Functional characterization of transcriptional regulatory elements in the upstream region of the yeast *GLK1* gene

Pilar HERRERO, Leticia FLORES, Tamara de la CERA and Fernando MORENO¹

Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Biotecnología de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain

The glucokinase gene *GLK1* of the yeast *Saccharomyces cerevisiae* is transcriptionally regulated in response to the carbon source of the growth medium. Northern-blot analysis shows that the *GLK1* gene is expressed at a basal level in the presence of glucose, de-repressed more than 6-fold under conditions of sugar limitation and more than 25-fold under conditions of ethanol induction. *lacZ* fusions of the *GLK1* gene promoter were constructed and a deletion analysis was performed in order to identify the *cis*-acting regulatory elements of the promoter that controls *GLK1* gene expression. First, the expression seemed to be mediated mainly by one GCR1 and three stress-responsive element (STRE) activating elements. Secondly, an ethanol repression autoregulation (ERA)/twelve-fold TA repeat (TAB) repressor element was identified within the promoter region of the *GLK1* gene. A specific and differential protein binding to the STRE was observed with extracts from de-repressed and repressed cells. No differential binding to the GCR1 or ERA/TAB elements was observed with extracts from de-repressed and repressed cells, but, in both cases, the binding was competed for

by an excess of the unlabelled *GLK1*_{GCR1} and *GLK1*_{ERA} sequence. The transcription factors Msn2 and Msn4, which bind to the *GLK1* upstream region through the STRE, contribute to inductive activation. The transcription factor Gcr1, which binds through the GCR1 element, contributes to constitutive activation. In order to achieve the severe glucose repression of *GLK1*, constitutive repressor factors acting through the ERA/TAB element must counteract constitutive activation generated by Gcr1 binding to the GCR1 element. Full expression of the *GLK1* gene is produced by inductive activation of three STRE when Msn2 and Msn4 proteins are translocated to the nucleus by covalent modification. The combinatorial effect of the entire region leads to the regulated transcription of *GLK1*, 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.

Key words: glucokinase, *Saccharomyces cerevisiae*, transcriptional regulation.

INTRODUCTION

Glycolysis is a major metabolic pathway in *Saccharomyces cerevisiae* and most of the genes are highly expressed in the presence of glucose, whereas the gluconeogenic genes are repressed. In *S. cerevisiae*, the phosphorylation of glucose at C6 can be catalysed by three enzymes, namely the isoenzymes 1 and 2 of hexokinase (encoded by the *HXK1* and *HXK2* genes) and glucokinase (encoded by the *GLK1* gene). These enzymes can catalyse the first irreversible step in the intracellular metabolism of glucose. However, only *HXK2* appears to play a role during *in vivo* glucose phosphorylation in *S. cerevisiae*. Thus Northern-blot analysis has shown that the *HXK2* gene is highly expressed when glucose, fructose or mannose is used as the carbon source. Conversely, the expression of *HXK1* and *GLK1* genes only takes place when the culture medium contains non-fermentable carbon sources or galactose [1]. Recently, this differential expression has been confirmed by exploring the metabolic and genetic control of gene expression on a genomic scale [2]. This observation opens new questions about the physiological role of Hxk1 and Glk1 proteins in the yeast cell.

Because of the interest in discovering the mechanism that controls the expression of the glucokinase gene, in the present work we report a systematic analysis of the 5'-untranslated region of *GLK1* and identify the elements that are required for its regulated transcription. We show that *GLK1* is regulated by a 739 bp region, which has 129 bp inside the coding region of the *PDII* gene (encoding protein disulphide isomerase) [3,4], and is

composed of positive and negative elements. The identification of three distinct DNA-binding proteins (Msn2, Msn4 and Gcr1), that interact specifically within the 5'-flanking region of the *GLK1* gene, is reported. The related Zn²⁺-finger transcription factors Msn2 and Msn4 are involved in the general stress-response pathway [5,6]. Strains lacking *MSN2* and *MSN4* genes fail to accumulate stress-regulated messages following heat and osmotic stress, as well as nutrient starvation. Moreover, Msn2 protein binds specifically to DNA sequences, referred to as the stress-responsive element (AGGGG) or STRE [5,6]. The STRE has been shown to mediate both stress induction and repression of a large number of genes [7,8] by the cAMP-signalling pathway [9–11]. The *GCR1* gene appears to play a central role in co-ordinate regulation of glycolytic gene expression, since null mutations in this gene cause substantial reductions in the specific activities of many glycolytic enzymes [12,13], (for review see [14]). The Gcr1 protein binds to the sequences containing a CTTCC motif, and was shown to be a *trans*-acting positive regulator of transcription of most of the glycolytic genes [15] and the retrotransposon Ty2-917 [16].

