Rgt1, a glucose sensing transcription factor, is required for transcriptional repression of the *HXK2* gene in *Saccharomyces cerevisiae*

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Expression of *HXK2*, a gene encoding a *Saccharomyces cerevisiae* bifunctional protein with catalytic and regulatory functions, is controlled by glucose availability, being activated in the presence of glucose and inhibited when the levels of the sugar are low. In the present study, we identified Rgt1 as a transcription factor that, together with the Med8 protein, is essential for repression of the *HXK2* gene in the absence of glucose. Rgt1 represses *HXK2* expression by binding specifically to the motif (CGGAAAA) located at −395 bp relative to the ATG translation start codon in the *HXK2* promoter. Disruption of the *RGT1* gene causes an 18-fold increase in the level of *HXK2* transcript in the absence of glucose. Rgt1 binds to the RGT1 element of *HXK2* promoter in a glucose-dependent manner, and the repression of target gene depends on binding of Rgt1 to DNA. The physiological significance of the connection between two glucose-signalling pathways, the Snf3/Rgt2 that causes glucose induction and the Mig1/Hxk2 that causes glucose repression, was also analysed.

Key words: glucose repression, glucose signalling, *HXK2* gene, Med_8, Rgt_1, *Saccharomyces cerevisiae*.

INTRODUCTION

Glucose is known to affect a variety of processes in *Saccharomyces cerevisiae*. These include glucose repression of genes for growth on alternative carbon sources and the induction of genes necessary for glucose transport [1–3]. Thus, depending on the availability of glucose, cells significantly change expression of many genes through mechanisms by which yeast cells sense the level of extracellular glucose [4]. Indeed, several signalling pathways governing these processes have been identified in yeast. In the Snf3/Rgt2 pathway, these two proteins act as glucose receptors. The Rgt2 and Snf3 proteins resemble hexose transporters in structure but have long cytoplasmic tails required for signal transduction [5]. Glucose binding to these transmembrane proteins initiates signals that activate the pathway and allow hexose transporter gene expression by repressing Rgt1 function [6]. Thus glucose in yeast acts similarly to a growth hormone in mammalian systems.

Glucose also activates another pathway involved in gene repression, which is not needed during the growth on glucose. In this pathway, both the Mig_1 and Hxk2 (hexokinase 2) proteins are necessary to generate the glucose repression signal. The mechanism by which Mig_1, a transcriptional repressor responsible for glucose repression of many genes, operates has been extensively investigated [7–9]. On the other hand, the mechanism by which Hxk2, in addition to its classical metabolic role, plays an important function in glucose signalling is only beginning to be understood [10,11]. Recently, it was described that, in *S. cerevisiae*, Hxk2 partially localizes to the nucleus in a glucose-dependent manner. Mig_1 is required to sequester Hxk_2 into the nucleus to generate a repressor complex during its growth in glucose medium [12].

So far, experimental evidence for a connection between the two glucose-sensing pathways is scarce. Only two main connections have been established [13]. These include, first, the effect of the Snf3/Rgt2 induction pathway on the glucose-repression pathway by the induction of the transcription of MIG_2, which encodes a secondary repressor of the Mig_1 repressor pathway [14]. Secondly, the Mig1/Hxk2 repression pathway contributes to glucose induction by repressing the expression of *SNF3* and *MTH1* genes [15–17] that encode a regulator of the Rgt1 transcription factor.

In the present study, we characterize the factors involved in the repression of *HXK2* gene in the absence of glucose. We present evidence that repression of *HXK2* requires a functional Rgt1 protein together with the product of the essential gene *MED8*. The Med8 protein specifically binds to a DRS (downstream repressing sequence) of the *HXK2* gene [18,19] and constitutes a component of the large multiprotein complex called Srb-mediator complex. The latter enhances basal and facilitates activated transcription by interacting with the C-terminal domain of RNA polymerase II [20,21]. Transcription cofactors, such as Med8, mediate access to genes in chromatin and they help to establish, maintain or activate regulatory networks. Thus a possible role of Med8 could be to affect the formation and activity of basal initiation complexes by linking the specific DNA–protein regulatory complexes to the RNA polymerase II holoenzyme transcriptional machinery. Since the Hxk2 protein is also necessary to generate the glucose-repression signal through the Mig1/Hxk2-mediated glucose-signalling pathway, a new connection between both the pathways could be deduced.

MATERIALS AND METHODS

Strains and growth media

S. cerevisiae strain DBY1315 (*MATα ura3-52 leu2-3,2-112 lys2-801 gal2*) was donated by D. Botstein (Stanford University, Stanford, CA, U.S.A.) and was used as a wild-type strain and recipient in the transformation experiments. An *RGTI*-deficient strain was obtained by using a *loxP-KlURA3-LoxP* disruption cassette as described in [22]. Bacterial transformation and preparation of recombinant plasmid DNA were performed in

Abbreviations used: ChIP, chromatin immunoprecipitation; DAPI, 4,6-diamidino-2-phenylindole; DRS, downstream repressing sequence; EMSA, electrophoretic mobility-shift assay; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin; Hxk2, hexokinase 2.

