

# The hexokinase 2 protein regulates the expression of the *GLK1*, *HXK1* and *HXK2* genes of *Saccharomyces cerevisiae*

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The key glycolytic *HXK2* gene, coding for the enzyme hexokinase 2 (Hxk2p), is expressed when cells of the yeast *Saccharomyces cerevisiae* are grown on a fermentable medium using glucose, fructose or mannose as a carbon source. After shifting the cells to a non-fermentable carbon source, the *HXK2* gene is repressed and the *HXK1* and *GLK1* genes are rapidly de-repressed, producing the enzymes hexokinase 1 (Hxk1p) and glucokinase (Glk1p) respectively. Because the *in vivo* functions of the Hxk1p and Glk1p enzymes have remained a mystery so far, we have investigated this glucose-induced regulatory process. Here we demonstrate the involvement of Hxk2p in the glucose-induced repression of the *HXK1* and *GLK1* genes and the glucose-induced expression of the *HXK2* gene. We have also demonstrated the involvement of Hxk1p as a negative factor in the expression of the *GLK1* and *HXK2* genes. Further experimental evidence, using mutant cells expressing a truncated version of

Hxk2p unable to enter the nucleus, shows that nuclear localization of Hxk2p is necessary for glucose-induced repression signalling of the *HXK1* and *GLK1* genes and for glucose-induced expression of the *HXK2* gene. Gel mobility-shift analysis shows that Hxk2p-mediated regulation is exerted through ERA (ethanol repression autoregulation)-like regulatory sequences present in the *HXK1* and *GLK1* promoters and in two downstream repressing sequences of the *HXK2* gene. These findings reveal a novel mechanism of gene regulation whereby the product of a glycolytic gene, normally resident in the cytosol, interacts directly with nuclear proteins to regulate the transcription of the *HXK1* and *GLK1* genes and to autoregulate its own transcription.

**Key words:** glucose-phosphorylating enzyme, glucose repression, transcriptional control, yeast.

## INTRODUCTION

In *Saccharomyces cerevisiae*, the phosphorylation of glucose at C6 can be catalysed by three enzymes, namely the hexokinases 1 and 2 (EC 2.7.1.1; encoded by the *HXK1* and *HXK2* genes) and glucokinase (EC 2.7.1.2; encoded by the *GLK1* gene). These enzymes can catalyse the first irreversible step in the intracellular metabolism of glucose. However, the *HXK2* gene product appears to play the main role during glucose phosphorylation *in vivo*, because it is the isoenzyme that predominates in growth on glucose [1,2]. The two other isoenzymes are also singly adequate for growth on glucose, but their normal expression is highest on other carbon sources [2,3]. Furthermore, various genetic and biochemical evidence has shown that the hexokinase 2 protein (Hxk2p) is also important in regulating glucose repression [4,5]; in particular, a *HXK2* mutant gene (*HXK2ΔK7M<sup>16</sup>*), with a 30 bp deletion between nucleotides +19 and +48, has been described in which glucose repression is absent but Hxk2 catalytic activity is intact [6]. The expression of this mutant gene generates a truncated Hxk2 protein with similar specific activity to the wild-type hexokinase 2 but without amino acids Lys<sup>7</sup>–Met<sup>16</sup>. The absence of this decapeptide in the Hxk2 protein affects its nuclear localization, which is required for glucose repression of the *SUC2* gene [6].

Therefore, cytoplasmic Hxk2p is a key enzyme in glycolysis, whereas nuclear hexokinase 2 is a factor involved in signalling glucose repression of several genes [7,8]. The present work proposes a new function for the Hxk2 protein, that as a key

factor controlling the gene expression of the glucose-phosphorylation system of *S. cerevisiae*.

Glucose-phosphorylating enzymes of *S. cerevisiae* are regulated mainly at the transcriptional level by mechanisms that can increase and decrease the amounts of these enzymes. Glucose has been shown to induce transcription of the *HXK2* gene but the mechanism through which it operates is not known. DNA–protein complexes involving two downstream-repressing sequences (DRSs) located within the coding region of the *HXK2* gene have been described [9]. The transcription factors that operate through these DRSs repress *HXK2* gene transcription under conditions of sugar limitation or when ethanol is used as a carbon source [10]. The combinatorial effect of three regulatory sequences of the *GLK1* promoter, STRE (stress-responsive element) [11,12], ERA/TAB (ethanol repression autoregulation/TA box) [13] and GCR1 (glucose-regulatory protein 1; [14,15], and see [16] for a review), leads to the regulated transcription of the *GLK1* gene, i.e. silent in media with glucose and other preferred carbon sources such as fructose or mannose and increased levels of expression upon glucose depletion [17]. The transcription of the *HXK1* gene is repressed in the presence of glucose by transcription factors operating through an ERA element, and, upon glucose depletion, a quick induction of transcription, through several STRE elements is observed (P. Herrero and F. Moreno, unpublished work). In this paper, the role of Hxk2 protein in the regulation of gene expression, through these *cis*-acting elements of the glucose-phosphorylation system, is discussed.

Abbreviations used: DRS, downstream-repressing sequence; YEPD, yeast extract/peptone/dextrose; YEPE, yeast extract/peptone/ethanol; ERA, ethanol repression autoregulation; Hxk2p, hexokinase 2 protein; Hxk1, hexokinase 1; Glk1p, glucokinase.