The binding sites for these factors were mapped by band-shift assays and transformation of several mutant strains and was found to correlate with essential regions of upstream activating sequence (UAS) and upstream repressing sequence (URS) elements, identified by deletion analysis. The role of these specific *trans*-acting factors in regulation of glucokinase gene expression in response to different nutritional and environmental stress signals is discussed.

Abbreviations used: DTT, dithiothreitol; ERA, ethanol repression autoregulation; STRE, stress-responsive element; TAB, a twelve-fold TA repeat; UAS, upstream activating sequence; URS, upstream repressing sequence; YEPD, yeast extract/peptone/dextrose; YEPE, yeast extract/peptone/ethanol.

¹ To whom correspondence should be addressed (e-mail fms@sauron.quimica.uniovi.es).

MATERIALS AND METHODS

Strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. AMW-13C⁺ and W303-1A were used as wild-type strains. For investigations with different mutants the strains HBY4 and Wmsn2msn4 were used. 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*).

Plasmid YIp358R is a yeast-*E. coli* shuttle vector suitable for fusing yeast promoters and coding sequences to the *lacZ* gene of *E. coli*, and can be used to integrate *lacZ* fusions into yeast chromosomal DNA [19].

Media, growth conditions and enzymic analysis

Yeasts were grown on 1% (w/v) yeast extract and 2% (w/v) peptone supplemented with 2% (w/v) glucose (YEPE) or 3% (v/v) ethanol (YEPE) at 28 °C until the *D*₆₀₀ reached 1.0 (early log phase). To select for transformants, synthetic medium with yeast nitrogen base, 2% glucose and adequate supplements was used. β -Galactosidase activity was assayed according to [20].

General DNA techniques

Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were from Amersham Pharmacia Biotech, Sequenase V2.0 was from United States Biochemical. Radioactively labelled compounds were from Amersham International. DNA was amplified by PCR using 0.1 μ g of the primer pair, 1 μ g of template, 2.5 units of *Taq* polymerase (Promega), 0.2 mM dNTPs (Pharmacia) in a total reaction volume of 25 μ l in reaction buffer, provided by the manufacturer, for 30 cycles at 94 °C for 30 s, 55 °C for 90 s and 72 °C for 60 s. The PCR product was isolated from 0.8% (w/v) agarose gels and ligated. The dideoxyribonucleotide chain-termination procedure was used for DNA sequencing analysis [21]. All other DNA manipulations were as described previously [22].

Constructions of reporter fusions, promoter deletions and substitutions

To construct a gene fusion between *GLK1* and the bacterial *lacZ* gene, encoding β -galactosidase, a 1563-bp fragment containing 63 bp of the *GLK1* coding region plus additional DNA upstream was amplified by PCR using, as primer, oligonucleotides with an *Pst*I site (OL1, ATCTGCAGTCGATGCTAACAAAGTTC) or an *Eco*RI site (OL2, ATGAATTCATCTGGTCCACGGCCTGG) at the 5' end. Genomic DNA from the wild-type strain, AMW-13C⁺, served as the *GLK1*-containing template. The amplified product was digested with *Pst*I and *Eco*RI and ligated to YIp358R digested with the same enzymes to form plasmid YIp1563. Other gene fusion between different lengths of *GLK1*-

Figure 1 Deletion analysis of the *GLK1* promoter

All constructs were integrated at the *URA3* locus in strain AMW-13C⁺, and β -galactosidase activity was assayed from cultures growing exponentially in YEPE and YEPD medium. The results of β -galactosidase activity for each derivative are from a minimum of five independent experiments. Only strains with single copy integrations were used to obtain the data presented. Numbers on the far left are those used to describe the constructs. Numbers to the left of the constructs designate the last bp still present in the promoter region of each deletion. Black boxes show the residual part of the promoter-region sequences. Values in the right-hand columns are the specific activities of β -galactosidase (m-units/mg of protein; mU/mg) measured in crude extracts obtained by breaking the cells with glass beads and centrifugation at 14 000 rev./min for 15 min at 4 °C, before enzymic determination.