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Escherichia coli MC1061 [*hsdR mcrB araD139Δ(araABC-leu)7679Δlacx74 galU galK rpsL thi*]. Expressions of fusion proteins were obtained in *E. coli* BL21(DE3)pLysS (Promega, Madison, WI, U.S.A.).

Yeast cells were grown in the following media: rich media based on 1 % (w/v) yeast extract and 2 % (w/v) peptone (YEP), 2 % (w/v) glucose (YEPD) or 3 % (v/v) ethanol (YEPE) were added as carbon sources. Synthetic media consisted of 0.67 % yeast nitrogen base without amino acids, supplemented with amino acids as required and 2 % glucose or 3 % ethanol. This medium was utilized to select for transformants when plasmids carrying *URA3* or *LEU2* were used. Solid media contained 2 % (w/v) agar in addition to the components described above.

General DNA techniques

Restriction enzymes and T_4 DNA ligase were from Roche (Indianapolis, IN, U.S.A.), and radioactive isotopes were from Amersham International (Arlington, IL, U.S.A.). DNA manipulations were performed as described previously [23].

Plasmid constructions

Plasmid pGEX/RGT1 was constructed by subcloning a BamHI fragment from plasmid pGBKT7/RGT1 in frame with pGEX-4T (Amersham Biosciences, Piscataway, NJ, U.S.A.). Plasmid pGBKT7/RGT1 carried a 3512 bp BamHI fragment with the complete coding region of the *RGT1* gene in pGBKT7 (ClonTech Laboratories, Basingstoke, U.K.). The *RGT1* insert was synthesized by PCR using genomic DNA as a template with the primer pair OL1 + OL2 (OL1: AAGGATCCATGAACGAGCTGAACACTGT and OL2: ATGGATCCTCAATACCAGCCTAACTCGG). DNA sequencing verified this PCR-generated construct.

The yeast expression vector YEp352/MIG1::gfp was constructed as follows: a 969 bp BamHI–BglII fragment containing the *GFP* (green fluorescent protein) gene was subcloned into a YEp352/MIG1 plasmid cleaved with BamHI. YEp352/MIG1 plasmid contains a 2.77 kb SacI–BamHI fragment with the complete *MIG1* gene under the control of its own promoter. The yeast expression vector YEp352/HXK2::gfp was constructed as indicated in [12]. All the clones used were verified by sequencing the fusion points.

The *HXK2* reporter plasmid YIp-HXK2$_{+404}$ was constructed by placing sequences from − 838 to + 404 bp relative to the *HXK2* translation start codon, upstream of a *lacZ* reporter gene on the integrative yeast vector YIp357. An *HXK2* reporter plasmid lacking the DRS elements was constructed by placing sequences spanning from − 838 to + 39 relative to the *HXK2* ATG, upstream of a *lacZ* reporter gene on an integrative yeast vector to create the yeast reporter plasmid YIp-HXK2$_{+39}$ [24].

Plasmid YIp and YEp are yeast-*E. coli* shuttle vectors suitable for use respectively as integrative or episomal vectors. These vectors have an *URA3* yeast selectable marker [25].

RNA preparation and Northern blotting

Total yeast RNA was isolated from cells grown in the indicated medium until the absorbance (*A*) at 600 nm was 1.0 as described previously [26]. The RNA samples were separated by formaldehyde gel electrophoresis and transferred on to a Hybond-N membrane (Amersham Biosciences). To ensure uniform loading and transfer of RNA, ethidium bromide was added to the samples, and the stained rRNA was visualized on the blots under UV illumination. Northern blots were probed with a 1.5 kb PstI fragment from *HXK2*. Blots were also probed with a radiolabelled 1.4 kb BamHI–HindIII fragment from the *ACT1* gene to confirm uniform loading. All probes were radiolabelled by using [α-^{32}P]-dCTP (3000 μCi/nmol) and random priming. Blots were analysed by using a Packard Instantimager and the Instantimager software.

Preparation of crude protein extracts

Yeast protein extracts were prepared as follows: yeasts were grown in 10–20 ml of rich medium (YEPD) at 28 °C to A_{600} 1.0. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl of 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.42 M NaCl and 1.5 mM $MgCl_2$. The cells were broken by vortex-mixing (6 × 20 s) in the presence of glass beads (0.5 g), and 400 μl of the same buffer was added to the suspension. After centrifugation at 19 000 ***g*** (14 000 rev./min) for 15 min at 4 °C, the supernatant was used as crude protein extract.

Enzyme assays

Invertase activity was assayed in whole cells as described previously [27] and expressed as (μmol of glucose released) · min^{-1} · [100 mg of cells (dry weight)]$^{-1}$. For β-galactosidase activity determinations, crude extracts were prepared with glass beads as described above and 2 mg/ml *o*-nitrophenol β-D-galactopyranoside was used as a substrate [23]. Specific activity was calculated in relation to total protein in the crude extract, using BSA as the standard.

