifted from

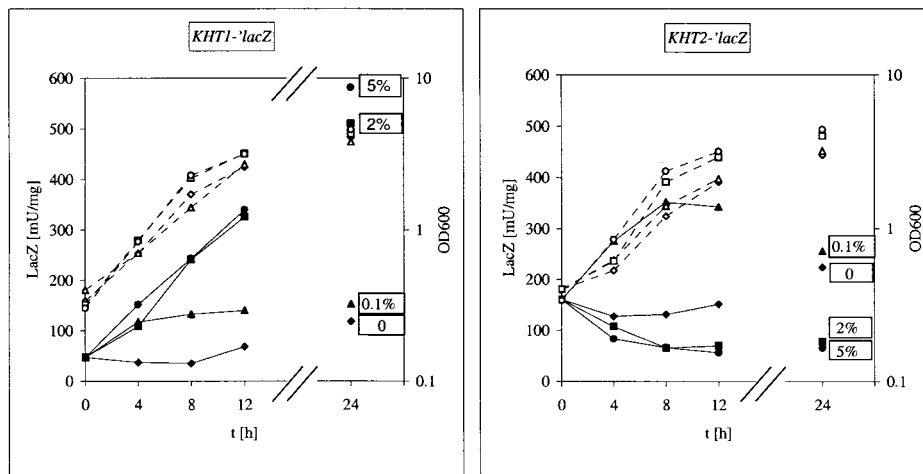


FIG. 2. Regulation by glucose of *KHT1* and *KHT2* promoter activity. Transformants of *K. lactis* strain JA6/DL4R (*KHT1 KHT2 lac4*) carrying single chromosomal insertions of *KHT1-lacZ* and *KHT2-lacZ* gene fusions, respectively, were grown to the exponential phase in YNB medium with 3% glycerol, collected by centrifugation, and resuspended at time 0 (t_0) in the same glycerol medium containing different amounts of glucose as additional carbon sources (no glucose \blacklozenge , 0.1% glucose \blacktriangle , 2% glucose \blacksquare , and 5% glucose \bullet). During batch cultivation at 30°C, samples were taken at the indicated time points for determination of *lacZ* activity (solid symbols) and cell density (open symbols with dotted lines) OD600, optical density at 600 nm.

glycerol to different concentrations of glucose (Fig. 2). For strain JA6/CM57 carrying the *KHT1-lacZ* fusion, β -galactosidase activity was low in glycerol and induced by glucose. Enzyme activity increased with the external glucose concentration up to a concentration of about 2% (100 mM) (Fig. 2) (data not shown) and proceeded slowly with about a twofold increase in 4 h.

In contrast, the regulation of *KHT2* is more complex. The *KHT2-lacZ* fusion (strain JA6/CM58) was expressed at a higher basal level in glycerol and induced about twofold by 0.1% (5 mM) glucose, whereas high glucose (2%) resulted in a weak repression of promoter activity.

The induction by glucose of *KHT1* is apparently identical to that of *RAG1*, a fact that is not surprising given the sequence identity between *KHT1* and *RAG1* in the 5'-upstream region and most of the coding region. The difference in the 3' ends between these genes has no obvious influence on regulation of promoter activity.

Northern blot analysis with a probe encompassing *KHT1* and *KHT2* showed that in logarithmic cells, reporter gene expression roughly reflected transcript levels (Fig. 3A). In wild-type cells grown in 2% glucose medium, the shorter *KHT1*-specific transcript dominated (lane 1), whereas in glycerol, the longer *KHT2*-specific signal was more prominent (lane 5), confirming a higher level of expression of this gene in the absence of glucose. The ratio between *KHT1* and *KHT2* mRNAs from glucose-grown cells varied from experiment to experiment, depending on the time in glucose medium and the density of the culture (data not shown). In most experiments, a very low *KHT2* transcript level was observed in cells grown in high-glucose medium for extended periods (Fig. 3A, lane 1).