5' flanking region and the *lacZ* gene were constructed using an identical PCR procedure, but with OL1 replaced by different primer oligonucleotides (OL3, ATCTGCAGGTGACAAAAGTCTCGTTA; OL4, ATCTGCAGGGCCAAGGTAATGTTT) both containing a *Pst*I site at the 5' end, to form plasmids YIp1063 and YIp944 respectively (Figure 1, construct 1).

Deletions which remove *GLK1* promoter sequences between the -881 bp (relative to the translational start codon) and specific endpoints downstream (Figure 1, constructs 2-5) were created taking advantage of internal restriction sites of the *GLK1* promoter. Construct 2 was created by digestion of construct 6 with *Bam*HI and *Eco*RI, the 765 bp fragment generated was subcloned into YIp358R, digested with the same enzymes, to form plasmid YIp765. Constructs 3, 4 and 5 were obtained by digestion of the *GLK1* promoter with *Dra*I/*Eco*RI, *Spe*I/*Eco*RI and *Hind*II/*Eco*RI respectively to generate 635, 472 and 247 bp fragments, which were ligated to YIp358R digested respectively with *Sma*I/*Eco*RI, *Xba*I/*Eco*RI and *Sma*II/*Eco*RI to form plasmids YIp635 (construct 3), YIp472 (construct 4) and YIp247 (construct 5).

Deletions which remove internal *GLK1* promoter sequences were created by PCR using suitable inside-out oligonucleotides. This procedure was used to amplify the entire promoter without a specific regulatory region. In all cases the primer oligonucleotides utilized contained a *Bam*HI site at the 5' end. Plasmid pUC944, a 944-bp fragment containing 881 nt from the 5' non-

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

Strain	Genotype	Reference
AMW-13C ⁺	<i>MATa trp1(fs) ura3(fs) leu2-3,112 his3-11,15 can1</i>	[17]
W303-1A	<i>MATa SUC2 ade2 can1 his3 leu2 trp1 ura3</i>	[5]
Wmsn2msn4	<i>MATa SUC2 ade2 can1 his3 leu2 trp1 ura3 msn2-Δ3::HIS3 msn4-1::TRP1</i>	[5]
HBY4	<i>MATα gcr1Δ::His3 leu2-3,112 his3Δ trp1-189 ura3-52</i>	[18]

Table 2 PCR primers used in this study

Construct number	Sense	Antisense
6	CATGGATCCACAGCCTTGTGGGGCG(–681)	CATGGATCCCGCTGTCGTTAAGTTGGC(–732)
8	GTCGGATCCAGGTTTGTGCTCCGAA(–301)	TATGGATCCTGTCGGGCGGCGCTCTT(–391)
10	CATGGATCCCGTGGTGTACACCACG(–138)	CATGGATCCCGCTCCGTCGGCACAC(–202)
11	CATGGATCCGTGGCCAACCTAACGACA(–718)	CATGGATCCGTACATTAGTCGCACG(–767)
12	CATGGATCCGTCCCATACGGGTTTCTC(–471)	CATGGATCCCGGTGCATTTTCTTGTG(–544)
13	CATGGATCCGTCCCATACGGGTTTCTC(–471)	CATGGATCCAAGCAGCATGTCCTA(–560)

Table 3 Complementary oligonucleotides carrying regulatory elements of the *GLK1* promoter used in this study

DNA fragment	Sense	Antisense
<i>GLK1</i> _{GCR1}	tcgaCTCATTGAAGGAGTCGG(–735)	tcgaCCGACTCCTTAATGAG(–751)
<i>GLK1</i> _{STRE}	tcgaCTTTCAGGGGGCCC(–167)	tcgaGGGCCCCCTGAAAG(–179)
<i>GLK1</i> _{ERA}	tcgaCAAGAAATGCACGCG(–526)	tcgaCGCGTGCATTTTCTTG(–541)

coding region and 63 bp of the *GLK1* coding sequence cloned in pUC18, served as the *GLK1* promoter-containing template for the PCR. The fragments synthesized were digested with *Bam*HI, ligated and digested with *Pst*I and *Eco*RI. The various DNA fragments were ligated to YIp358R, previously digested with the same enzymes, to form constructs 6, 8 and 10–13 (Figure 1). The oligonucleotides utilised as PCR primers to create internal deletions in the *GLK1* promoter are listed in Table 2.