Preparation of yeast nuclear extracts

Nuclear extracts were prepared by a method described in [28] with the modifications indicated in [29].

Heterologous expression of *S. cerevisiae RGT1* gene in *E. coli* and purification of Rgt1 protein

E. coli cells containing the expression plasmid pGEX/RGT1 were used to produce the *S. cerevisiae* protein Rgt1 as a fusion protein with the *Schistosoma japonicum* GST (glutathione S-transferase) using the procedure described previously [18]. Rgt1 was obtained from GST–Rgt1 fusion protein coupled with glutathione–Sepharose beads by site-specific separation of the GST affinity tag using 2.5 units of thrombin.

DNA probes and EMSA (electrophoretic mobility-shift assay)

To investigate the interaction of Rgt1 with the sequence carrying the RGT1 element of the *HXK2* promoter, we reconstituted the fragment from two complementary oligonucleotides (RGT1$_{HXK2}$ sense, 5′-tcgaGCAGTTTTTCCGGTCGAT-3′; and RGT1$_{HXK2}$ antisense, 5′-tcgaATCGACCGGAAAAACTGC-3′). The complementary strands were annealed and either end was labelled with [α-^{32}P]dCTP by fill-in, using the Klenow fragment of DNA polymerase I. The labelled double-stranded DNA was used as a probe and the unlabelled DNA was used as a specific competitor in gel-retardation assays. Calf-thymus DNA was used as a non-specific competitor.

Binding reaction mixtures contained 10 mM Hepes (pH 7.5), 1 mM dithiothreitol, 1–5 μg of poly(dI-dC) · (dI-dC) and 0.5 ng of end-labelled DNA in a volume of 25 μl. The amount of unlabelled competitor DNA added is indicated in the Figure legends. The binding reaction mixtures included 12 μg (6 μl) of protein from a nuclear extract, and after 30 min of incubation at room temperature (20 °C), they were loaded on to a 4 % (w/v) non-denaturing polyacrylamide gel. Electrophoresis was performed at 10 V/cm of gel for 45 min to 1 h in 0.5 × TBE buffer

(45 mM Tris/borate and 1 mM EDTA). Gels were dried and autoradiographed at −70 °C with an intensifying screen.

ChIP (chromatin immunoprecipitation) assays

Rgt1 and Med8 binding to the *HXK2* promoter *in vivo* was assayed by ChIP as described previously [30,31]. Genomic DNA fragments cross-linked to HA–Rgt1 (where HA stands for haemagglutinin) or HA–Med8 were immunoprecipitated with anti-HA antibody and Protein A–Sepharose beads (Amersham Biosciences). The DNA sequences upstream (containing the RGT1 element) and downstream (containing the MED8 element) of the *HXK2* translation start codon were amplified by PCR using respectively the primer pairs: OL3 + OL4 (OL3, ACTACGAGTTTTCTGAACCTCC; and OL4, TAATTTCGTGGATCTCGAATC) and OL5 + OL6 (OL5, GGAATTGATGCAACAAATTGAG; and OL6, GATTGAGTGGTGTCAAAGGTAC).

Fluorescence microscopy

An *rgt1* mutant strain expressing the Hxk2–GFP or Mig1–GFP fusion proteins was grown to early-log phase ($A_{600} < 0.7$) in a synthetic medium with the carbon sources indicated and with selection for maintenance of plasmids. The cells (25 μl) were loaded on to poly(L-lysine)-coated slides and the remaining suspension was immediately withdrawn by aspiration. An aliquot (2 μl) of DAPI (4,6-diamidino-2-phenylindole; 2.5 μg/ml in 80 % glycerol) was added and a covert slide was placed over the microscope slide. GFP and DAPI localization in live cultures was monitored by direct fluorescence using a Leica DMR-XA fluorescent microscope. Images were taken with a Leica Q550 camera using Leica QWin software and processed in Adobe Photoshop 6.0.

RESULTS

Rgt1 negatively regulates *HXK2* gene expression in response to extracellular glucose

To gain more insight into the mode of *HXK2* gene expression, we have analysed the upstream sequence of the *HXK2* promoter using the yeast reporter plasmid YIp-HXK2$_{+404}$. Since this promoter drives a glucose-regulated expression and its *in silico* analysis revealed the presence of a putative RGT1 element located at −395 bp, we measured reporter gene expression in an *rgt1* null mutant. We found that in ethanol-grown cells, *lacZ* expression increased 18-fold, whereas in glucose-grown cells, Rgt1 did not affect β-galactosidase activity (Figure 1A).

To confirm these results, we also determined the transcription levels of *HXK2*. For this purpose, we isolated RNA from wild-type and *rgt1* mutant cells grown with glucose or ethanol as carbon sources. As shown in Figure 1(B), strong signals were obtained in a Northern blot when wild-type or *rgt1* mutant cells were grown on glucose at a size of approx. 1.45 kb and a weak signal was detected with RNA extracted from wild-type cells grown on ethanol medium. These results indicate, in accordance with previous results [32–34], that the expression of the *HXK2* gene is regulated by the carbon source present in the culture medium. However, a high amount of mRNA from *HXK2* was detected in the *rgt1* strain grown on ethanol medium, confirming the essential role played by Rgt1 in the repression of the *HXK2* gene.