The regulation of *KHT2* resembles that of the *S. cerevisiae* *HXT6/7* genes (6, 23). Interestingly, in a Clustal analysis, *HXT6* and *HXT7*, which are nearly identical, turned out to be the closest relatives to *KHT2* among the *HXT* genes. Like the

KHT1-KHT2 locus in *K. lactis* (38), the *HXT6-HXT7* locus seems to be recombinogenic, and some strains contain an *HXT6/7* chimeric gene (10, 23). In the *S. cerevisiae* genome, *HXT3* and *HXT6/7* form a gene cluster similar to *KHT1-KHT2*, with respect to regulation and properties of the gene products. In a strain retaining only *HXT3*, *HXT6*, and *HXT7*, no growth deficiencies on glucose, fructose, and mannose could be observed (40). We speculate that this gene cluster evolved before the separation of *K. lactis* and *S. cerevisiae* species and represents another example of synteny between *S. cerevisiae* and *K. lactis* genomes as found at many loci (2, 41).

The *kht1 kht2* double mutation eliminates the high glucose response of the *KHT1* and *KHT2* genes. To examine the dependence of gene regulation on the structural integrity of the *KHT* transporter genes, the *kht1*, *kht2*, and *kht1 kht2* mutants described previously (38) were analyzed in parallel to the wild type (Fig. 3). Interpretation of the banding pattern in Northern blots is complicated by the fact that the *kht2* mutant had been generated by introducing the *RAG1* gene into the *kht1 kht2* deletion instead of *KHT1*. Since *RAG1* differs at the very 3' end from *KHT1*, it gives rise to an mRNA that is slightly longer than in the parent strain and migrates at the same position as the *KHT2* transcript (Fig. 3A, lane 3). With a short 5' probe, the *KHT1* and *RAG1*-specific signals were identified (Fig. 3B). *KHT1* promoter activity could be monitored even in *kht1* mutants, since a small *kht1::URA3* fusion was formed. This fusion transcript migrated close to the *HHT1* transcript used as a loading control (Fig. 3A); therefore, the two probes were applied sequentially to the same filter (Fig. 3B, top and middle panels).

The intensity of the *KHT1*- and *kht1::URA3* band was stronger on glucose (2%) than on glycerol in all four strains examined (Fig. 3B, lanes 1, 3, 5, and 7). However, in the *kht1 kht2* double mutant, the induced level was reduced compared to those of the wild type and single mutants, indicating that at