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## MATERIALS AND METHODS

### Strains and plasmids

The *S. cerevisiae* strains used in this study are listed in Table 1 and are isogenic to the wild-type strain DBY1315, a gift from David Botstein (Stanford University, Stanford, CA, U.S.A.). The host strains for transformation are summarized in Table 1; the remaining strains are one of the host strains with a particular integrated plasmid, always carrying an allele of the *HXX2* gene. Bacterial transformation and large-scale preparation of plasmid DNA were performed in *Escherichia coli* MC1061 (*hsdR mcrB araD139Δ(araABC-leu)7679Δlacx74 galU galK rpsL thi*).

Plasmids YIp and YE<sub>p</sub> are yeast–*E. coli* shuttle vectors suitable for use respectively as integrative or episomal vectors. These vectors have a *URA3* yeast-selectable marker [19]. Plasmid pJH215, a gift from Jürgen Heinisch (University of Hohenheim, Stuttgart, Germany), is also a yeast–*E. coli* shuttle vector suitable for use as an episomal vector with a *LEU2* yeast-selectable marker.

### Media and growth conditions

Rich media were based on 1% yeast extract/2% peptone, with either 2% glucose (YEPD) or 3% ethanol (YEPE) 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. These media were utilized to select for transformants when plasmids carrying *URA3* or *LEU2* were used.

### General DNA techniques

Routine DNA manipulations were performed essentially as described previously [20].

### Construction of reporter fusions and plasmids carrying alleles of the *HXX2* gene

To construct a gene fusion between *HXX1* and the bacterial *lacZ* gene, encoding β-galactosidase, a 621 bp *Sau3A*–*XmnI* fragment containing 582 nucleotides from the 5′-non-coding region and 39 bp of the *HXX1* coding sequence was ligated in frame to *lacZ* into YIp358R [19], digested previously with *Bam*HI and *Sma*I to form plasmid YIp621. To construct a gene fusion between *HXX2* and *lacZ*, a 1242 bp *Bam*HI–*Hind*III fragment containing 838

nucleotides from the 5′-non-coding region and 404 bp of the *HXX2* coding sequence was cloned in frame to *lacZ* into YIp356 [19] to form plasmid YIp1242 [9]. To construct a gene fusion between *GLK1* and *lacZ*, a 944 bp fragment containing 63 bp of the *GLK1* coding region plus additional DNA upstream was amplified by PCR using, as a primer, an oligonucleotide with either a *Pst*I site (OL1, 5′-ATCTGCAGTCGATGCTAACAAAGTTC-3′) or an *Eco*RI site (OL2, 5′-ATCTGCAGGGCCAGGTAATGTTTT-3′) at the 5′ end (the restriction sites are underlined). Genomic DNA from the wild-type strain DBY1315 served as the *GLK1*-containing template. The amplified product was digested with *Pst*I and *Eco*RI and ligated to YIp358R [19] digested with the same enzymes to form plasmid YIp944 [17].

Plasmids YIp621, YIp944 and YIp1242 were used to transform the yeast strains DBY1315, DBY2052, DBY2184 and DBY2053. The plasmids were integrated into the *URA3* locus by digestion with *Stu*I prior to transformation. Single-copy integration was confirmed by Southern-blot analysis of genomic DNA digested with *Bgl*II and by probing with a 1.1 kb *Hind*III fragment containing the *URA3* gene. The yeast strains generated are listed in Table 1.

A DNA fragment containing the complete *HXX2* promoter was isolated from the vector pRS-HXX2 [9] as an 0.88 kb *Sph*I–*Nco*I fragment and subcloned into a *Sph*I–*Nco*I previously cleaved vector pSP73-HG (this plasmid contains the complete coding region of the *HXX2* gene and 254 bp of the 5′-non-coding region in a 2.75 kb fragment). The resulting plasmid pSP73-*HXX2* contains the complete *HXX2* gene under the control of its own complete promoter in a 3.35 kb *Sph*I–*Eco*RI fragment. The 3.35 kb fragment was cloned into YE<sub>p</sub>352 [21] and pJH215, and the resulting plasmids were named YE<sub>p</sub>352-*HXX2* and pJH215-*HXX2*.

Oligonucleotides 5′-ATGAACCATTATT-3′ and 5′-GGT-TCCATGGCCGATGTG-3′ were used to generate construction pSP73-*HXX2ΔK<sup>7</sup>M<sup>16</sup>* by PCR. The resulting plasmid (pSP73-*HXX2ΔK<sup>7</sup>M<sup>16</sup>*) was cleaved by *Sph*I and *Eco*RI to obtain an approx. 3.35 kb fragment, which was subcloned into vectors pJH215 and YE<sub>p</sub>352, cleaved previously by *Sph*I/*Eco*RI. The plasmids obtained, pJH215-*HXX2ΔK<sup>7</sup>M<sup>16</sup>* and YE<sub>p</sub>352-*HXX2ΔK<sup>7</sup>M<sup>16</sup>*, have a 30-nucleotide deletion between nucleotides +19 and +48 of the *HXX2* gene. Expression of this mutant gene generates a truncated Hxk2 protein with similar specific activity to the wild-type hexokinase 2 but lacking amino acids Lys<sup>7</sup>–Met<sup>16</sup> [6].