Constructs 7 and 9 (Figure 1) were created using internal restriction sites of constructs previously obtained by PCR. Thus construct 7 was obtained by inserting a *Pst*I/*Spe*I fragment obtained from construct 6 into construct 13 previously digested with the same enzymes. Construct 9 was obtained by inserting a *Pst*I/*Spe*I fragment obtained from construct 6 into construct 10 previously digested with the same enzymes. The DNA sequence of the *GLK1* promoter in each construct was determined to detect any mutations resulting from PCR amplification and to verify that cloning junctions were correct.

The YIp358R derivatives were then integrated at the *URA3* locus. Recombinant plasmids were digested with *Stu*I, which cuts the plasmids at a unique site in *URA3*, and the linear DNAs were then used to transform strain AMW-13C⁺ to uracil prototrophy. Since tandem integration events can occur, transformants were screened by Southern-blot analysis in order to ensure that transformants used for further study had only one copy of the fusion construct integrated into the genome.

DNA probes

To investigate interaction of proteins with the sequences carrying regulatory elements in the *GLK1*-5' non-translated region, we reconstituted the fragment from two complementary oligonucleotides. The complementary strands were annealed and either end-labelled with [α -³²P]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 nt overhang at the 5'-terminal end. The synthetic DNA fragments used in gel-retardation experiments are listed in Table 3.

Preparation of protein extracts

Yeast protein extracts were prepared as follows: yeast was grown on 10–20 ml of rich medium (YEPD or YEPE) at 28 °C until a D_{600} of 1.0 was reached. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μ l of buffer C [20 mM Hepes, pH 7.9, containing 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.42 M NaCl, 1.5 mM MgCl₂ and 25 % (v/v) glycerol]. The cells were broken by vortex mixing (6 $\times$ 20 s) in the presence of glass beads (0.5 g), and 400 μ l buffer C was added to the suspension. After centrifugation at 19000 *g* (14000 rev./min) for 15 min at 4 °C, the supernatant was used as crude protein extract.

Gel-retardation assays

Binding reaction mixtures contained 10 mM Hepes (pH 7.5), 1 mM DTT, 1–5 μ g of poly(dI-dC) and 0.5 ng of end-labelled DNA in a volume of 25 μ l. When unlabelled competitor DNA was added, the amount is shown in the Figure legends. Protein extract (60 μ g contained in 6 μ l) was added and, after incubation for 30 min at room temperature, samples were loaded on to a non-denaturing 4% polyacrylamide gel. Electrophoresis was carried out at 10 V/cm of gel for 45–60 min in 0.5 $\times$ TBE buffer (45 mM Tris/borate/1 mM EDTA). Gels were dried and autoradiographed at –70 °C, using an intensifying screen.

RESULTS

Expression levels of the *GLK1* gene in various nutritional and environmental conditions

Previous studies have suggested that the *GLK1* gene has a differential expression depending on the carbon source used for growth [1]. To determine if the steady-state level of *GLK1* mRNA was affected by both the nutritional and environmental conditions of the culture medium, total RNA was prepared from cells grown on different carbon sources, at different osmotic pressures and at different temperatures and Northern-blot analysis was performed (Figure 2). By using a *GLK1* probe, a strong hybridization band was obtained when cells were incubated at 28 °C in media with ethanol (YEPE) as the only carbon source or in media without a carbon source. Only minor bands were detected in preparations from cells grown on YEPD and on YEPD containing 0.4 M NaCl, and a very weak band was observed in preparations from cells grown on YEPD at 37 °C.

Delineation of positively and negatively acting *GLK1* promoter regions

A *GLK1* ::*lacZ* gene fusion was prepared for use in determining the 5' boundary of the *GLK1* controlling region. Since upstream regulatory sequences may be quite some distance away from the transcriptional start site of the gene, our initial studies were

Figure 2 Comparison of steady-state *GLK1* mRNA levels in cells cultured in different nutritional and environmental conditions

Total RNA was prepared from wild type AMW-13C⁺ strain after incubation as follows. NC, yeast cells from an overnight culture grown in YEPE medium were washed and resuspended in 1% (w/v) yeast extract and 2% (w/v) peptone medium, without added glucose, to a D_{600} of 1.0. The cells were then incubated at 28 °C for 6 h. Glc and EtOH, yeast cells were grown respectively in YEPE and YEPE medium at 28 °C until D_{600} of 1.0 was reached. NaCl, yeast cells were grown at 28 °C on YEPE medium supplemented with 0.4 M NaCl to a D_{600} of 1.0. 37 °C, yeast cells were grown at 37 °C in YEPE medium to a D_{600} of 1.0. The isolated RNA was electrophoresed through a 0.8% (w/v) agarose/37% (v/v) formaldehyde gel and transferred to a nylon filter. Each lane was loaded with 10 µg of total RNA. After transfer, the filter was hybridized with radiolabelled probe complementary to the *GLK1* transcript. Following autoradiography the filter was stripped and rehybridized with a radiolabelled probe complementary to the actin gene (*ACT1*) transcript, this probe was used as an internal reference. Arrows indicate the positions of the *GLK1* and *ACT1* transcripts.