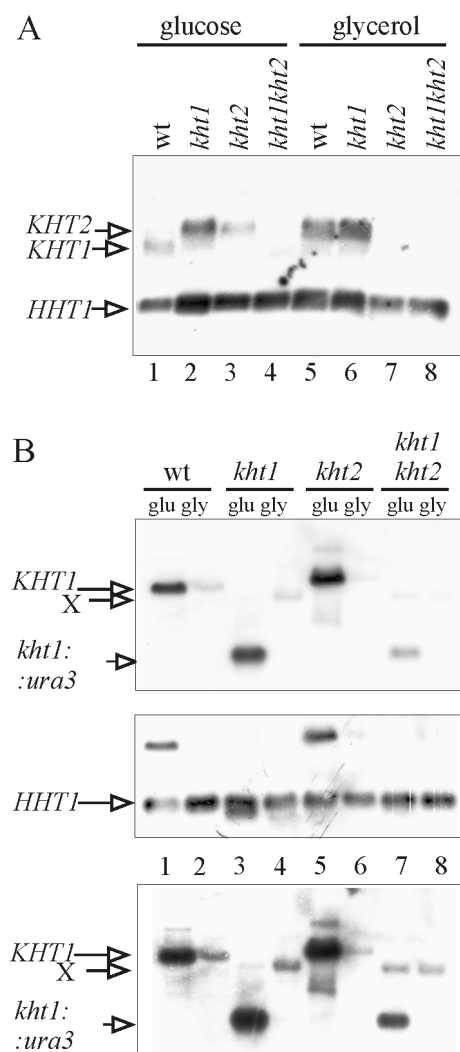


FIG. 3. Northern blot analysis of *KHT1*- and *KHT2*-specific transcription in wild-type (WT) and *kht* mutant cells. *K. lactis* strain JA6 and congenic *kht* disruption mutants were grown to the exponential phase in YNB minimal medium containing 2% glucose (glu) (lanes 1 to 4) or 3% glycerol (gly) (lanes 5 to 8), and poly(A)⁺ RNA was isolated. Five micrograms of poly(A)⁺ RNA samples was fractionated by electrophoresis on a 1.3% agarose gel in the presence of formaldehyde. Plasmid pJW10-23 carrying the entire *KHT-KHT2* locus (38) (A) and a small *KHT1* 5'-specific probe (*Hpa*I-*Xho*I fragment of pJW10-7 [38] [B]) were used as probes. An *HHT1* (histone H3) probe was added as a loading control. Note that in panel A, a weak *kht1::ura3* fusion transcript overlaps with the *HHT1* signal in lanes 2 and 7. In panel B, the two probes were applied sequentially (top and middle panels). A longer exposure of the top panel is shown at the bottom to visualize the weak cross-hybridizing transcript X.

least one of the *KHT* genes is required for full induction of *KHT1* and that the two genes can substitute for one another.

KHT2 transcription could not be analyzed in the same way, since no *KHT2*-specific transcript was formed in *kht2* mutants. (Note that the signal in Fig. 3A, lane 3, is *RAG1*.)

Therefore, the *KHT-lacZ* fusions were integrated into the genome of the *kht1 kht2* strain JA6/DT12R, and the influence of the mutations on *KHT1* and *KHT2* promoter activities was determined by reporter gene assays (Table 3). For the *KHT1*

promoter, no influence of the *kht1 kht2* mutations was detectable in low glucose (0.1%). The wild type and mutant had roughly the same ratio of β -galactosidase activity in glycerol versus glucose (2.7- and 3.3-fold, respectively). However, in 2% glucose, induction in the double mutant was weaker than in the wild type (3.3-fold versus 5.1-fold, respectively), in agreement with the results obtained by Northern analysis (Fig. 3).

For the *KHT2* promoter, the influence of the mutations was even more striking. Whereas in the wild type, a modest induction by low glucose (2.1-fold) and a slight repression (0.4-fold induction) in high glucose were observed, induction was high (4.8-fold) in low glucose and even higher (5.4-fold) in high glucose. These results suggested that Khtp transporter activity was not required for induction but for repression of *KHT2* gene expression at a high glucose concentration.

In the double mutant, another transcript of unknown origin, labeled "X" in Fig. 3B, was also released from glucose repression (compare lanes 3 and 4 to lanes 7 and 8 in Fig. 3B, bottom panel).

Taken together, the results indicate that in the absence of both *KHT* transporters, the specific high-glucose response of the *KHT1* and *KHT2* promoters does not occur, whereas the responses to low glucose were not affected by the mutations.

Several transporter genes restore the high-glucose response in *kht1 kht2* double mutants. To examine whether complementation of the glucose uptake deficiency was sufficient to restore the high-glucose response in the double mutant, transporter genes were introduced on centromeric plasmids into the *kht1 kht2* strains carrying the integrated *KHT1-lacZ* and *KHT2-lacZ* fusions, respectively. (Table 3). In transformants with the *KHT1-KHT2* tandem genes (plasmid pY10-23), the wild-type regulation of the *KHT1* and *KHT2* promoters was completely (*KHT1-lacZ*) or almost completely (*KHT2-lacZ*) restored. Partial induction of the *KHT1* promoter and partial repression of the *KHT2* promoter by glucose were observed with *RAG1* (with plasmid pY10-7), and a weaker effect was seen with the *S. cerevisiae HXT1* gene (on pVSH1), encoding a low-affinity hexose transporter.