Plasmids YE<sub>p</sub>352-*HXX2*, pJH215-*HXX2*, YE<sub>p</sub>352-*HXX2ΔK<sup>7</sup>M<sup>16</sup>* and pJH215-*HXX2ΔK<sup>7</sup>M<sup>16</sup>* were used to transform the different host strains, as indicated in each Figure.

**Table 1** Strains of *S. cerevisiae* used in this study

Strain	Genotype	Reference
DBY1315	<i>MATα. ura3-52 leu2-3,2-112 lys2-801 gal2</i>	[18]
DBY2052	<i>MATα. hxx1::LEU2 hxx2-202 ura3-52 leu2-3,2-112 lys2-801 gal2</i>	[18]
DBY2053	<i>MATα. hxx1::LEU2 ura3-52 leu2-3,2-112 lys2-801 gal2</i>	[18]
DBY2184	<i>MATα. hxx2-202 ura3-52 leu2-3,2-112 lys2-801 gal2</i>	[18]
FMY151	Isogenic to DBY1315, with <i>HXX1p::lacZ</i>	This work
FMY521	Isogenic to DBY2052, with <i>HXX1p::lacZ</i>	This work
FMY531	Isogenic to DBY2053, with <i>HXX1p::lacZ</i>	This work
FMY841	Isogenic to DBY2184, with <i>HXX1p::lacZ</i>	This work
FMY152	Isogenic to DBY1315, with <i>HXX2p::lacZ</i>	This work
FMY522	Isogenic to DBY2052, with <i>HXX2p::lacZ</i>	This work
FMY532	Isogenic to DBY2053, with <i>HXX2p::lacZ</i>	This work
FMY842	Isogenic to DBY2184, with <i>HXX2p::lacZ</i>	This work
FMY153	Isogenic to DBY1315, with <i>GLK1p::lacZ</i>	This work
FMY523	Isogenic to DBY2052, with <i>GLK1p::lacZ</i>	This work
FMY533	Isogenic to DBY2053, with <i>GLK1p::lacZ</i>	This work
FMY843	Isogenic to DBY2184, with <i>GLK1p::lacZ</i>	This work

### Preparation of protein extracts and enzymic determinations

Enzyme activities were determined on crude extracts. These were prepared as follows: yeast was grown on 10–20 ml of rich medium (YEPD or YEPE) at 28 °C until a *D*<sub>600</sub> value of 1.0. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl of buffer C (20 mM Hepes buffer, pH 7.9, containing 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub> and 25% glycerol). The cells were broken by vortexing (6 × 20 s) in the presence of glass beads (0.5 g), and 400 μl of buffer C was added to the suspension. After centrifugation at 19000 g for 15 min at 4 °C, the supernatant was used as crude protein extract.

This fraction was used directly for enzyme assays of hexokinase and total protein determinations. Hexokinase was assayed at 28 °C with either glucose or fructose as the substrate (in the latter case, 2 μg of phosphoglucosomerase was added), as described

**Table 2** Complementary oligonucleotides carrying regulatory elements of the *GLK1*, *HXK1* and *HXK2* promoters used in this study

Lower-case letters indicate 5' overhangs. Bold letters indicate regulatory elements of the respective promoters.

DNA fragment	Sense (5'–3')	Antisense (5'–3')
ERA <sub>GLK1</sub>	tcgaCAAG <b>AAATGCACGC</b> CGTAACAAAATATATATATATA (–526)	tcgaTATATATATATATATATTTTGTACG <b>CGTGCATTT</b> TCTTG (–541)
ERA <sub>HXK1</sub>	tcgaTAAGAACT <b>TATGCATTT</b> TTTTTTAA (–334)	tcgaTAAAAA <b>AAATGCATAG</b> TTCTTA (–357)
DRS1 <sub>HXK2</sub>	tcga <b>CTTCATTT</b> CCGAATTGAAAAGGG (+163)	tcgaCCCTTTTCCAATTCG <b>AAATGAAG</b> (+140)
DRS2 <sub>HXK2</sub>	tcgaGAATCCG <b>GTGATT</b> CTTGGC (+251)	tcgaGCCAAG <b>AAATCAC</b> CGGATTC (+231)

previously [22]. Protein concentration was determined with the Lowry protein assay.  $\beta$ -Galactosidase activity was assayed according to [23]. Reagents and auxiliary enzymes were from Sigma.

### Northern-blot analysis

Yeast cells were harvested in mid-exponential phase. Total RNA was isolated as described in [24], and separated on 1.5% agarose/formaldehyde gels. After transfer to Hybond-N nylon membranes (Amersham Pharmacia Biotech), the RNA was UV cross-linked and hybridized with probes for the *HXK1*, *GLK1* and *ACT1* (actin) genes as described previously [2]. The amount of the hybridizing RNA was quantified in an Instantimager Packard and normalized to the Act1 mRNA level as described previously [2].

### DNA probes

To investigate interaction of proteins with the sequences carrying regulatory elements in the *GLK1*, *HXK1* and *HXK2* promoter regions, we reconstituted the fragment from two complementary oligonucleotides. The complementary strands were annealed, and either end was labelled with [ $\alpha$ -<sup>32</sup>P]dCTP by fill-in using the Klenow fragment of DNA polymerase I or used as unlabelled competitors in gel-retardation assays. In all cases the oligonucleotides were synthesized with an added TCGA nucleotide overhang at the 5' end. The synthetic DNA fragments used in gel-retardation experiments are listed in Table 2.

