MOLECULAR AND CELLULAR BIOLOGY, Oct. 1996, p. 5536–5545 Vol. 16, No. 10
0270-7306/96/$04.00+0

Two Different Repressors Collaborate To Restrict Expression of the Yeast Glucose Transporter Genes *HXT2* and *HXT4* to Low Levels of Glucose

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Received 5 May 1996/Returned for modification 2 July 1996/Accepted 17 July 1996

Transcription of the yeast *HXT2* and *HXT4* genes, which encode glucose transporters, is induced only by low levels of glucose. This low-glucose-induced expression is mediated by two independent repression mechanisms: in the absence of glucose, transcription of both genes is prevented by Rgt1p, a C_6 zinc cluster protein; at high levels of glucose, expression of *HXT2* and *HXT4* is repressed by Mig1p. Only at low glucose concentrations are both repressors inactive, leading to a 10- to 20-fold induction of gene expression. Mig1p and Rgt1p act directly on *HXT2* and *HXT4* by binding to their promoters. This transcriptional regulation is physiologically very important to the yeast cell because it causes these glucose transporters to be expressed only in low-glucose media, in which they are required for growth.

Transcriptional repression is a common mechanism used to restrict gene expression in response to different environmental signals (for reviews, see references 7, 15, and 41). Several DNA-binding transcriptional repressors have been identified in the yeast *Saccharomyces cerevisiae*. They include Mig1p, a Cys_2His_2 zinc finger protein that binds to the promoters of several glucose-regulated genes and represses their transcription in the presence of high levels of glucose (14, 31, 32, 40); Rox1p, which is involved in the repression of several oxygen-regulated genes; and α2p, which represses cell-type-specific genes (3, 17). These repressors require the function of two other proteins, Ssn6p and Tup1p, for repression of transcription (43, 44, 47, 49, 50, 55). Neither Ssn6p nor Tup1p appears to be a DNA-binding protein, but they both repress transcription when bound to DNA through an attached DNA-binding domain (23). This has led to the view that the DNA-binding repressors recruit Ssn6p and Tup1p to different promoters to achieve repression. Ssn6p and Tup1 may act by organizing repressive regions of chromatin (6).

Glucose repression is an important gene regulatory mechanism in *S. cerevisiae*. Glucose causes repression of the expression of many genes that are dispensable or unimportant to cells growing on it, including genes for the utilization of other carbon sources (e.g., *GAL* and *SUC*) and for oxidation of glucose (e.g., genes encoding cytochromes and tricarboxylic acid cycle enzymes). Glucose also induces the expression of several genes involved in its utilization, including many of the genes for glycolysis (reviewed in reference 20) and several of the *HXT* genes encoding glucose transporters (4, 34, 46, 53, 54). These two regulatory mechanisms ensure that glucose is preferentially and efficiently utilized by fermentation.

Glucose repression has been studied extensively. A central component of the regulatory mechanism is Mig1p, the DNA-binding repressor of many glucose-repressed genes (22, 32, 40). Several components of the signal transduction pathway responsible for regulation of Mig1p function by glucose, including the Snf1p protein kinase and several of its regulators, have also been identified (reviewed in references 5, 20, 40, and 48).

Much less is known about how glucose induces gene expression. We recently identified a repressor, Rgt1p, that is responsible for glucose-induced expression of several *HXT* genes (28, 34a). Rgt1p, together with Ssn6p and Tup1p, prevents expression of *HXT* genes when glucose is absent; its function is inhibited in the presence of glucose, leading to derepression of *HXT* transcription. Expression of *HXT2* and *HXT4* is induced only by low levels of glucose, and this is achieved by superimposing on these two genes repression by the high-glucose-activated repressor Mig1p (34). Thus, two independent repression mechanisms cause the expression of *HXT2* and *HXT4* to occur only in low-glucose media, the condition to which these two glucose transporters are presumably best adapted. We show here that Rgt1p and Mig1p act directly on *HXT2* and *HXT4* promoters, and we define their binding sites in these promoters. This provides a clear and simple example of how multiple regulatory proteins that respond differently to environmental signals can combine to provide a specific and unique pattern of gene expression.

MATERIALS AND METHODS

Strains and plasmids. Growth and medium conditions have been described previously (34). The strains used in this study are YM4127 and YM2062 (wild type), YM4509 (*rgt1*Δ), YM4553 (*mig1*Δ) and YM4554 (*ssn6*Δ). For the genotype of these strains, see reference 34. The promoter fusions of *HXT2* (pBM2717) and *HXT4* (pBM2800) to *lacZ* in vector YEp357R have been described previously (34). *HXT2* promoter deletions were constructed by subcloning PCR products as *Bam*HI (5′) and *Eco*RI (3′) fragments into the vector YEp357R (30). The reporter plasmid pBM2832, which has the upstream activator sequence (UAS) fragment of the *LEU2* gene and the TATA box with part of the *HIS3* coding region fused to *lacZ* in YEp356, was constructed in two steps. First, the *HIS3* portion was inserted as *Sph*I (−79 from the *HIS3* initiation codon) and *Hin*dIII (+306, codon 102) in YEp356 in frame to *lacZ* (pBM 2829). Then a 288-bp fragment containing the *LEU2*-UAS (−415 to −127) was subcloned as a *Mun*I-*Eco*RI fragment into the *Eco*RI site of pBM2829. pBM3260 contains the *HXT2* promoter region from −618 to −339 as a *Bam*HI fragment (amplified by PCR with primers OM572 and OM648) in the reporter plasmid pBM2832. The same fragment of the *HXT2* upstream region was inserted into the *Bam*HI site of Bluescript, creating pBM3281 and pBM3282 (in reverse orientation; −339 to −618). Plasmids pBM3261 and pBM3262 were constructed by insertion of two annealed oligonucleotides (in both tandem and native orientations) containing the wild-type Rgt1p-binding site (OM871, gatcCAAAAT ATAATTTTCCGTGAAA; OM872, gatcTTTCACGGAAAATTATATTTTG; *HXT2* promoter sequences are in capital letters, and restriction sites added to the primers [*Bam*HI and *Bgl*II, respectively] are in lowercase letters) or by insertion

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TABLE 1. Expression of *HXT2* and *HXT4* is inducible by low levels of glucose