carried out with fusions which contained 1500, 1000 and 881 bp, 5' to the translational start site of *GLK1*. Subcloning experiments of these PCR-generated DNA fragments were used to map the 5'-boundary of the *GLK1* controlling region. First, no effect on β -galactosidase activity was observed with constructs that removed DNA 5' to position -881 (results not shown). Thus all sequences necessary for a high expression level of *GLK1* reside within 881 bp preceding the start codon of *GLK1* (Figure 1, construct 1). Secondly, we found that β -galactosidase activity increased 7-fold in ethanol-grown (YEPE) cells as compared with those grown in glucose (YEPE) (Figure 1, construct 1). Thus the expression ratio between cells grown on ethanol and glucose was very similar to that observed for the genuine *GLK1* gene in Northern-blot experiments (Figure 2). Thirdly, sequences involved in the function of the *GLK1* promoter were delineated by removing additional DNA downstream of the -881 bp position (Figure 1). This deletion analysis was carried out taking advantage of internal restriction sites of the *GLK1* promoter. On the basis of β -galactosidase activity in the yeast strains containing these deletions (Figure 1, lines 2–5), the *GLK1* promoter was divided into three functional regions, designated UAS1, URS and UAS2. We have designated the region contained between nucleotides -881 and -702 UAS1 because its upstream location and apparent role in stimulating transcription indicate that it is analogous to UAS elements in typical yeast promoters. This region is broadly defined by the -881 to -702 deletion (Figure 1, line 2), which leads to a 60% reduction of the expression level

Table 4 β -Galactosidase levels in regulatory mutants

β -Galactosidase levels expressed from a *GLK1::lacZ* fusion integrated in unit copy at the *URA3* locus in wild-type (W303-1A), *gcr1* (HBY4) and *msn1/msn4*-deletion (Wmsn2msn4) mutant strains.

Strain	Plasmid	β -Galactosidase activity (m-units/mg of protein)	
		YEPE	YEPE
W303-1A	Ylp944	1449	216
HBY4	Ylp944	475	220
Wmsn2msn4	Ylp944	211	110

in YEPE medium. Further downstream of the UAS1 lies a region, designated the URS because it mediates a 12-fold decrease in β -galactosidase activity, defined by the -572 to -409 deletion (Figure 1, line 4). The region adjacent to the URS, between nucleotides -408 and -104, appears to contain a strong activating element of promoter activity. We have chosen to designate this region UAS2.

Functional elements of the UAS regions

The two functional regions designated UAS1 and UAS2 were analysed with the promoter database of *S. cerevisiae* (SCPD), revealing several features that could be involved in transcriptional regulation. At -163, -170, -178 and -702 bp (relative to the ATG translation start codon) reside four putative Msn2/Msn4 binding sites, designated STRE. At -317 and -349 bp two putative Pho4 binding sites were detected; the -349 bp Pho4 binding site was flanked by two putative Pho2 binding sites at -334 and -360 bp. Finally, at -739 bp, a putative Gcr1 binding site was localised. To determine the functional role of these elements they were deleted from construct 1 (Figure 1) by inverse PCR, and the effects of internal deletions spanning the consensus sequence of each element were examined. The results are shown in Figure 1 (lines 6 to 11). Whereas removal of the STRE at -702 bp (Figure 1, lines 6, 7 and 9) and the PHO4 and PHO2 elements from -317 to -373 bp (Figure 1, lines 7 and 8) had no significant effect on the β -galactosidase activity in cells growing in YEPE medium, the deletion that spans the region from -155 to -187 bp, containing the STRE at -163, -170 and -178 bp, resulted in a severe decrease in expression (lines 9 and 10), indicating that the three STRE are essential in all cases for full expression, and thus appear to be the most important activating element of the *GLK1* promoter. Removal of the Gcr1 binding site at -739 bp by deleting the region from -735 to -751 bp (line 11) caused a significant decrease in expression, suggesting that the functional element of the UAS1 region is a Gcr1 binding site.