We also assayed the ability of the *S. cerevisiae GAL2* gene to complement the glucose uptake deficiency of the *kht1 kht2* mutant strain. The galactose permease encoded by *GAL2* had been shown to be able to mediate glucose uptake (23, 31), but the regulation of *GAL2* (induction by galactose) suggested that it is not a component of a glucose signaling pathway. To overcome the requirement of galactose for the Gal4p-regulated *GAL2* gene, the *KIGAL80* gene was disrupted in the *kht1 kht2* mutant background. The resulting triple mutant was Rag⁻ like the parent, but a centromeric *GAL2* plasmid clearly improved growth on 2% glucose plus antimycin A, an effect not observed when the *KIGAL80* gene was functional (data not shown). Thus, Gal2p was able to function as a glucose transporter in *K. lactis*.

The triple mutant was used to analyze regulation of the *LAC4* promoter (Table 3). The *KHT1 KHT2 Kigal80* strain showed fivefold-lower *LAC4*-encoded β -galactosidase activity on glucose compared to glycerol (13,500 mU/mg of protein). This implies that glucose not only inhibits galactose induction of the *LAC/GAL* genes, but also represses the induced promoter to some extent. This form of glucose repression was abolished in the *kht1 kht2 Kigal80* triple mutant. Again, repres-

TABLE 3. Influence of hexose transporters on glucose induction and repression

| Genetic background | Gene on plasmid | Glucose induction at glucose concn ^a : | | | | | |
|--------------------|------------------|---|-----------------|---------------------------------|-----------------|------------------------------------|----------------------------|
| | | <i>KHT1</i> '-lacZ ^b | | <i>KHT2</i> '-lacZ ^b | | <i>LAC4</i> ^{b,c} (2%) | PDC ^{c,e} (2%) |
| | | 0.1% ^d | 2% ^d | 0.1% ^d | 2% ^d | | |
| Wild-type | Vector | 2.7 | 5.1 | 2.1 | 0.4 | 0.2 | 5.2 |
| <i>kht1 kht2</i> | Vector | 3.3 | 3.3 | 4.8 | 5.4 | 0.9 | 1.0 |
| <i>kht1 kht2</i> | <i>KHT1 KHT2</i> | ND ^f | 6.2 | ND | 1.0 | ND | ND |
| <i>kht1 kht2</i> | <i>RAG1</i> | ND | 4.6 | ND | 1.7 | 0.4 | 3.4 |
| <i>kht1 kht2</i> | <i>ScHXT1</i> | ND | 4.3 | ND | 3.3 | 0.6 | 1.0 |
| <i>kht1 kht2</i> | <i>GAL2</i> | ND | ND | ND | ND | 0.3 | 5.8 |

^a Fold-induction relative to glycerol (3%) cultures, mean of at least three independent experiments, deviations <15%.

^b β -galactosidase activity.

^c Pyruvate decarboxylase activity.

^d Glucose (0.1 or 2%) was added to 3% glycerol medium.

^e In *gal80* mutant background.

^f ND, not determined.

sion could be restored by transformation with the *RAG1*- as well as with the *GAL2*-containing centromeric plasmid; *ScHXT1* gave a weaker effect.

We also analyzed glucose induction of the glycolytic enzyme pyruvate decarboxylase encoded by the unique glucose-inducible gene, *KIPDC1* (4, 12). In glycerol-grown cells, pyruvate decarboxylase activities varied between 60 and 260 mU/mg of protein. A fivefold induction by glucose observed in the *Klgal80* mutant was completely abolished in the *kht1 kht2 Klgal80* mutant and could be fully or partially restored by *GAL2* and *RAG1*, respectively. No effect was observed with *S. cerevisiae HXT1* in this case.

Thus, the *kht1 kht2* mutation had a broad influence on glucose-regulated gene expression in general and was not restricted to the inducible *LAC/GAL* regulon (38). Formally, the possibility exists that the *KHT* gene products have regulatory activity in addition to their transport function, like the *S. cerevisiae* Snf3p and Rgt2p proteins, which serve as membrane-bound high- and low-affinity glucose receptors, respectively (30).