Genotype[a]	Mean β-galactosidase activity (U) ± SD[b]					
	HXT2			*HXT4*		
	gly	gly + 0.1% glu	4% glu	gly	gly + 0.1% glu	4% glu
WT	20 ± 1.7	290 ± 19	18 ± 2.4	17 ± 3.3	277 ± 31	14 ± 3
*rgt1*Δ	239 ± 53	312 ± 20	19 ± 2	203 ± 15	313 ± 18	15 ± 1.5
*mig1*Δ	18 ± 2.5	270 ± 31	86 ± 17	12 ± 2.3	292 ± 45	221 ± 37
*ssn6*Δ	312 ± 27	388 ± 36	414 ± 52	310 ± 56	430 ± 62	561 ± 80

[a] WT, wild type.
[b] gly, 5% glycerol + 0.5% galactose; gly + 0.1% glu; 5% glycerol + 0.1% glucose; 4% glu, 4% glucose.

of three copies of mutated (CGG-to-TTT) oligonucleotides (two copies in tandem and native orientation, and the third in reverse orientation) (OM995 and OM996) into the *Bam*HI site of the reporter pBM2832. The number and the orientation of the oligonucleotides were verified by DNA sequencing. pBM3331 and pBM3332 were created by insertion into the Bluescript *Bam*HI site of two (in tandem) or three (two in tandem, and the third in reverse orientation) copies of oligonucleotides (OM871 and OM872) containing the wild-type Rgt1p-binding site. pBM 3264 contains the *HXT4* promoter region from −790 to −374 as a PCR product (primers OM987 and OM988) inserted into the *Bam*HI site of the reporter plasmid pBM2832.

β-Galactosidase assays. β-Galactosidase activity was assayed in permeabilized cells grown to mid-log phase as described previously (12, 13). Activities are given in Miller units and are the mean results of four to six assays of three to six independent transformants. Cells were pregrown on yeast nitrogen base (YNB) containing 5% glycerol plus 0.5% galactose but lacking uracil and transferred to YNB containing 4% glucose, 5% glycerol plus 0.5% galactose, or 5% glycerol plus 0.1% glucose but lacking uracil and incubated for 4 to 5 h before being assayed for β-galactosidase activity. Precultures for *rgt1*Δ and *mig1*Δ mutants were grown on YNB containing 4% glucose and 5% glycerol with 0.5% galactose, respectively.

Primer extension analysis. Total yeast RNA was isolated from the wild-type strain YM2062 as described previously (11). Cultures were pregrown on minimal medium (YNB) containing 5% glycerol plus 0.5% galactose with the appropriate amino acids and transferred for 5 h to minimal medium containing 4% glucose, 5% glycerol, plus 0.5% galactose or 5% glycerol plus 0.1% glucose prior to RNA isolation. Primer extension analysis was performed with 5′-end-labelled oligonucleotides +46 to +69 for *HXT2* (OM771), +47 to +70 for *HXT4* (OM773), and +13 to +40 for *LEU2* (OM28) (2) with 50 μg of total RNA per reaction, as described previously (42). The products of the primer extension reaction were separated on a 6% polyacrylamide–7.0 M urea gel.

Preparation of purified Rgt1p and Mig1p from bacteria. Purified Rgt1p from bacteria was obtained as a fusion with the bacterial maltose-binding protein. For construction of the fusion plasmid (pBM3183), the complete open reading frame of the *RGT1* gene was amplified by PCR with oligonucleotides OM738 and OM740. Several independent PCR products were combined and digested with *Bam*HI and then subcloned into the *Bam*HI site of p*MalE* (New England BioLabs). Cells were grown and induced and proteins were prepared as specified by the manufacturer. Mig1p was generated as a fusion to the bacterial Gstp. This fusion plasmid (pBM2445) was constructed by PCR amplification of the *MIG1* coding region with primers OM380 and OM377. The products of several independent reaction were pooled, digested with *Bam*HI, and subcloned into the *Bam*HI site of pGEX 3X (Pharmacia) to generate pBM2421. The resultant construct was digested with *Sty*I, and the vector was religated to generate pBM2445. This results in a 654-bp deletion of *MIG1*. Protein preparation and purification were performed as specified by the manufacturer.

Gel mobility retardation assays. The 282-bp *Bam*HI fragment of pBM3281 or pBM3282 containing the *HXT2* upstream region (−618 to −339) was labelled by filling in 5′ overhangs with [α-^{32}P]dATP and Klenow enzyme and used as a probe for gel shift DNA-binding assays with purified Rgt1p or Mig1p. The probes used for the gel retardation assays in Fig. 5B (two or three copies of the Rgt1p-binding site) were obtained by cutting out the inserts of pBM3331 and pBM3332 with *Eco*RI and *Xba*I (both sites are within the polylinker) and labelled by filling in 5′ overhangs with [α-^{32}P]dATP. Binding-reaction mixtures contained 10 μl of retardation buffer (50 mM Tris-HCl [pH 7.5], 75 mM KCl, 5 mM $MgCl_2$), 2.5 mM dithiothreitol, 10 μM $ZnSO_4$, 10% (vol/vol) glycerol, 1 μg of salmon sperm DNA, 0.5 to 2 ng (20,000 to 40,000 cpm) of radiolabelled probe, and 50 to 300 ng of purified Rgt1p or Mig1p in a final volume of 20 μl. After incubation for 10 min at 4°C, the protein-DNA complexes were separated on a native 5% polyacrylamide gel.

DNase I protection assay. The probe used for the DNase I protection assay was a 282-bp fragment containing the *HXT2* upstream region from −618 to −339 subcloned into Bluescript. The top strand was labelled with [α-^{32}P]dATP by cutting the plasmid pBM3281 (−618 to −339) with *Eco*RI and by filling in with Klenow enzyme. The plasmid was then digested with *Xba*I (within the polylinker), and the resultant fragment was isolated from the agarose gel. The binding reactions were performed as for the gel retardation assays, and 0.2 or 0.5 U of DNase I was added for 1 min at room temperature. The digestion was stopped by addition of 20 μl stop buffer (20 mM EDTA, 0.2 M NaCl, 0.5% sodium dodecyl sulfate). The samples were extracted with phenol-ethanol and electrophoresed on a 7% polyacrylamide gel.