### Preparation of yeast nuclei

Nuclei were prepared by a method based on that of [25] with the modifications indicated in [26].

### Gel-retardation assays

Binding reactions contained 10 mM Hepes (pH 7.5), 1 mM dithiothreitol, 1–5  $\mu$ g of poly(dI-dC) and 0.5 ng of end-labelled DNA in a volume of 25  $\mu$ l. Where unlabelled competitor DNA was added, its amount is indicated in the Figure legends. The binding-reaction mixtures included 24  $\mu$ g (6  $\mu$ l) of nuclear protein extract and, after 30 min of incubation at room temperature, they were loaded on to a 4% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 10 V/cm of gel for 45 min–1 h in 0.5  $\times$  TBE buffer (45 mM Tris/borate/1 mM EDTA). Gels were dried and autoradiographed at –70 °C with an intensifying screen.

## RESULTS AND DISCUSSION

### Differential expression of the *HXK1* and *GLK1* genes in the presence and absence of Hxk2 protein

The expression of the *HXK1* and *GLK1* genes was investigated by Northern-blot analysis of total RNA isolated from a wild-

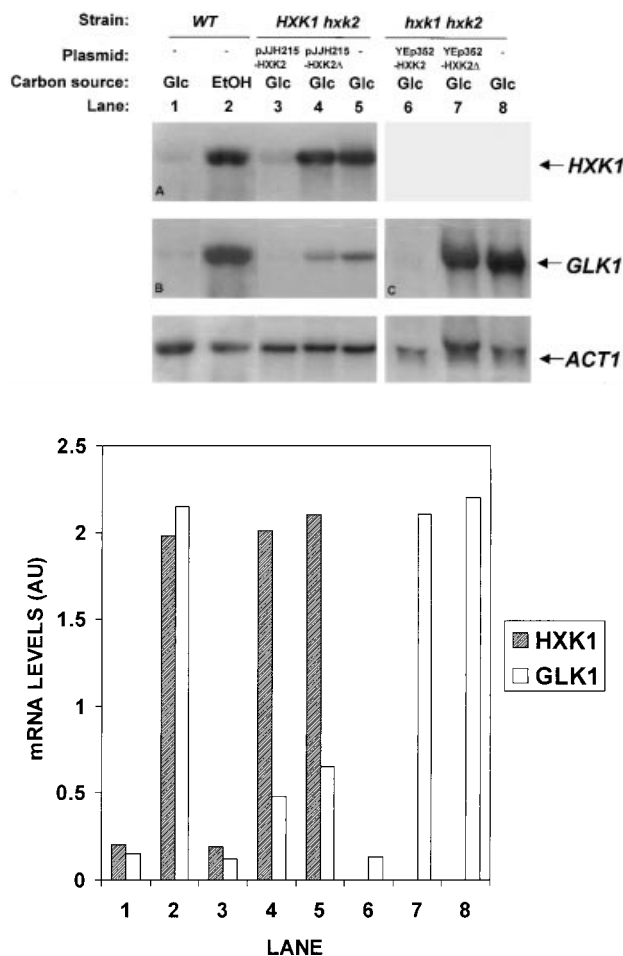
type strain (*HXK1 HXK2 GLK1*) and two isogenic, *hxx2* $\Delta$  and *hxx1* $\Delta$  *hxx2* $\Delta$ , mutant strains. The probes utilized were specific for the Hxk1 and Glk1 mRNAs expressed by these strains, as was demonstrated by Northern-blot analysis using, as RNA sources, null mutants of the *HXK1* and *GLK1* genes and the corresponding probe. Wild-type yeast cells were grown in glucose (repressed conditions) or ethanol (de-repressed conditions) media; the mutant strains and transformed derivative strains were grown only in glucose medium. The results are shown in Figure 1. The mRNA levels reported were quantified by an Instantimager and normalized to the levels of actin mRNAs.

As was previously reported [2], the *HXK1* and *GLK1* transcripts are repressed by glucose and de-repressed in the absence of a preferential sugar or when the cells are grown using ethanol as a carbon source (Figure 1, lanes 1 and 2). Deletion of the *HXK2* gene increased the level of the *HXK1* transcript 10-fold in the repressed glucose medium (Figure 1A, lane 5). Thus Hxk1 mRNA levels are similar under the de-repressed conditions of the wild-type strain and the repressed conditions of the *hxx2* $\Delta$  strain. This pattern of *HXK1* regulation is opposite to that in *HXK2* cells and indicates that the *hxx2* $\Delta$  mutation converts *HXK1* from a glucose-repressed gene into a glucose-non-repressed gene.

In order to confirm that Hxk2 protein is necessary for *HXK1* regulation, we transformed the *hxx2* $\Delta$  mutant strain with two different plasmids. One contained the native *HXK2* gene under the control of its own promoter and the other contained a *HXK2* $\Delta$ K<sup>M16</sup> (*HXK2* $\Delta$ ) gene under the control of the *HXK2* promoter. In fact, the wild-type phenotype was restored after transformation of the *hxx2* $\Delta$  mutant strain with a *HXK2*-containing plasmid (Figure 1A, lane 3). However, the wild-type phenotype was not restored after transformation of the *hxx2* $\Delta$  mutant strain with a *HXK2* $\Delta$ -containing plasmid (Figure 1A, lane 4), and the Hxk1 mRNA levels remained as in the de-repressed conditions of the wild-type strain.