In previous works, the Med8 protein was identified as a transcription factor that binds to DRS within the *HXK2* gene and participates in the regulation of *HXK2* expression [18,35]. A typical result of the *HXK2* gene expression in the absence of

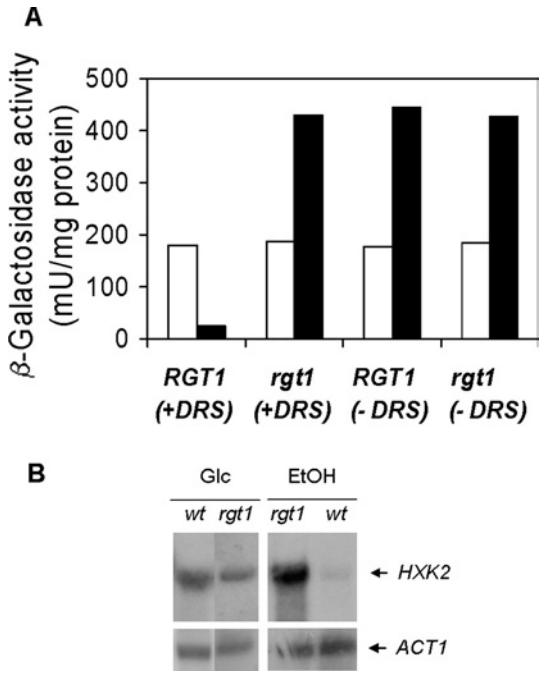

Figure 1 Rgt1 and Med8 proteins repress *HXK2* expression in the absence of glucose

(**A**) Hxk2 expression was measured by using the *lacZ* expression as reporter gene. One copy of the *HXK2*$_{+404}$*::lacZ*, containing the Med8 binding downstream regulatory sequence (+DRS), or the *HXK2*$_{+39}$*::lacZ*, lacking the Med8 binding downstream regulatory sequence (−DRS), constructs were integrated in the chromosome at *URA3* locus of the wild-type strain DBY1315 or the mutant strain *rgt1*. β-Galactosidase activities are averages of the results obtained for four to five independent experiments. Average values have standard errors of 10 % or less. Yeasts were grown on YEPD medium (open bars) or YEPE medium (solid bars) until the A_{600} reached 1.0 [3.0 mg (wet weight)/ml]. β-Galactosidase activity was assayed in crude extracts. (**B**) Effect of *RGT1* gene disruption on the expression of *HXK2*. A wild-type yeast strain (DBY1315) and the isogenic *rgt1* mutant strain were grown using glucose (YEPD) or ethanol (YEPE) as carbon source as described above each lane. Total RNA was isolated, size-separated using a horizontal agarose gel and transferred on to a nylon membrane that was then hybridized to probes derived from *HXK2* as described in the Materials and methods section. The probe used is indicated at the side of each panel.

Med8 binding sites in the *HXK2* gene regulatory region is shown in Figure 1(A). To assay the contribution of Med8 to *HXK2* gene expression, both in the presence and in the absence of the Rgt1 protein, we transformed the wild-type and the *rgt1* mutant strains with the YIp-HXK2$_{+39}$ plasmid and then measured β-galactosidase activity. We found that Med8 repressed *lacZ* expression 18 times in a wild-type strain containing the HXK2$_{+39}$ reporter gene in response to growth in ethanol, whereas in glucose-grown cells, Med8 did not affect *HXK2* transcription (Figure 1A). Moreover, a double defect in DRS function and Rgt1 protein also increases *lacZ* expression 18 times in cells growing in ethanol (Figure 1A).

Rgt1 binds to a specific site within the *HXK2* promoter

To test whether the Rgt1 protein binds specifically to the putative RGT1 element located at −395 bp in the *HXK2* promoter, one synthetic oligonucleotide from −390 to −410 bp of the *HXK2* promoter, containing the RGT1 consensus sequence, was used as a probe for gel EMSA. First, we tested the ability of a recombinant Rgt1 protein from *E. coli* cells to bind to the DNA probe. Two significantly shifted bands are produced when Rgt1 was added, indicating that the protein indeed binds to the RGT1$_{HXK2}$ element (Figure 2A). The fact that two complexes, CI and CII, are observed