RESULTS

Expression of *HXT2* and *HXT4* is induced by low levels of glucose. Expression of *HXT2* and *HXT4* is induced about 10- to 20-fold by low levels of glucose (Table 1) (4, 34, 53, 54). This regulation is mediated by two separate repression mechanisms. In the absence of glucose, expression of both genes is inhibited by the Rgt1p repressor: deletion of *RGT1* causes constitutive expression of *HXT2* and *HXT4* in the absence of glucose but has no effect on expression of these genes at high concentrations of glucose (Table 1). In the presence of high levels of glucose, expression of both genes is inhibited by Mig1p, a Cys_2His_2 zinc finger protein involved in glucose-mediated repression of several genes (32): deletion of *MIG1* causes expression of these genes to become inducible by high levels of glucose but has no effect on Rgt1p-dependent repression in the absence of glucose (Table 1). However, unlike *HXT4*, expression of *HXT2* at high concentrations of glucose is not completely derepressed in a *mig1*Δ mutant. Both repression mechanisms are inactive only at low concentrations of glucose, resulting in a 10- to 20-fold induction of *HXT2* and *HXT4* expression. Interestingly, both Rgt1p- and Mig1p-mediated repression requires the function of Ssn6p. Thus, expression of *HXT2* and *HXT4* is constitutive (carbon source independent) in an *ssn6* mutant (Table 1).

To confirm these results obtained with plasmid-borne *HXT2* and *HXT4* promoter fusions to *lacZ*, we used primer extension analysis to compare the levels of *HXT2* and *HXT4* transcripts in cells grown on different carbon sources (Fig. 1). Consistent with the β-galactosidase data, *HXT2* and *HXT4* transcripts are most abundant in cells grown on media containing low levels of glucose (5% glycerol with 0.1% glucose). This analysis also revealed the RNA start sites for *HXT2* and *HXT4* at approximately −39 and −76, respectively, relative to the translation initiation codon (data not shown).

Deletion analysis of the *HXT2* upstream regulatory region. To identify the *HXT2* regulatory elements through which Rgt1p and Mig1p prevent expression, we performed a deletion analysis of a plasmid-borne *HXT2* promoter fused to *lacZ* (Fig. 2A). All regulatory sequences are within 618 bp of the ATG codon, since these sequences are sufficient for proper regulation. The region between −618 and −495 appears to contain repression sites for both Mig1p and Rgt1p, because deletion of this region substantially relieves repression both in the absence of glucose and in the presence of high levels of glucose. Fur-

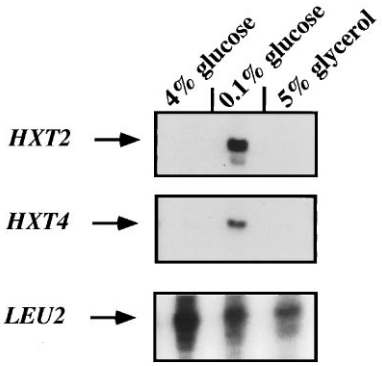
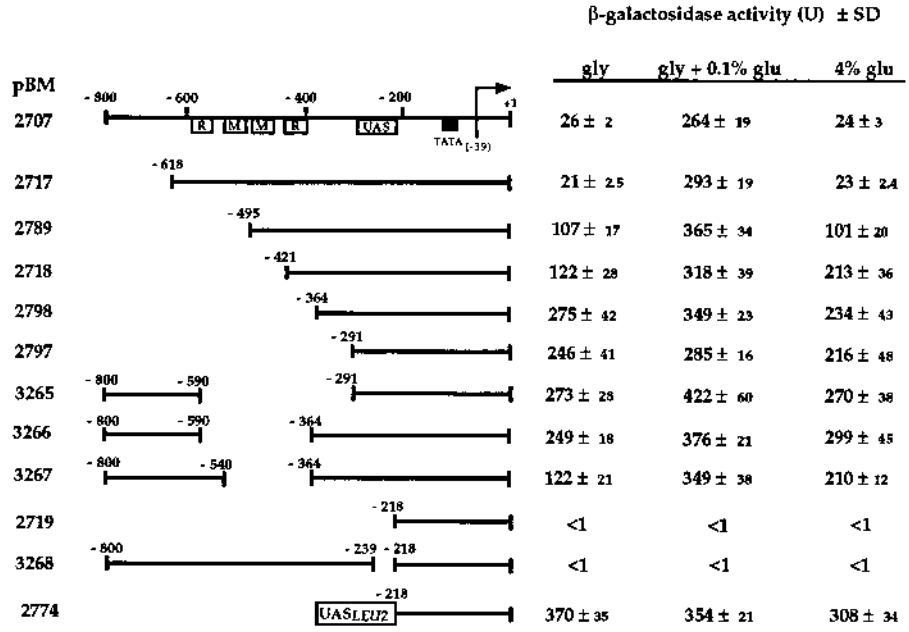


FIG. 1. Primer extension analysis of *HXT2* and *HXT4* transcript levels in cells grown on different carbon sources: 4% glucose, 0.1% glucose (5% glycerol + 0.1% glucose), and 5% glycerol (5% glycerol + 0.5% galactose). The transcript level of the *LEU2* gene was also determined to demonstrate that approximately equal amounts of total RNA (50 μg) were loaded in each reaction.

ther deletion to −421 increased expression about twofold on 4% glucose, suggesting the presence of an additional repression site in this region for Mig1p. Further deletion to −364 causes a twofold increase in expression only on glycerol, suggesting the presence of a second repression element within this region for Rgt1p. Further deletion to −291 did not significantly change the level of *HXT2* expression. Results obtained with the internal deletions support the view that Rgt1p- and Mig1p-mediated repression sites lie between −618 and −364.

A UAS element appears to lie between −291 and −218 (compare pBM2797 with pBM2719), since deletion of this region completely abolishes expression. This UAS must include sequences between −239 and −218, since this region is required for promoter function (pBM3268). Insertion of the UAS element of *LEU2* at −218 restores expression, supporting the conclusion that diminished expression caused by these deletions is due to lack of a UAS element. The fact that expres-

A



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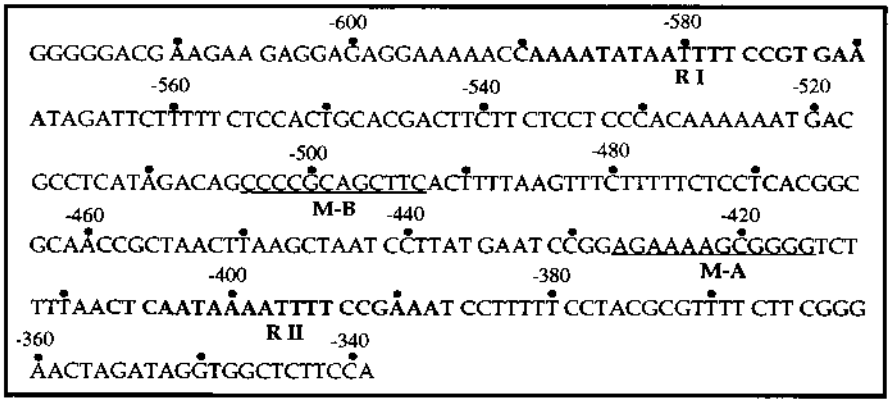
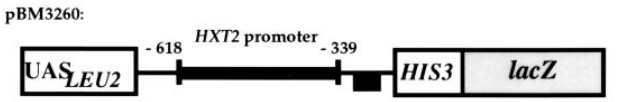