However, there is no indication that Kht1p and Kht2p are such glucose sensors. (i) Based on sequence similarity (65% identity), *KHT1* and *KHT2* clearly belong to the *HXT* gene family, with much weaker resemblance to *SNF3* and *RGT2* (25% identity), and the cytoplasmic tail characteristic of the latter gene products is not found in Kht1p and Kht2p. (ii) Both *KHT1* and *KHT2* encode functional glucose transporters, and the regulatory phenotype displayed by the *kht1 kht2* double mutant could be restored by *S. cerevisiae* hexose transporter genes such as *HXT1* and *GAL2*. (iii) There is a correlation between glucose uptake capacity and glucose signaling. The Hxt1p transporter, which has a very low affinity for glucose and requires high glucose concentrations for induction (29), had a weaker effect on glucose repression and induction than *RAG1* and *GAL2*. The correlation between glucose uptake and the glucose response of gene expression is consistent with recent results from *S. cerevisiae* showing a correlation between relief from glucose repression and the decrease in glucose transport capacity. Even under high-glucose conditions, an increase in *SUC2* expression was detected when cells displayed reduced glucose uptake (31, 42).

We conclude that Kht1p and Kht2p influence regulation

through their transport function and that glucose sensing occurs intracellularly.

An intracellular sensing mechanism for glucose does not exclude an additional extracellular sensor. The *RAG4* gene conferring a Rag⁻ phenotype when mutated encodes an Snf3/Rgt2-related protein with a long C-terminal cytoplasmic tail containing the conserved sequence motif (3a). It is a regulator of *RAG1* and may mediate the low glucose response of *KHT1/RAG1*, which we have shown here to be independent of *KHT1* and *KHT2*. A combination of extracellular and intracellular sensing mechanisms would ensure that the high-glucose response is only triggered if the cell is able to metabolize the available glucose.

We propose that in *K. lactis* glucose repression correlates with fermentative metabolism. Both forms of regulation require the transport capacity of the cell to exceed a threshold level. This level cannot be reached in the *kht1 kht2* or *rag1* mutant. The presence of either *KHT1/RAG1* or *KHT2* alone is sufficient to overcome the threshold. By a self-sustaining process caused by further induction of *KHT1/RAG1* and other fermentative genes (like the pyruvate decarboxylase gene *KIPDC1*) and by favoring the utilization of glucose over other carbon sources through glucose repression, the metabolism is shifted towards fermentation. The presence of both genes *KHT1* and *KHT2* favors this metabolic shift due to the high basal expression of *KHT2* resulting in a higher uptake capacity in noninduced cells. Since *KHT2* itself is subject to glucose repression, the transporter with the higher K_m , Kht2p, is replaced by the one with the lower K_m if there is a high and constant supply of glucose. The slow and concentration-dependent induction of Kht1p/Rag1p enables the cells to monitor the availability of glucose over time; its low-affinity ensures a concentration-dependent rate of transport at subsaturating glucose levels.

In *S. cerevisiae*, the abundance of hexose transporter genes and their specific regulation indicate that this yeast tries to sustain the highest possible glucose transport activity, such that the glycolytic flux is largely determined by the extracellular glucose concentration. In contrast, the respirofermentative yeast *K. lactis* does not fully exploit its glucose uptake capacity during oxidative growth. Sugar uptake may therefore be pri-

marily controlled by the availability of oxygen and by the cellular demand rather than by the extracellular supply (8).