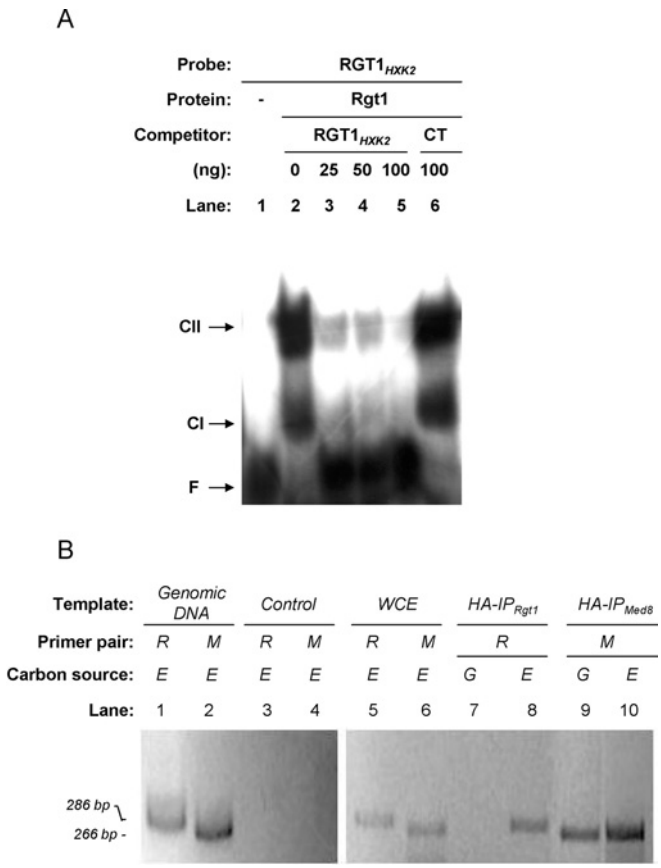

Figure 2 Rgt1 protein binds to the RGT1 element of the *HXK2* promoter *in vitro* and *in vivo*

(**A**) Gel mobility-shift analysis of Rgt1 binding to the RGT1 regulatory element of the *HXK2* promoter. The specific competitor for binding was the unlabelled RGT1-annealed oligonucleotides. The non-specific competitor for binding was 100 ng of calf-thymus DNA (CT). Nucleoprotein complexes were resolved from free DNA by non-denaturing PAGE. For the control (lane 1), the radiolabelled DNA fragment was added alone. F, unbound fragment; CI and CII, positions of shifted bands observed with purified Rgt1 protein. (**B**) ChIP analysis of Rgt1 binding to the RGT1 regulatory element of the *HXK2* promoter. Chromatin was prepared from wild-type yeast cells containing HA-tagged Rgt1 or HA-tagged Med8 proteins. The cells were grown on YEPG (G) or YEPE (E) media as indicated above each lane, and their chromatin was immunoprecipitated using anti-HA monoclonal antibodies. The *HXK2* regulatory regions in the immunoprecipitated DNA (IP) was detected by ethidium bromide staining after amplifying it in a PCR by using the primer pair OL3 + OL4 (R) or OL5 + OL6 (M) and resolving it by electrophoresis on a 2% agarose gel. Lanes show DNA amplified from genomic DNA, whole-cell extract before immunoprecipitation or extracts from cells without tagged protein (Control).

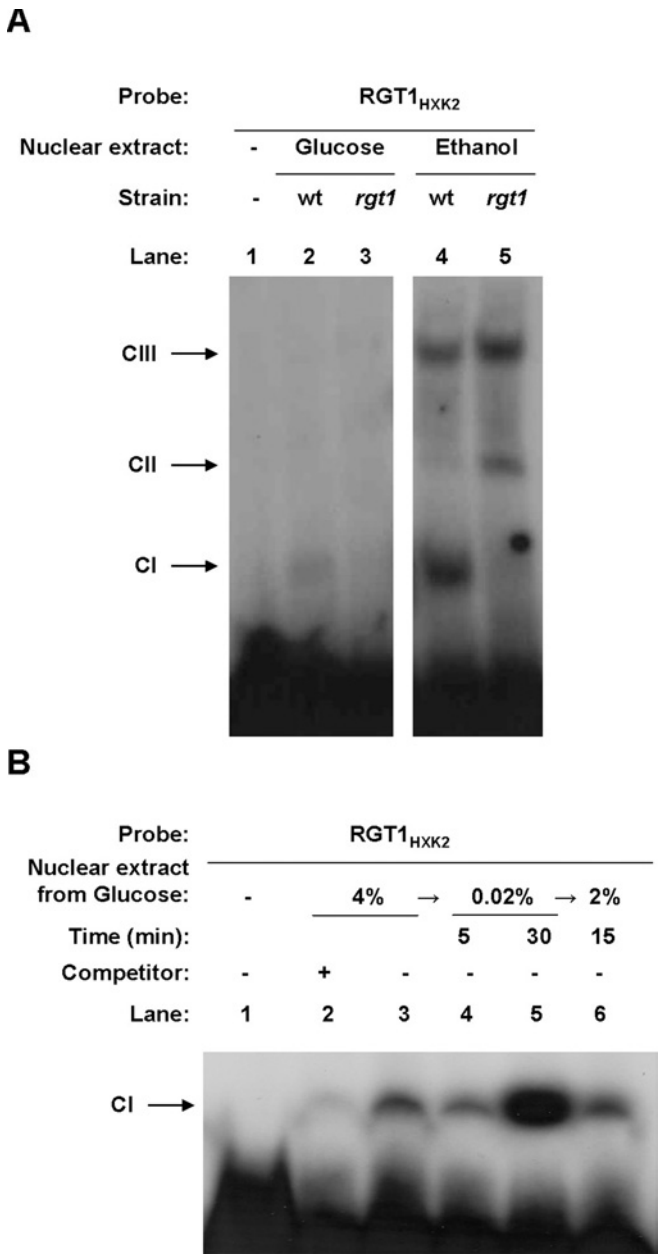

Figure 3 The Rgt1 protein binds to the RGT1 element of the *HXK2* promoter in a glucose-dependent manner