FIG. 2. (A) Deletion analysis of the *HXT2* upstream regulatory region. The boxes (at −364 to −618) represent the regions through which Rgt1p (R) and Mig1p (M) mediate repression. The UAS (−291 to −218) is indicated. Deletion endpoints are numbered with respect to the *HXT2* translation initiation codon (+ 1). The RNA start site is at approximately −39. The small solid box upstream of the RNA start site represents the putative TATA element (at −122). (B) Sequence of the *HXT2* promoter region from −618 to −339, which is sufficient to provide glucose-induced expression to a heterologous promoter. The putative binding sites for Rgt1p, RI and RII (bold type), and for Mig1p, M-A and M-B (underlined), are indicated.



genotype		Mean ß-galactosidase activity (U) ± SD gly	gly + 0.1% glu	4% glu
WT	vector	187 ± 19	207 ± 43	229 ± 37
WT -	pBM3260	7 ± 0.8	134 ± 19	3.5 ± 0.6
rgt1Δ -	"	159 ± 28	127 ± 11	4.1 ± 0.8
mig1Δ -	"	4.8 ± 1.3	156 ± 4	46 ± 10

FIG. 3. The *HXT2* promoter region from −618 to −339 is sufficient to mediate induction by low levels of glucose. WT, wild type; vector, the reporter construct pBM2832 without insert. For definitions of gly and glu, see Table 1, footnote *b*.

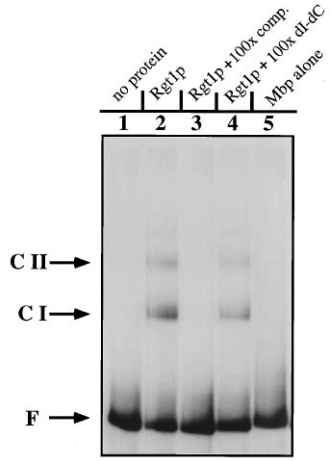


FIG. 4. Gel mobility retardation assays with labelled *HXT2* promoter containing the sequences from −618 to −339 as a probe and Rgt1p from bacteria. The probe was mixed with purified Rgt1p (50 ng) and electrophoresed on a native 5% polyacrylamide gel. The arrows indicate the mobility of free DNA (F) and the mobility of Rgt1-DNA complexes (C I and C II). Binding reactions were performed as described in Materials and Methods.

sion of the UAS_{LEU2}-containing plasmid is constitutive demonstrates that no repression elements lie downstream of −218.

To determine whether this regulatory region of *HXT2* is sufficient to mediate expression induced by low glucose levels, we examined its effect on a heterologous promoter (Fig. 3). A fragment containing the *HXT2* upstream regulatory region from −618 to −339 (Fig. 2B) was inserted between the UAS element of *LEU2* and the TATA element and part of *HIS3* fused to *lacZ*. Expression driven by this promoter is repressed about 20-fold on glycerol, mediated by the Rgt1p repressor. The function of this promoter is repressed at high concentrations of glucose as a result of Mig1p. These data establish that the 280-bp region of the *HXT2* upstream regulatory region from −618 to −339 contains all of the regulatory elements required for repression by Rgt1p and Mig1p.

Determination of the Rgt1p-binding sites in the *HXT2* promoter. The 280-bp fragment (−618 to −339) that mediates induction at low glucose levels was used as a probe in gel mobility retardation assays with Rgt1p expressed in bacteria. Addition of purified Rgt1p (fused to the maltose-binding protein) yielded predominantly two complexes with different mobilities (Fig. 4, lane 2). Binding of Rgt1p to this probe is specific, since the addition of unlabelled fragment (lane 3) but not of a nonspecific competitor (lane 4) competes for Rgt1p binding. This demonstrates that Rgt1p binds directly to the *HXT2* promoter, possibly to at least two different binding sites on this fragment.

To define more precisely the region to which Rgt1p binds to the *HXT2* promoter, we carried out DNase I footprint analysis (Fig. 5). Rgt1p clearly protects a small region near the nucleotide triplet CGG (nucleotides −596 to −594) that is known to be required for DNA binding of this type of zinc cluster protein (Cys_6Zn_2 Gal4p family [1, 18]) and may span as much as 33 bp (nucleotides −601 to −569). To confirm that Rgt1p binds to the footprinted region, a synthetic oligonucleotide containing the first 22 bp of the protected region was used as probe for gel shift DNA-binding assays with purified Rgt1p (Fig. 6A). In addition, we used a second synthetic oligonucleotide, in which the triplet CGG was changed to TTT (mutant oligonucleotide). We observed specific binding of Rgt1p to the oligonucleotide containing the wild-type sequence (Fig. 6A). Since binding of Rgt1p to the wild-type oligonucleotide yielded only one complex in gel shift assays, it seems likely that the protected 33-bp region contains only one binding site for Rgt1p. By contrast, purified Rgt1p did not bind to the mutant oligonucleotide and binding to the wild-type oligonucleotide was inhibited only by the wild-type, not by the mutant oligonucleotide, indicating a requirement of the CGG triplet for DNA

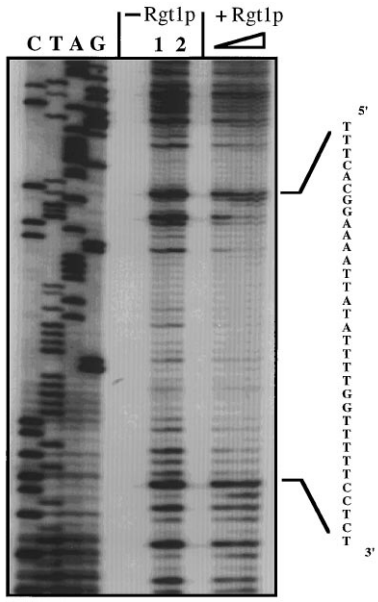


FIG. 5. DNase I footprint analysis of the Rgt1p-DNA complex. The probe included the *HXT2* upstream regulatory region from −618 to −339 containing the Rgt1p binding sites and was labelled on the top strand. The reaction mixtures without Rgt1p were partially digested with 0.2 U (lane 1) or 0.5 U (lane 2) of DNase I for 1 min at room temperature. The binding-reaction mixtures containing increasing amounts of purified Rgt1p (0.1, 0.4, and 1 μg) were treated with 0.5 U of DNase I for 1 min. A Bluescript-based plasmid (pBM3281) containing the same fragment was sequenced with primer OM855 (anneals to sequences in the polylinker of Bluescript). The protected region extends from approximately −601 to −569.