In a parallel experiment, the level of Glk1 RNA was examined by using the same filters utilized in the Hxk1 RNA experiment, but now hybridizing with a *GLK1* probe. The Glk1 regulation pattern is very similar to that described for Hxk1 RNA. The transcript is repressed by glucose and de-repressed in the absence of glucose when the cells are grown using ethanol as a carbon source (Figure 1B, lanes 1 and 2). However in this case, deletion of the *HXK2* gene only increased the levels of the Glk1 transcript 4-fold in the repressed glucose medium, which represents 30% of the Glk1 mRNA levels detected in the de-repressed conditions of the wild-type strain (Figure 1B, lane 5).

It is known that glucose-dependent transcriptional repression exerted through the main glucose-repression pathway is primarily dependent on hexokinase 2 [4,27]. It has also been described that overexpression of *HXK1*, but not of *GLK1*, partially relieves the requirement for Hxk2p in establishing glucose repression [18]. Our results indicate that *HXK1* gene expression is repressed 10-fold by glucose and that this inhibition is Hxk2p-dependent. Since disruption of the *HXK2* gene resulted in very high



**Figure 1** Effect of *hxx2* gene disruption on the expression of the *HXX1* (A) and *GLK1* (B) genes and effect of *hxx1 hxx2* double-gene disruption on the expression of the *GLK1* (C) gene

Strains DBY1315 (wild-type strain, relevant genotype *HXX1 HXX2 GLK1*), DBY2184 (*HXX2Δ* mutant strain, *HXX1 hxx2 GLK1*), DBY2184 transformed with *HXX2*- and *HXX2Δ<sup>K7M16</sup>*-containing plasmids, DBY2052 (*hxx1Δ hxx2Δ* mutant strain, *hxx1 hxx2 GLK1*) and DBY2052 transformed with *HXX2*- and *HXX2Δ<sup>K7M16</sup>*-containing plasmids were grown using glucose (Glc; YEPD) or ethanol (EtOH; YEPE) as a carbon source, as shown above each lane. Total RNA was isolated, size-separated using a horizontal agarose gel, and transferred to a nylon membrane that was then hybridized to probes derived from *HXX1* and *GLK1* as described in the Materials and methods section. The probes used are indicated at the side of each panel. mRNA levels were quantified in an Instantimager, and normalized using hybridization to an Act1 probe. For each mRNA, the level is expressed in arbitrary units (AU).

**Table 3** Regulation of *HXX1*, *HXX2* and *GLK1* expression in wild-type and mutant strains

One copy of the *HXX2::lacZ*, *HXX1::lacZ* or *GLK1::lacZ* fusions was integrated into the chromosome at the *URA3* locus of the wild-type strain DBY1315 and the mutant strains DBY2184, DBY2052 and DBY2053 containing the indicated *HXX2* allele.  $\beta$ -Galactosidase activities, assayed in crude extracts, are the means of results obtained from five to six independent experiments (S.E.M.  $\leq 10\%$ ). Yeasts were grown on YEPD medium until the  $D_{600}$  reached 1.0 (3.0 mg wet weight/ml).

		$\beta$ -Galactosidase activity (m-units/mg of protein)							
		<i>hxx1 hxx2</i>		<i>hxx1 HXX2</i>		<i>HXX1 HXX2</i>		<i>HXX1 hxx2</i>	
Fusion gene integrated	Gene in plasmid ...	–	<i>HXX2Δ</i>	<i>HXX2</i>	–	–	<i>HXX2</i>	<i>HXX2Δ</i>	–
<i>HXX1::lacZ</i>		900	930	115	118	128	125	920	920
<i>HXX2::lacZ</i>		102	110	1103	1445	306	290	106	86
<i>GLK1::lacZ</i>		925	965	121	114	116	110	310	302

intracellular levels of Hxx1p, which could partially relieve the absence of Hxx2p in *hxx2Δ* mutants, the effect of Hxx2p on the expression of the *Glk1* mRNA was studied using a *hxx1Δ hxx2Δ* double-mutant strain.