(**A**) Yeast cells were grown in YEPD-2% glucose medium or in YEPE-3% ethanol to an A_{600} 1.0. Nuclear extracts were prepared as indicated in the Materials and methods section and used for EMSA analysis. (**B**) Yeast cells were grown in YEPD-4% glucose medium to an A_{600} of 1.0 and shifted to YEPD-0.02% glucose medium for 5 or 30 min; then 2% glucose was added to the medium and cells were collected after 15 min of incubation. Nuclear extracts were prepared as described in the Materials and methods section and used for EMSA analysis. Unlabelled $RGT1_{HXK2}$ probe (25 ng) was used as a specific competitor in lane 2.

in band-shift experiments with purified Rgt1 protein suggests that Rgt1 can bind to the $RGT1_{HXK2}$ element both as a monomer and as a dimer.

To confirm that Rgt1 and Med8 control *HXK2* transcription by direct binding to the RGT1 and DRS elements of the gene regulatory region, we also used ChIP analyses. Our results showed that Rgt1 was recruited to *HXK2* promoter in the absence of glucose (Figure 2B, lane 8). However, binding of Rgt1 to the *HXK2* promoter was abolished in the presence of glucose (Figure 2B, lane 7). To test whether Med8 was also present at the DRS regulatory regions of the *HXK2* gene, we also utilized ChIP analyses. As shown in Figure 2 (B), lanes 9 and 10, Med8 was present at the DRS in cells growing both in the presence and in the absence of glucose.

Thus our results indicate that, in the absence of glucose, Rgt1 and Med8 are present at the *HXK2* promoter and co-repress gene transcription. Moreover, when glucose is present in the culture medium, Rgt1 has no regulatory function in the *HXK2* gene expression. Previous studies showed that in glucose-rich media, Rgt1 becomes phosphorylated and dissociates from the repressor complex involved in the expression of *HXT* genes [36,37]. To examine whether the presence or absence of glucose could determine a differential pattern of band shifting with nuclear extracts prepared from wild-type and *rgt1* mutant strains, we use the RGT1 element of the *HXK2* promoter in EMSA assays. As shown in Figure 3(A), we observed three main DNA–protein

complexes with nuclear extracts from cells grown in ethanol medium (Figure 3A, lanes 4 and 5). Among these complexes, the CI complex was formed with extracts from wild-type cells grown in the presence or absence of glucose. In extracts from cells grown in ethanol medium, comparatively strong signals appeared. In contrast, signal strength is greatly decreased by utilizing nuclear extracts from cells grown in high levels of glucose. The CI complex was not detected in nuclear extracts from *rgt1* mutant cells grown in the presence or absence of glucose. Moreover, CI complex formation is reversible in response to glucose. If glucose was added back to cells after a shift to low glucose, the CI complex rapidly decreased (Figure 3B). The specificity of the binding was demonstrated by competition assays with the non-labelled oligonucleotide (Figure 3B, lane 2). Together, these results indicate that the Rgt1 protein is required in the CI complex formation and that, in cells growing in glucose, the Rgt1 protein dissociates from the repressor complex involved in the *HXK2* gene expression.

Rgt1 is not involved in the glucose-repression-signalling pathway controlling *SUC2* repression

The Hxk2 protein phosphorylates glucose and also regulates glucose repression [12]. Since the *rgt1* mutant cells growing in a non-fermentable carbon source have high levels of Hxk2 protein, it was interesting to investigate whether under these growth conditions, the *rgt1* mutant cells still respond to glucose repression. To address this issue, we tested the extracellular invertase activity in *rgt1* mutants under repressing (high glucose) and inducing (low glucose) conditions. As can be seen in Figure 4(A), invertase activity was not affected by the *RGT1* gene deletion either under repressing or inducing growth conditions. This indicates that a high level of Hxk2 under low-glucose growing conditions is not sufficient for repression signalling in yeast.

To investigate why a high level of Hxk2 protein is not sufficient for repression signalling under low-glucose growing conditions, we studied the intracellular localization of Hxk2 and Mig1 in *rgt1* mutants by using Hxk2–GFP and Mig1–GFP fusion proteins expressed from multicopy plasmids. As shown in Figure 4(B), in *rgt1* mutant cells, grown in the presence of glucose, Hxk2–GFP is predominantly localized in the cytoplasm although a fraction is located in the nucleus (Figure 4B, column a). In contrast, Mig1–GFP is mainly located in the nucleus (Figure 4B, column c). In *rgt1* mutant cells grown overnight on 3 % ethanol, condition under which repression does not occur, both Hxk2 and Mig1 proteins are found in the cytoplasm, apparently excluded from the nucleus (Figure 4B, columns b and d).