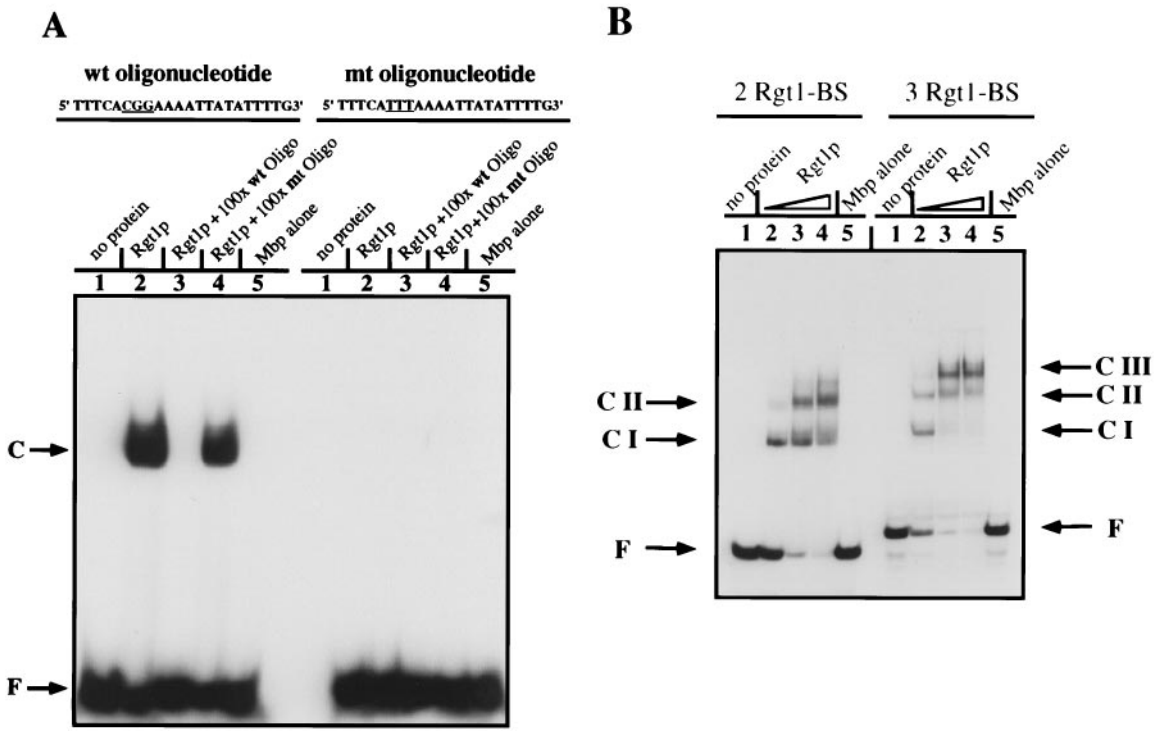


FIG. 6. Gel shift DNA-binding assays with purified Rgt1p and with oligonucleotides containing wild-type or mutated Rgt1p-binding sites (A) or with multiple Rgt1p-binding sites (B) as the probe. (A) Binding-reaction mixtures contained no protein (lane 1), 50 ng of purified Rgt1p (lanes 2 to 4), or 60 ng of purified Mbp (lane 5). Wild-type (wt) (lane 3) or mutant (mt) oligonucleotide (lane 4) in 100-fold molar excess was added to the binding-reaction mixtures. The arrows indicate the mobility of the unbound probe (F) and the Rgt1p-DNA complex (C). (B) Binding-reaction mixtures contained no Rgt1p (lane 1), increasing amounts of Rgt1p (50, 100, and 500 ng) (lanes 2 to 4), or Mbp (lane 5).

binding. These results confirm that the protected region determined by DNase I footprint indeed consists of one Rgt1p-binding site. A fragment containing two or three annealed oligonucleotides with the Rgt1p-binding site as the probe yielded two or three shifted complexes, respectively (Fig. 6B), also in agreement with the view that the 33-bp region contains one binding site for Rgt1p.

Deletion analysis of the *HXT2* promoter suggests the location of a second repression site for Rgt1p between −421 and −364. However, we were unable to detect a significant protection of this region from DNase I by Rgt1p. To determine the second binding site, we used different regions of the *HXT2* promoter as probes for gel retardation assays with purified Rgt1p (Fig. 7A). As demonstrated above (Fig. 4), the 280-bp fragment sufficient for low-glucose-induced expression of a reporter construct yields two DNA-Rgt1p complexes (Fig. 7A, lanes 1 and 2). Purified Rgt1p gave rise to only one complex, with the region extending from −421 to −339 as the probe (lanes 3 and 4) but failed to bind to a 25-bp fragment containing sequences from −364 to −339 (lanes 5 and 6). This suggests that the second Rgt1p-binding site lies between −421 and −364. We found within this 58-bp region only one potential Rgt1p-binding site that fits the binding site determined by the DNase I protection assay (Fig. 7B).

The Rgt1p-binding site mediates repression in vivo. To demonstrate that the Rgt1p-binding site mediates repression of gene expression, a double-stranded oligonucleotide containing the wild-type sequence of the protected region was inserted between the UAS_{LEU2} and the TATA box of *HIS3* fused to *lacZ* (Fig. 8). Expression from a promoter carrying two copies of the Rgt1p-binding site is repressed in the absence of glucose, and this requires *RGT1*. The presence of three copies of the mutant Rgt1p-binding site does not provide Rgt1p-mediated repression. Thus, the oligonucleotide covering the *HXT2* promoter sequences from −590 to −569 contains an Rgt1p-binding site that is sufficient for Rgt1p-dependent repression.