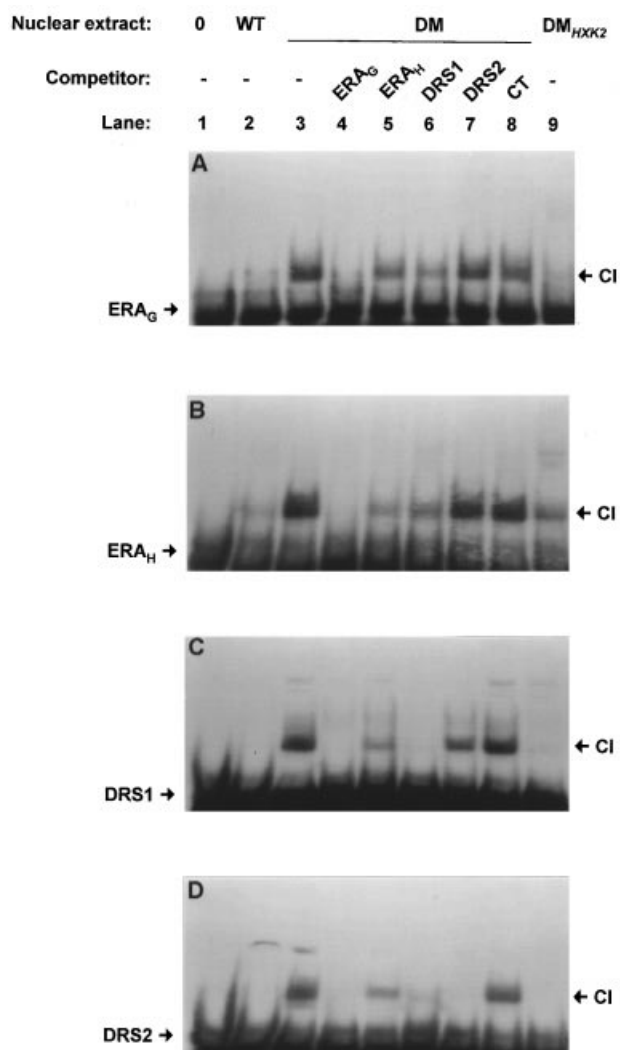
The results reported in Figure 1(C) strongly suggest that disruption of both *HXX1* and *HXX2* genes leads to an approx. 14-fold increase in the levels of *Glk1* transcription under repressed conditions (Figure 1C, lane 8). Thus *Glk1* mRNA levels are similar under the de-repressed conditions of the wild-type strain and the repressed conditions in the *hxx1Δ hxx2Δ* strain. This pattern of *Glk1* regulation is opposite to that in wild-type cells and indicates that the *hxx1Δ hxx2Δ* mutation converts *GLK1* from a glucose-repressed gene into a glucose-non-repressed gene. These results also confirm that high intracellular levels of Hxx1p can partially relieve the absence of Hxx2p in signalling glucose repression.

Additionally, the wild-type phenotype was completely restored after transformation of the *hxx1Δ hxx2Δ* double-mutant strain with a *HXX2*-containing plasmid (Figure 1C, lane 6), indicating that the Hxx2 protein is enough to signal glucose-repression of the *GLK1* gene in a *hxx1Δ hxx2Δ* double-mutant strain. However, the wild-type phenotype was not restored after transformation of the *hxx1Δ hxx2Δ* double-mutant strain with a *HXX2Δ*-containing plasmid (Figure 1C, lane 7), suggesting again that nuclear localization of the Hxx2p is necessary in signalling glucose repression of *Glk1* transcription. mRNA levels of the control gene, *ACT1*, are unaffected by either Hxx2p or Hxx1p function and serve as loading controls.

Thus we conclude that glucose repression of the *HXX1* and *GLK1* genes requires Hxx2p, which operates at the nuclear level, suggesting that the Hxx2p is a direct or indirect *trans*-acting regulatory factor of the molecular machinery involved in the transcriptional repression of the *HXX1* and *GLK1* genes.

### Hxx2p is required for glucose-induced expression of *HXX2* and for glucose-repressed expression of the *HXX1* and *GLK1* genes *in vivo*

The finding that the glucose-controlled expression of the *HXX1* and *GLK1* genes failed in *hxx2Δ* mutant strains suggested that these phenotypes were mediated by the Hxx2 protein. To analyse this and to create an analytical system to study *HXX2* gene expression, we used a set of strains containing integrated reporter plasmids with the *lacZ* gene cloned downstream of the *HXX1*, *HXX2* and *GLK1* promoters in single copy (Table 3). In order to complete the analytical system, we transformed the *hxx2Δ* and the *hxx1Δ hxx2Δ* mutant strains with two different plasmids.



**Figure 2** Gel-retardation analysis of complexes formed with the ERA<sub>GLK1</sub>, ERA<sub>HXK1</sub>, DRS1<sub>HXK2</sub> and DRS2<sub>HXK2</sub> sequences

Gel mobility-shift assays were performed with <sup>32</sup>P-labelled ERA<sub>GLK1</sub> (A), ERA<sub>HXK1</sub> (B), DRS1<sub>HXK2</sub> (C) and DRS2<sub>HXK2</sub> (D) oligonucleotides (see Table 2), in the presence of nuclear extracts from glucose-grown yeast cells of the indicated strains, prepared as described in the Materials and methods section. Unlabelled DRS1, DRS2, ERA<sub>HXK1</sub> and ERA<sub>GLK1</sub> oligonucleotides and calf thymus DNA (CT) were used as competitors. Lane 1, no protein added; lane 2, protein from nuclear extracts obtained from wild-type (WT) repressed cells; lane 3–8, protein from nuclear extracts obtained from *hxx1Δ hxx2Δ* double-mutant (DM) repressed cells; lane 9, protein from nuclear extracts obtained from *hxx1Δ hxx2Δ* double-mutant repressed cells transformed with plasmid YEp352-*HXK2*. Binding-reaction mixtures contained no competitor DNA (–), a 100-fold molar excess of the indicated competitor DNA (lanes 4–7) or a 300-fold molar excess of the calf thymus DNA (lane 8). The arrows indicate the locations of the differential DNA–protein complex (CI) formed with each oligonucleotide probe and with nuclear extracts prepared from wild-type or *hxx1Δ hxx2Δ* mutant strains.