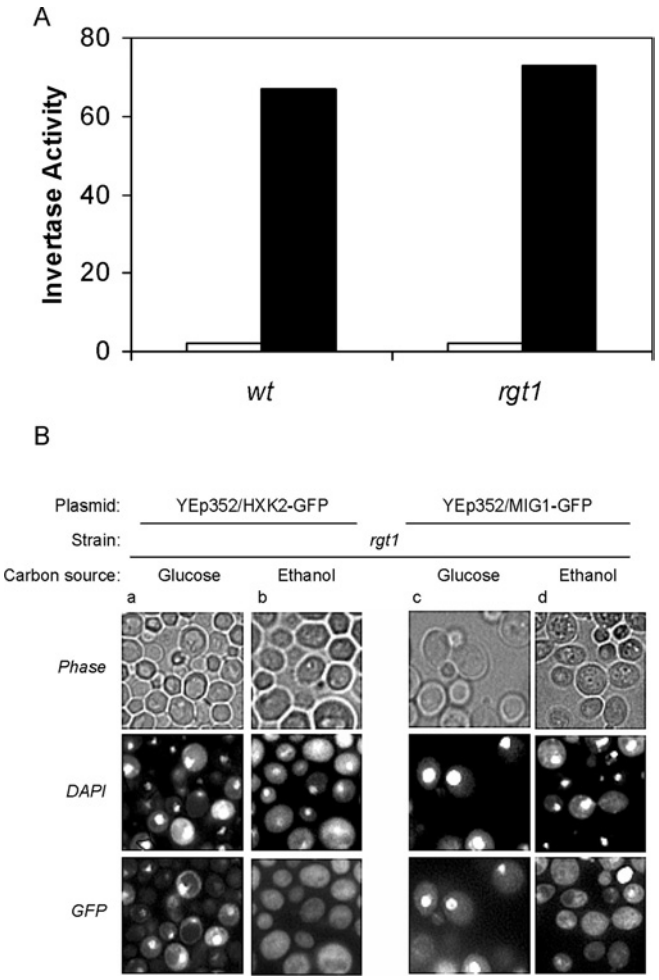

Figure 4 Effect of *RGT1* gene disruption on the expression of *SUC2*

(**A**) The wild-type yeast strain (DBY1315) and its isogenic *rgt1* null mutant were grown using 2 % glucose (open bars) or 3 % ethanol plus 0.05 % glucose (solid bars) as carbon sources, until the A_{600} reached 1.0 [3.0 mg (wet weight)/ml]. Invertase activity was assayed in whole cells. The average values of the results obtained for four independent experiments have standard errors $\leqslant$ 10 %. (**B**) Yeast *rgt1* mutant strain expressing Hxk2–GFP (a and b) from the plasmid YEp352/HXK2::gfp and Mig1–GFP (c and d) from the plasmid YEp352/MIG1::gfp were grown on SD-Ura$^-$ medium supplemented with glucose or ethanol as carbon source and fluorescence was imaged. The cells were stained with DAPI for DNA and then imaged for GFP fluorescence and for DAPI fluorescence by phase-contrast optics.

DISCUSSION

Several lines of evidence provided in the present study suggest that Rgt1 and Med8 are repressors regulating transcription of the *HXK2* gene in the absence of glucose. First, *rgt1* null mutants, when grown in ethanol medium, exhibit intensely increased β-galactosidase activities from a *lacZ* gene used as a reporter for *HXK2* gene expression. Identical results were obtained for a promoter lacking the Med8 binding sites to control the transcriptional expression of the *lacZ* reporter gene. However, a strain lacking both the Rgt1 protein and the Med8 binding sites in the *HXK2* promoter does not show any synergistic effect on β-galactosidase activity. Thus the results presented in this paper support the conclusion that both Rgt1 and Med8 proteins repress HXK2 expression probably by interacting directly with repressing elements located upstream and downstream respectively in the *HXK2* gene in the absence of glucose. Apparently, both factors are essential to repress *HXK2* expression, because deletion of either the DRS element of the *HXK2* gene or the *RGT1* gene abolished to the same extent *HXK2* gene repression in the absence of glucose. Secondly, Northern-blot analyses show an increased *HXK2* transcription in *rgt1* mutants growing in ethanol medium. These experiments confirm the results obtained by using *lacZ* as a fusion reporter gene. Thirdly, EMSA experiments demonstrated that Rgt1 binds specifically to a motif of the *HXK2* gene promoter located at -395 bp (relative to the ATG translation start codon) containing the sequence CGGAAAA in accordance with the consensus sequence recently proposed for Rgt1 binding to DNA [37]. Finally, both ChIP and EMSA experiments demonstrated that the binding of Rgt1 to the *HXK2* gene promoter element is regulated by external glucose concentrations. Rgt1 binds to the *HXK2* gene promoter in the absence of glucose and has low affinity for its binding element at high levels of glucose. This result could be explained if glucose regulates the transcriptional activity of Rgt1 towards *HXK2* expression by inducing changes