Mig1p binds to two different sites within the *HXT2* upstream region. To determine whether Mig1p also prevents *HXT2* expression by direct binding to its promoter, we used the same 280-bp *HXT2* fragment as a probe for gel mobility shift assays with purified Gst-Mig1p. Like Rgt1p, purified Mig1p yielded two complexes, suggesting that it binds to two different sites in the *HXT2* promoter (Fig. 9, lane 2). Competition experiments with unlabelled fragment and dI-dC as the nonspecific competitor demonstrated that Mig1p binding is specific (lanes 3 and 4). Since the consensus sequence to which Mig1p binds is known (26), we searched the *HXT2* upstream regulatory region for putative Mig1p-binding sites. The 280-bp fragment used for gel mobility retardation assays contains two potential Mig1p-binding sites (site A, −416 to −427; site B, −493 to −504). We confirmed the binding of Mig1p to site A and site B by DNase I footprint analysis. Purified Gst-Mig1p clearly protected site A from DNase I digestion, while protection of site B was less obvious (Fig. 10). The sequence of both regions conforms to the consensus sequence defined for Mig1p (26).

Rgt1p and Mig1p inhibit *HXT4* expression directly. Like *HXT2*, the expression of *HXT4* is induced only at low levels of glucose, because of repression by Rgt1p and Mig1p (Table 1) (34, 46). The *HXT4* promoter region from −790 to −374 (416 bp) provides more than 10-fold induction of gene expression by low concentrations of glucose (Fig. 11), and this is due to Rgt1p-mediated repression. Repression caused by this frag-

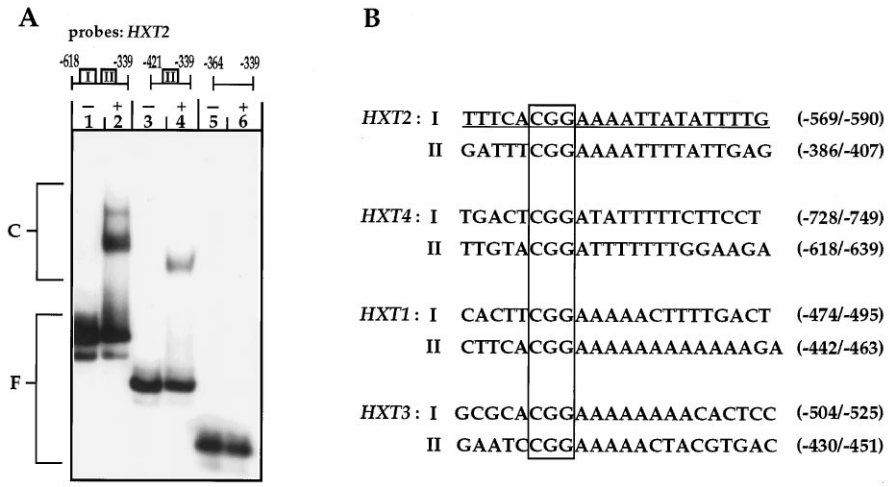


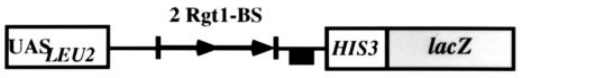
FIG. 7. (A) Gel shift DNA-binding assays with different regions of the *HXT2* promoter as probe and Rgt1p. The binding reactions were performed as described in Materials and Methods, and the mixtures contained no protein (−) or 50 ng of purified Rgt1p (+). (B) Putative Rgt1p-binding sites at different *HXT* promoters are compared with the footprinted binding site in the *HXT2* promoter (underlined sequence).

ment on high levels of glucose is due to Mig1p (Fig. 11). Thus, this region contains sequences through which Mig1p and Rgt1p act to inhibit *HXT4* expression. The 416-bp fragment of the *HXT4* promoter was used for gel retardation assays with Rgt1p and Mig1p (Fig. 12). Both repressors, Mig1p and Rgt1p, gave rise to two shifted complexes, suggesting that this region of the *HXT4* promoter contains two different sites for both Rgt1p and Mig1p. The 416-bp fragment contains two potential binding sites for Rgt1p (Fig. 7B) and for Mig1p. Thus, Rgt1p and Mig1p also inhibit the expression of *HXT4* directly by binding to its upstream regulatory region.

DISCUSSION

The glucose transporter genes *HXT2* and *HXT4* are expressed only in cells growing on low concentrations of glucose. This is due to two different transcriptional repressors: Mig1p prevents expression at high levels of glucose, and Rgt1p inhibits its expression in the absence of glucose. Low concentrations of glucose cause inhibition of Rgt1p function but are insufficient to activate the Mig1p repressor, resulting in 10- to 20-fold induction of gene expression. Both repressors act by directly binding to *HXT2* and *HXT4* promoters.

Analysis of regulatory elements in the *HXT2* promoter. The *HXT2* UAS element appears to reside between −291 and −218, because deletion of this region strongly impairs *HXT2* expression. A smaller deletion, which removes sequences from −239 to −218, is also defective in *HXT2* expression. Interestingly, this region contains a perfect inverted repeat of six nucleotides ($CCACGCN_5GCGTGG$) between −226 and −210. Perhaps this sequence is the binding site for a transcriptional activator protein. The *HXT2* promoter contains three potential TATA elements (at −14, −47, and −122) (25). Since the RNA start site is at approximately −39, it is likely that the most



relevant genotype	inserted oligonucleotide	Mean ß-galactosidase activity (U) ± SD gly	gly + 0.1% glu	4% glu
WT	wild type	7 ± 0.7	52 ± 12	46 ± 7
rgt1Δ	wild type	67 ± 10	96 ± 11	50 ± 9
WT	mutant	95 ± 13	90 ± 6	103 ± 22
rgt1Δ	mutant	84 ± 8	82 ± 19	83 ± 13

FIG. 8. Oligonucleotides containing Rgt1p binding site mediate Rgt1p-dependent repression. WT, wild type. For definitions of gly and glu, see Table 1, footnote *b*.

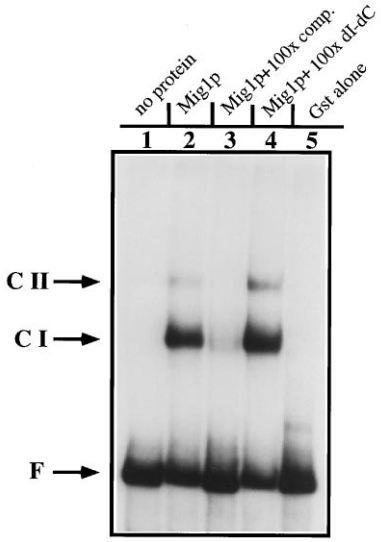


FIG. 9. Gel mobility retardation assays with labelled *HXT2* promoter (−618 to −339) and purified Mig1p. The binding-reaction mixtures were electrophoresed on a native 5% polyacrylamide gel and contain no protein (lane 1), 300 ng of purified Mig1p (lanes 2 to 4), or 250 ng of purified Gstp as a negative control for binding (lane 5).