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REFERENCES

- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1992. Current protocols in molecular biology. Green Publishing Associates and Wiley-Interscience, New York, N.Y.
- Bao, W. G., and H. Fukuhara. 2000. The ubiquitin-encoding genes of *Kluyveromyces lactis*. *Yeast* **16**:343-351.
- Bergmeyer, H. U. 1974. Methoden der enzymatischen Analyse. Verlag Chemie, Weinheim, Germany.
- Betina, S., P. Goffrini, I. Ferrero, and M. Wesolowski-Louvel. 2001. *RAG4* gene encodes a glucose sensor in *Kluyveromyces lactis*. *Genetics* **158**:541-548.
- Bianchi, M. M., L. Tizzani, M. Destruelle, L. Frontali, and M. Wésolowski-Louvel. 1996. The 'petite-negative' yeast *Kluyveromyces lactis* has a single gene expressing pyruvate decarboxylase activity. *Mol. Microbiol.* **19**:27-36.
- Billard, P., S. Ménart, J. Blaisonneau, M. Bolotin-Fukuhara, H. Fukuhara, and M. Wésolowski-Louvel. 1996. Glucose uptake in *Kluyveromyces lactis*: role of the *HGT1* gene in glucose transport. *J. Bacteriol.* **178**:5860-5866.
- Boles, E., and C. P. Hollenberg. 1997. The molecular genetics of hexose transport in yeasts. *FEMS Microbiol. Rev.* **21**:85-111.
- Breunig, K. D. 1989. Glucose repression of LAC gene expression in yeast is mediated by the transcriptional activator LAC9. *Mol. Gen. Genet.* **216**:422-427.
- Breunig, K. D., M. Bolotin-Fukuhara, M. M. Bianchi, D. Bourgarel, C. Falcone, I. Ferrero, L. Frontali, P. Goffrini, J. J. Krijger, C. Mazzoni, C. Milkowski, H. Y. Steensma, M. Wesolowski-Louvel, and A. M. Zeeman. 2000. Regulation of primary carbon metabolism in *Kluyveromyces lactis*. *Enzyme Microb. Technol.* **26**:771-780.
- Breunig, K. D., and P. Kuger. 1987. Functional homology between the yeast regulatory proteins GAL4 and LAC9: LAC9-mediated transcriptional activation in *Kluyveromyces lactis* involves protein binding to a regulatory sequence homologous to the GAL4 protein-binding site. *Mol. Cell. Biol.* **7**:4400-4406.
- Brown, C. J., K. M. Todd, and R. F. Rosenzweig. 1998. Multiple duplications of yeast hexose transport genes in response to selection in a glucose-limited environment. *Mol. Biol. Evol.* **15**:931-942.
- Chen, X. J., M. Wésolowski-Louvel, and H. Fukuhara. 1992. Glucose transport in the yeast *Kluyveromyces lactis*. II. Transcriptional regulation of the glucose transporter gene *RAG1*. *Mol. Gen. Genet.* **233**:97-105.
- Destruelle, M., R. Menghini, L. Frontali, and M. M. Bianchi. 1999. Regulation of the expression of the *Kluyveromyces lactis* *PDC1* gene: carbon source-responsive elements and autoregulation. *Yeast* **15**:361-370.
- De Winde, J. H., M. Crauwels, S. Hohmann, J. M. Thevelein, and J. Winderickx. 1996. Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. *Eur. J. Biochem.* **241**:633-643.
- Dohmen, R. J., A. W. M. Strasser, C. B. Höner, and C. P. Hollenberg. 1991. An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. *Yeast* **7**:691-692.
- Entian, K.-D. 1980. Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in catabolite repression in yeast. *Mol. Gen. Genet.* **178**:633-637.
- Gietz, R. D., and A. Sugino. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast lacking six-base pair restriction sites. *Gene* **74**:527-534.
- Gödecke, A., W. Zachariae, A. Arvanitidis, and K. D. Breunig. 1991. Co-regulation of the *Kluyveromyces lactis* lactose permease and β -galactosidase genes is achieved by interaction of multiple LAC9 binding sites in a 2.6 kbp divergent promoter. *Nucleic Acids Res.* **19**:5351-5358.
- Goffrini, P., A. A. Algeri, C. Donnini, M. Wésolowski-Louvel, and I. Ferrero. 1989. *RAG1* and *RAG2*: nuclear genes involved in the dependence/independence on mitochondrial respiratory function for growth on sugars. *Yeast* **5**:99-106.
- Hohmann, S., J. Winderickx, J. H. De Winde, D. Valckx, P. Cobbaert, K. Luyten, C. de Meirman, J. Ramos, and J. M. Thevelein. 1999. Novel alleles of yeast hexokinase PII with distinct effects on catalytic activity and catabolite repression of *SUC2*. *Microbiology* **145**:703-714.