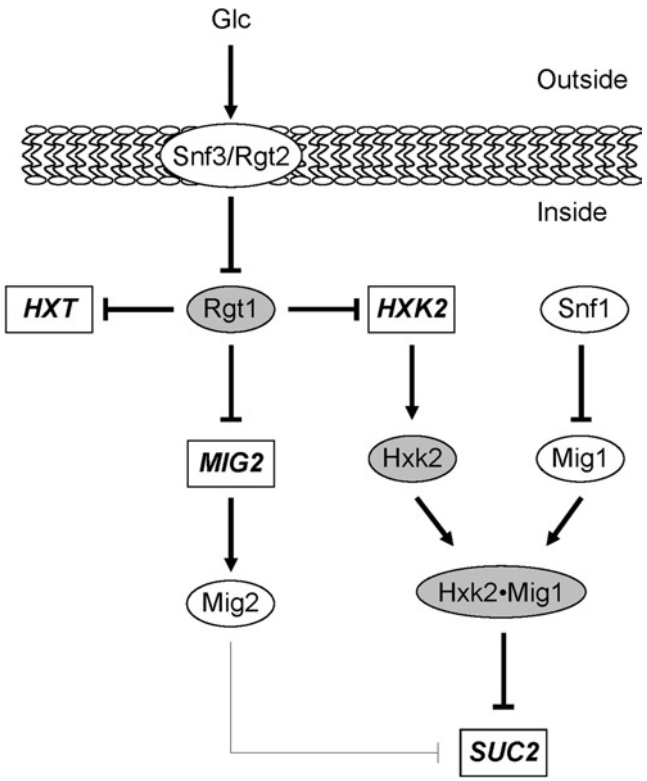

Figure 5 Model for the integration of the glucose signal through distinct signalling pathways

Glucose signals could take several possible routes, namely Snf3/Rgt2 and Mig1/Hxk2 pathways. Binding of glucose to the transmembrane proteins Snf3/Rgt2 inactivates the Rgt1 repressor function, leading to expression of several genes (*HXT*s, *MIG2* and *HXK2*). Rgt1 is the main downstream repressor of the Snf3/Rgt2 pathway controlling expression in the absence of glucose of several targets genes. Two of the targets, Hxk2 and Mig2, are directly involved, together with Mig1, in glucose signalling through the Mig1/Hxk2 pathway. Genes are depicted by rectangles, proteins by ovals and shaded proteins are discussed in the present work.

in the phosphorylation state of the protein in a similar manner as reported for the transcriptional control of the *HXT1* gene [36,37].

These observations and previous results [13,38] suggest that the Rgt1 repressor together with the classical function dedicated to regulating the expression of *HXT1* genes [5] is also involved in the repression of the *HXK2* gene, connecting two glucose signalling pathways that cause glucose induction and glucose repression of gene expression. Earlier results demonstrated that the *MIG2* gene, encoding a repressor that collaborates with Mig1 in some target promoters, is induced by glucose through the Rgt2/Snf3-Rgt1 signalling pathway [13]. On the basis of our observations that the transcription of *HXK2* is regulated by Rgt1, we suggest a simplified model (Figure 5) of how the glucose signal reaches Rgt1 through the Snf3/Rgt2 pathway and could then be transferred to the Mig1/Hxk2 repression pathway. Rgt1, in the absence of glucose, binds to the upstream sequence of *HXK2* gene to repress its expression. Since Hxk2 levels are regulated by Rgt1, new possibilities of cross-talk between these two glucose-signalling pathways could be opened. To test these possibilities, we examined whether Rgt1 is involved in the glucose-repression-signalling pathway controlling *SUC2* repression. Our results show that *rgt1* mutant cells respond to glucose repression in a similar manner as was reported for wild-type cells. This observation could be explained by the fact that the intracellular distribution of both Hxk2 and Mig1, two essential factors of the glucose repression signalling pathway, in *rgt1* mutant cells is identical as in a wild-type strain [12]. These results suggest that the increase in Hxk2 levels in the *rgt1* mutant strain in the absence of glucose is not sufficient to establish a complete glucose repression, because Mig1 localization was not affected by *RGT1* gene deletion and, in the absence of glucose, is localized in the cytoplasm [39]. This observation, in accordance with previous ones, suggests that the cell modulates the Hxk2 regulatory function by controlling the amount of protein localized in the nucleus and that Hxk2 is sequestered in the nucleus through its interaction with Mig1 [12]. Thus Rgt1 does not contribute to glucose repression of the *SUC2* gene because the Hxk2 protein is excluded from the nucleus in the absence of high levels of glucose in the culture medium. Thus, in accordance with the results we obtained for *SUC2* expression, the physiological significance of the proposed cross-communication between the Snf3–Rgt2 induction pathway and the Mig1–Hxk2 repression pathway remains to be clarified.

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