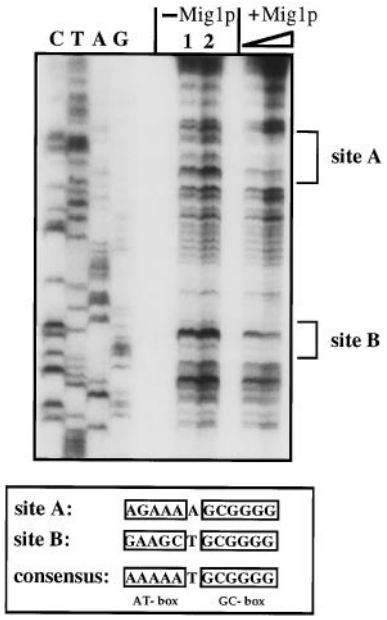
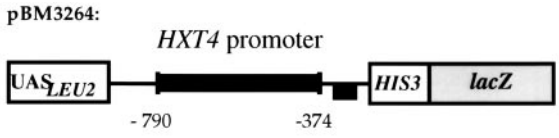


FIG. 10. DNase I footprint analysis of Mig1p-DNA complex. A fragment containing the *HXT2* promoter region from −618 to −339 was labelled on the top strand. DNA-binding reaction mixtures without Mig1p were partially digested with either 0.2 (lane 1) or 0.5 (lane 2) U of DNase I for 1 min. The binding-reaction mixtures containing increasing amounts of Mig1p (1 and 4 μg) were partially digested with 0.5 U of DNase I for 1 min. The same fragment used as the probe on a plasmid (pBM3281) was sequenced with the oligonucleotide OM855 as the primer. The sequences of the two Mig1p-binding sites (sites A and B) are compared with the defined consensus sequence (26).

upstream TATA element located at −122 is the functional one.

The promoter sequences through which Rgt1p and Mig1p inhibit *HXT2* expression were defined in several ways. First, replacement of *HXT2* sequences upstream of −218 with UAS_{LEU2} leads to constitutive expression, demonstrating that all repression elements are probably located upstream of this site. Second, deletion of the region from −618 to −364 results in an almost complete loss of repression by Mig1p and Rgt1p.



		Mean ß-galactosidase activity (U) ± SD		
genotype	plasmid	gly	gly + 0.1% glu	4% glu
WT	vector	187 ± 19	207 ± 43	229 ± 37
WT	pBM3264	8 ± 1	153 ± 30	11 ± 3
rgt1Δ	"	113 ± 19	147 ± 12	18 ± 5
mig1Δ	"	5 ± 2	140 ± 22	136 ± 18

FIG. 11. Transcription of *HXT4* is repressed by Mig1p and Rgt1p. WT, wild type; vector, the reporter construct pBM2832 without insert. For definitions of gly and glu, see Table 1, footnote *b*.

Finally, a 280-bp fragment covering this region (−618 to −339) is sufficient to provide low-glucose-induced expression to a heterologous promoter. Thus, this region of the *HXT2* promoter contains both Rgt1p- and Mig1p-dependent repression sites, and two binding sites for each of these repressors were identified (Fig. 2B).

Rgt1p-binding sites. The Rgt1p DNase I footprint spans 33 bp, and purified Rgt1p binds to a synthetic oligonucleotide containing the first 22 bp of this sequence. This Rgt1p-binding site is sufficient to support Rgt1p-dependent repression of a heterologous promoter. Thus, Rgt1p directly represses *HXT2* expression. Introduction of multiple copies of the oligonucleotide with the Rgt1p-binding site appears to titrate Rgt1p and thus causes increased *HXT* gene expression (45a).

Rgt1p contains an amino-terminal C_6 zinc cluster motif similar to the DNA-binding domain of the Gal4p family of DNA-binding proteins (18, 37). Gal4p residues Lys-17 and Lys-18 make base-specific contacts via hydrogen bonds (27), and these lysine residues are conserved in Rgt1p. A significant difference between Gal4p and Rgt1p is in the proline residue at position 26, which is critical for zinc binding (19, 21) and is conserved in all transcription factors of this family. This residue is a glycine in Rgt1p.

Most members of this family of DNA-binding proteins function as transcriptional activators (e.g., Gal4p, Ppr1p, Put3p, and Arg81p). The only other family member that functions as a repressor is Ume6p, which is a negative regulator of early meiotic genes and arginine catabolic enzymes (35, 45). The carboxy-terminal location of the DNA-binding domain of the Ume6p repressor raised the possibility that this location has functional significance in repression, but this seems unlikely, since the C_6 domain of the Rgt1p repressor is near its amino terminus.

Most C_6 zinc cluster proteins recognize two CGG triplets as inverted palindromes (CGG-CCG) separated by a characteristic spacing for each protein (1, 38). For example, 11 nucleotides separate the CGG triplets in Gal4p-binding sites and 6 nucleotides separate the triplets bound by Ppr1p. One C_6 zinc cluster protein, Hap1p, recognizes two CGG sequences as direct repeats (36). The Rgt1p-binding site contains only a single CGG triplet that is essential for Rgt1p binding in vitro and for repression in vivo. Since Gal4p binds to DNA as a dimer, with each monomer recognizing one CGG triplet, we believe that Rgt1p contacts its site as a monomer. Consistent with this idea is the fact that Rgt1p does not contain any obvious dimerization domains like those found in most other members of this family. One other member of the C_6 zinc cluster family, ArgRIIp, appears to bind to only one CGG half site (9, 10).

Two other Rgt1p-regulated glucose transporter genes, *HXT1* and *HXT3* (34), each contain two possible binding sites for Rgt1p (Fig. 7B). Indeed, purified Rgt1p forms at least two complexes with fragments of these regions of the *HXT1* and *HXT3* promoters (reference 34a and unpublished data). A striking feature of these real and potential Rgt1p-binding sites is the presence of an AT-rich region 3′ to the CGG triplet (Fig. 7B). The C_2H_2 zinc finger Mig1p also requires AT-rich sequences flanking its conserved GC box for binding (26).