- Johnston, M. 1999. Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. *Trends Genet.* **15**:29-33.
- Klebe, R. J., J. V. Harris, Z. D. Smart, and M. G. Douglas. 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* **25**:333-341.
- Kraakman, L., K. Lemaire, P. Ma, A. W. Teunissen, M. C. Donaton, P. Van Dijk, J. Winderickx, J. H. De Winde, and J. M. Thevelein. 1999. A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32**:1002-1012.
- Liang, H., and R. F. Gaber. 1996. A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. *Mol. Biol. Cell* **7**:1953-1966.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Meyer, J., A. Walker-Jonah, and C. P. Hollenberg. 1991. Galactokinase encoded by *GAL1* is a bifunctional protein required for induction of the *GAL* genes in *Kluyveromyces lactis* and is able to suppress the *gal3* phenotype in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**:5454-5461.
- Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty. 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. *Gene* **45**:299-310.
- Özcan, S., and M. Johnston. 1999. Function and regulation of yeast hexose transporters. *Microbiol. Mol. Biol. Rev.* **63**:554-569.
- Özcan, S., J. Dover, A. G. Rosenwald, S. Wülfel, and M. Johnston. 1996. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. *Proc. Natl. Acad. Sci. USA* **93**:12428-12432.
- Reifenberger, E., E. Boles, and M. Ciriacy. 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. *Eur. J. Biochem.* **245**:324-333.
- Rolland, F., J. H. De Winde, K. Lemaire, E. Boles, J. M. Thevelein, and J. Winderickx. 2000. Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. *Mol. Microbiol.* **38**:348-358.
- Rose, M., W. Albig, and K.-D. Entian. 1991. Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *Eur. J. Biochem.* **199**:511-518.
- Sheetz, R. M., and R. C. Dickson. 1980. Mutations affecting synthesis of β -galactosidase activity in the yeast *Kluyveromyces lactis*. *Genetics* **95**:877-890.
- Sreekrishna, K., and R. C. Dickson. 1985. Construction of strains of *Saccharomyces cerevisiae* that grow on lactose. *Proc. Natl. Acad. Sci. USA* **82**:7909-7913.
- Versele, M., J. H. De Winde, and J. M. Thevelein. 1999. A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. *EMBO J.* **18**:5577-5591.
- Weirich, J. 1992. Ph.D. thesis. Isolierung und Charakterisierung des Glukose Transporters RAG1 aus *Kluyveromyces lactis* und Untersuchungen zu dessen Rolle bei der Glukoserepression der β -Galaktosidase. Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany.
- Weirich, J., P. Goffrini, P. Kuger, I. Ferrero, and K. D. Breunig. 1997. Influence of mutations in hexose-transporter genes on glucose repression in *Kluyveromyces lactis*. *Eur. J. Biochem.* **249**:248-257.
- Wesolowski-Louvel, M., P. Goffrini, I. Ferrero, and H. Fukuhara. 1992. Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporter gene. *Mol. Gen. Genet.* **233**:89-96.
- Wiczorke, R., S. Krampe, T. Weierstall, K. Freidel, C. P. Hollenberg, and E. Boles. 1999. Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett.* **464**:123-128.
- Wolfe, K. H., and D. C. Shields. 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387**:708-713.
- Ye, L., A. L. Kruckeberg, J. A. Berden, and K. Van Dam. 1999. Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. *J. Bacteriol.* **181**:4673-4675.
- Zachariae, W., P. Kuger, and K. D. Breunig. 1993. Glucose repression of lactose/galactose metabolism in *Kluyveromyces lactis* is determined by the concentration of the transcriptional activator LAC9 (KIGAL4). *Nucleic Acids Res.* **21**:69-77.
- Zenke, F., W. Zachariae, A. Lunkes, and K. D. Breunig. 1993. Gal80 proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are highly conserved but contribute differently to glucose repression of the galactose regulon. *Mol. Cell. Biol.* **13**:7566-7576.
- Zenke, F. T., R. Engels, V. Vollenbroich, J. Meyer, C. P. Hollenberg, and K. D. Breunig. 1996. Activation of Gal4p by galactose-dependent interaction of galactokinase and Gal80p. *Science* **272**:1662-1665.