One contained the native *HXK2* gene under the control of its own promoter and the other contained a *HXK2ΔK<sup>r</sup>M<sup>16</sup>* (*HXK2Δ*) gene under the control of the *HXK2* promoter. Both genes were expressed efficiently in the host strains as judged by enzymic determinations of fructose-phosphorylating activities and by immunoblotting analysis using polyclonal antiserum raised against hexokinase 2 from *S. cerevisiae* in rabbits (results not shown). The cells were grown in glucose medium (YEPD) until the *D*<sub>600</sub> reached 1.0. The results are shown in Table 3.

Approximately a 7–8-fold increase in Hxk1-lacZ and Glk1-lacZ fusion enzyme activities were observed in the *hxx1Δ hxx2Δ* double null-mutant strains over the activity of the same fusion in the wild-type strains (Table 3, compare columns 1 and 5). These results could be attributed to a Hxk2p-dependent regulatory mechanism, because expression of the *HXK2* gene in a *hxx1Δ hxx2Δ* double null-mutant background (Table 3, column 3) or a *hxx2Δ* null-mutant background (Table 3, column 6), led to a dramatic decrease of Hxk1-lacZ and Glk1-lacZ fusion enzyme activities, restoring the basal levels of the reporter gene in the wild-type strains (Table 3, column 5).

Moreover, comparison of the activities observed in column 1 with 3 and 4, and column 5 with 8 of Table 3 indicated that Hxk2p not only represses *HXK1* and *GLK1* but also induces *HXK2* expression. Comparing column 3 with 4 and column 5 with 6 in Table 3, it is observed that there is no difference between chromosomal *HXK2* or *HXK2* on a plasmid, although it was a multi-copy plasmid.

Thus the *HXK2* gene, which encodes the major glucose-phosphorylating enzyme in *S. cerevisiae* and is also required for repression of several glucose-regulated genes and for induction of *HXT1* expression at high concentrations of glucose [27,28], can also be considered a repressor of major importance in the control of the *HXK1* and *GLK1* and an important inducer in the control of *HXK2* expression. One would expect that if Hxk2p plays a role in the transcriptional machinery that controls *HXK1*, *GLK1* and *HXK2* expression, it should be present in the nucleus. Recently, it has been reported from subcellular-localization studies that Hxk2p is localized in both the cytosol and nucleus [29]. Moreover, the essential part of the protein-mediating nuclear localization has been reported [6]. Thus comparison of  $\beta$ -galactosidase activities observed in Table 3, columns 1 and 2 and in columns 7 and 8, demonstrated that *HXK2Δ* is not functional in the regulatory process. Thus the Hxk2-mediated repression of *HXK1::lacZ* and *GLK1::lacZ* fusion genes together with the Hxk2-mediated induction of *HXK2::lacZ* fusion gene are dependent on the presence of the Hxk2 protein in the nucleus.

Finally, the reduction in  $\beta$ -galactosidase-specific activity in transformants carrying the *hxx2Δ* mutation and the *GLK1::lacZ* fusion gene (Table 3, column 8) suggests that the activity observed is caused by the high level of Hxk1 protein present in the strain. Our results in Table 3 confirm that when Hxk2p was present, Hxk1p repressed *HXK2-lacZ* expression (compare column 4 with 5). In the absence of Hxk2p, Hxk1p partially repressed *GLK1-lacZ* expression (compare column 1 with 8).

### The importance of Hxk2p for protein–DNA interactions

We were interested to determine whether we could detect a differential pattern of band shifting with nuclear protein extracts prepared from wild-type and *hxx1Δ hxx2Δ* double-mutant strains grown in the presence of glucose and the different regulatory elements identified previously in the *GLK1*, *HXK1* and *HXK2* promoters. As shown in Figure 2, we observed that the *hxx1Δ hxx2Δ* mutation influences DNA–protein complex formation with four nearly homologous regulatory sequences from these genes: the ERA elements of the *GLK1* (position –536, AAATGCA) and *HXK1* (position –341, AAATGCA) genes and the DRS elements of *HXK2* (DRS1, +147, AAATGAA, and DRS2, +245, AAATCAC). To carry out these experiments we synthesized double-stranded oligonucleotides containing the ERA<sub>GLK1</sub>, ERA<sub>HXK1</sub>, DRS1<sub>HXK2</sub> and DRS2<sub>HXK2</sub> sequences (see Table 2) and used them as probes in a band-shift experiment. The four probes gave rise to a positive-differential band-shift reaction

(CI, DNA–protein complex, on Figure 2) with extracts prepared from the *hxx1Δ hxx2Δ* double-mutant strain (Figure 2, lane 3). However, a band shift was not observed with extracts prepared from the wild-type strain (Figure 2, lane 2). After transformation of the *hxx1Δ hxx2Δ* mutants with plasmid-encoded *HXX2*, the signal representing the CI complex disappeared (Figure 2, lane 9). These results suggested that Hxk2p impairs CI complex formation, which only appears in the absence of Hxk2p.