Mig1p-binding sites. *MIG1* encodes a C_2H_2 zinc finger protein involved in glucose repression of several genes (32). The Mig1p-binding site was defined by saturation mutagenesis and found to be GCGGGG (GC box) with 5′-flanking A or T nucleotides (AT box) (26). On the basis of the structure of similar zinc finger proteins, it is proposed that each of the two zinc fingers interacts with base triplets in the GC box. The two Mig1p-binding sites within the *HXT2* promoter fit well with the defined Mig1p consensus sequence. While both sites contain a

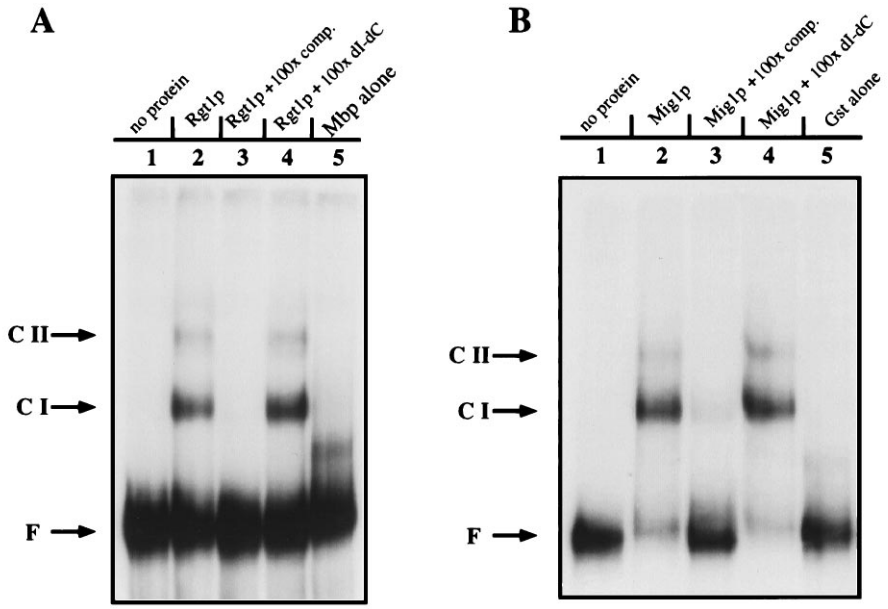


FIG. 12. Gel shift DNA-binding assays with either purified Rgt1p (A) or Mig1p (B) and a 416-bp fragment (−374 to −790) of *HXT4* upstream region as the probe. Binding-reaction mixtures contained no protein (lane 1), 50 ng of Rgt1p or 300 ng of Mig1p (lanes 2 to 4), or 60 ng of Mbp or 250 ng of Gstp (lane 5). Unlabelled fragment in 100-fold molar excess (lane 3) or the same amount of dI-dC as nonspecific competitor (lane 4) was added to the binding reactions. Arrows indicate unbound DNA (F) and complexed DNA (C I, C II).

perfect GC box, they differ from the consensus sequence in their AT-rich region. It has been demonstrated that more than one C within the AT-box prevents Mig1p binding in vitro; therefore, site B is predicted to be a weaker binding site than site A. This fits with our observation that the Mig1p footprint of site A is more apparent than that of site B. The spacing of the two Mig1p-binding sites in the *HXT2* promoter (64 bp) is similar to that found in the *SUC2* (42-bp) and *GAL1* (59-bp) promoters. However, the location of the Mig1p-binding sites is downstream of the UAS element in *SUC2* and *GAL1* (31, 32) but upstream of the *HXT2* UAS.

Deletion of *MIG1* does not completely abolish glucose repression of *HXT2*, suggesting that additional repressors may contribute to glucose repression of *HXT2* expression. Indeed, two other Mig1p-related proteins that contribute to glucose repression of *HXT2* transcription have been identified (26a).

Repression of *HXT4* expression. Mig1p and Rgt1p also collaborate to limit the expression of *HXT4* to occur only at low levels of glucose. We found that a 416-bp fragment of the *HXT4* promoter is sufficient to provide Mig1p- and Rgt1p-dependent repression to a heterologous promoter. Results of DNA-binding assays indicate the presence of at least two Rgt1p- and Mig1p-binding sites in this region of the *HXT4* promoter, and it also contains two potential Rgt1p-binding sites (at −749 to −728 and −639 to −618 [Fig. 7B]) and two Mig1p consensus sites (at −566 to −554 and −498 to −486). Although *HXT4* and *HXT2* regulation is similar, *HXT4* is more glucose repressed than is *HXT2* and deletion of *MIG1* completely abolishes its repression. This suggests that Mig1p is solely responsible for glucose repression of *HXT4* expression.

The transcriptional regulation of *HXT2* and *HXT4* is a simple example of combinatorial control of gene expression, in which each repression element functions independently of the other and responds to a different environmental signal. The combination of both repression modules at the same promoter causes expression to be restricted to low-glucose media. Interestingly, at the promoter of the *HXT1* gene, the combination of Rgt1p repression sites with other regulatory modules limits its expression to high concentrations of glucose. This resembles in a simple way the regulation of certain key genes involved in the determination of cell fate. The combinatorial effect of several different transcription factors at these promoters can change the time and the place of their expression and thus determine the fate of the cell (8, 57).

Physiological relevance of transcriptional repression of *HXT2* and *HXT4* genes. *HXT2* and *HXT4* clearly encode glucose transporters (4, 25, 46). Because they are expressed only on low-glucose-containing media (0.1%), it seems likely that *HXT2* and *HXT4* encode high-affinity glucose transporters. Therefore, we are not surprised that Hxt2p and Hxt4p are required for growth on low concentrations of glucose (24, 39). Like most eukaryotes, yeast cells prefer glucose over other sugars. We believe that they adapt to different concentrations of glucose by turning on the expression of glucose transporters with affinities and capacities appropriate to the amount of available glucose.

Induction of glucose transporter gene expression by glucose is a common regulatory mechanism that is also found in mammalian cells. Like *HXT2* and *HXT4* expression in *S. cerevisiae*, transcription of the mammalian glucose transporter genes *GLUT1* and *GLUT2* is induced in the presence of glucose (51, 56). The *GLUT2* gene is expressed in pancreatic β cells and is required for the first step of glucose-induced insulin secretion. Insulin functions as a hormone that stimulates the translocation of the glucose transporter Glut4p to the cell surface of fat and muscle cells (16, 29). Therefore, the expression level of the *GLUT2* gene plays a critical role in the regulation of glucose uptake in mammals (33, 52). Thus, both yeasts and mammals ensure efficient metabolism of glucose by inducing the transcription of specific glucose transporter genes in response to glucose availability.

ACKNOWLEDGMENTS

We thank Jim Dover for providing purified Rgt1p and Mig1p and John Majors for helpful discussions.

This work was supported by NIH grant GM32540 and funds provided by the McDonnell Foundation. S.Ö. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (DFG).

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