The apparently identical gel mobility observed for the complexes, independent of the probe utilized in the experiment, suggested that the DNA–protein interactions could occur with the same protein(s). To test this hypothesis, we carried out a series of competition experiments with a 100-fold excess of unlabelled double-stranded oligonucleotides containing the ERA<sub>GLK1</sub>, ERA<sub>HXX1</sub>, DRS1<sub>HXX2</sub> and DRS2<sub>HXX2</sub> elements as specific competitors and with a 300-fold excess of unlabelled calf thymus DNA as non-specific competitor. Figure 2 shows that the excess of calf thymus DNA was an ineffective competitor in band-shift assays with radiolabelled probes (lane 8). However, the oligonucleotide containing the ERA<sub>GLK1</sub>-binding site was an effective competitor in the band-shift assays that utilized the ERA<sub>HXX1</sub>, DRS1<sub>HXX2</sub> and DRS2<sub>HXX2</sub> elements, and itself as probe. The fact that the oligonucleotide containing the ERA<sub>GLK1</sub>-binding site was able to compete with all these probes suggested that identical proteins are responsible for the band shifts that we observed with the differently labelled oligonucleotides.

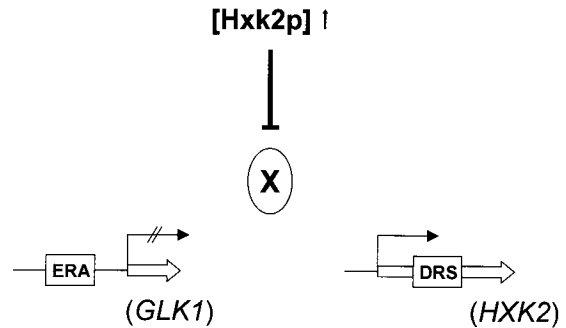
The oligonucleotides containing the ERA<sub>HXX1</sub>, DRS1<sub>HXX2</sub> and DRS2<sub>HXX2</sub>-binding sites were also effective competitors in the band-shift assays that utilized these probes, but higher amounts of competitor were necessary to completely displace the complexes (results not shown).

Taken together, this information is consistent with a model (Figure 3) in which the participation of Hxk2 and Hxk1 proteins is required for the glucose-induced modulation of *GLK1*, *HXX1* and *HXX2* gene expression. In the presence of glucose, the ERA and DRS elements do not operate in the control of *GLK1* and *HXX2* expression. When glucose is present in the culture medium the *GLK1* gene is either not expressed or expressed at a very low level. We did not observe differences between yeast strains with or without the ERA element of the *GLK1* promoter, in terms of *GLK1* gene expression [17]. Similarly, in rich medium with glucose the presence or absence of the DRS element in *HXX2* does not affect its gene expression [9]. Thus the ERA and DRS elements do not have a regulatory function under these metabolic conditions and the CI DNA–protein complex was not detected (Figure 2, lane 2). However, several non-carbon-source-dependent DNA–protein complexes were observed, as described previously [10,17]. Upon glucose exhaustion or even in the presence of glucose when the *HXX2* gene was deleted (*hxx2Δ*), the ERA and DRS elements were operative and functioned in the negative control of *GLK1* and *HXX2* gene expression, respectively [9,17]. A high-mobility DNA–protein complex (CI) was detected under these metabolic conditions (Figure 2, lane 3), suggesting that unknown proteins (X in Figure 3) interact with the regulatory elements. We believe that this DNA–protein complex should be a functional complex under the negative control of *GLK1* and *HXX2* gene expression.

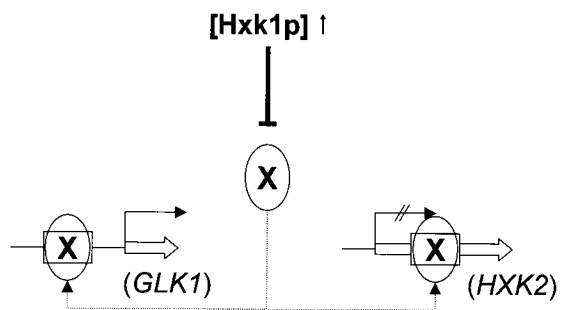
Deletion of the *HXX2* gene induces an overexpression of *HXX1* (Figure 1), and this has a marked repressing effect on *HXX2* and *GLK1* gene expression (Table 3, compare lane 4 with 5 and lane 1 with 8). A possible explanation would be that Hxk1p acts as an Hxk2p analogue with regard to the ERA element and as an Hxk2p competitor with regard to the DRS element.

In summary, we have shown that a cytosolic protein (Hxk2p) that participates in an essential metabolic pathway (glycolysis)

### Glucose ↑



### Glucose ↓ or Glucose ↑ and *hxx2Δ*



**Figure 3** A model describing the role of Hxk2p and Hxk1p under high- and low-glucose conditions in the control of *GLK1* and *HXX2* gene expression

Arrows with double bars across them denote negative regulation. A high amount of protein is indicated by bold lettering and an upwards arrow. X denotes unknown proteins that operate through the ERA and DRS regulatory elements. Regulatory elements involved in the control of gene expression are represented by rectangles and genes by open arrows.

can also be targeted to the nucleus, where it functions in transcription, alluding to a potentially important pathway of nuclear–cytosolic dialogue. This dual capacity suggests that Hxk2p plays a multifunctional role in the cell. The simplest interpretation of our findings is that Hxk2p is involved in a positive-feedback loop that serves to amplify its own expression and in a negative-feedback loop that represses the expression of *HXX1* and *GLK1* in response to high glucose levels in the culture medium. Our data also suggest that Hxk1p acts to dampen Hxk2p-mediated regulatory functions.

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