To confirm that the Gcr1 binding site and the three STRE are the functional *cis*-acting elements contained in the UAS1 and the UAS2 regions of the *GLK1* promoter, we used plasmid Ylp944 to transform the wild-type strain W303-1A and a *gcr1* and a *msn2 msn4*-deletion mutant strains (Table 4). The level of expression of *GLK1::lacZ* fusion, which exhibited maximal levels of expression in the wild-type, was reduced by more than 67% in the *gcr1* mutant and by more than 85% in the *msn2 msn4* double mutant. Thus the *gcr1* and the *msn2 msn4* lesions have the same effect on *GLK1* wild-type promoter-directed *lacZ* expression as deleting the Gcr1 binding site and the three STRE of the *GLK1* promoter respectively (Figure 1, constructs 11 and 10).

Figure 3 Influence of *gcr1*-deletion mutation on protein binding to the *GLK1* promoter UAS1 element

Gel mobility-shift assays were performed with ^{32} P-labelled *GLK1_{GCR1}* double-stranded oligonucleotide (see Table 3) and protein extracts from repressed or de-repressed cells of the indicated strains, prepared as described in the Materials and methods section. Lane 1, no protein added; lanes 2–5, protein from repressed cells; lanes 6–9, protein from de-repressed cells. WT, wild-type (AMW-13C⁺ strain); *gcr1*, *gcr1*-deletion mutation (HBY4 strain); CT, calf-thymus DNA used as competitor; *GLK1_{GCR1}*, unlabelled *GLK1_{GCR1}* double-stranded oligonucleotide used as competitor; ng, amount, in ng, of competitor DNA added. The complexes isolated were designated CI, CII, CIII and CIV and are marked with arrows.

First, these results confirm that the Gcr1 binding site (–739 bp) and the three STRE (–163, –170 and –178 bp) are the functional *cis*-acting elements contained the UAS1 and the UAS2 regions of the *GLK1* promoter. Secondly, our results strongly suggest that the Gcr1, Msn2 and Msn4 proteins are *trans*-acting factors involved in the control of *GLK1* gene expression.

Gel-shift assays using the *GLK1_{GCR1}* element (Table 3) and total proteins extracted from yeast cells grown under the conditions described above for the *lacZ* experiments revealed the formation of three specific DNA–protein complexes (Figure 3). Non-specific competition with calf-thymus DNA did not reduce the formation of these complexes (Figure 3, lanes 4 and 8), whereas specific competition caused a decrease (Figure 3, lanes 3 and 7). The results shown in Figure 3 (lanes 2 and 6) demonstrate that the formation of the *GLK1_{GCR1}*–protein complexes is not subject to nutrient regulation. The fact that the double-stranded oligonucleotide used in the band-shift assays contained the consensus Gcr1 binding site suggested that this transcription factor was responsible for the band shift observed. To test this hypothesis, a protein extract prepared from a *gcr1*-deleted mutant strain, grown under the conditions described above, was used in band-shift assays (Figure 3, lanes 5 and 9). When protein extracts from cells grown on YEPD or YEPE medium were used, four DNA–protein complexes were formed in the wild-type (Figure 3, lanes 2 and 6), but only one complex (CIV) was detected with protein extracts from the *gcr1* mutant strain (Figure 3, lanes 5 and 9). This observation confirms that the *GCR1* gene product binds to the *GLK1* controlling region designated UAS1.

Gel-shift assays, using the *GLK1_{STRE}* element (Table 3) and total proteins extracted from yeast cells grown under the con-

Figure 4 Influence of *msn2/msn4*-deletion mutations on protein binding to the *GLK1*-promoter UAS2 element

Gel mobility-shift assays were performed with ^{32} P-labelled *GLK1_{STRE}* double-stranded oligonucleotide (see Table 3) and protein extracts from repressed or de-repressed cells of the indicated strains, prepared as described in the Materials and methods section. (A) Specificity of the DNA–protein complexes formed. Lane 1, no protein added; lanes 2–4, protein from repressed cells; lanes 5–7, protein from de-repressed cells. (B) Influence of mutations on protein binding. Lane 1, no protein added; lanes 2 and 3, protein from repressed cells; lanes 4 and 5, protein from de-repressed cells. wt, wild-type (AMW-13C⁺ strain); dm, *msn2/msn4* double mutant (Wmsn2msn4 strain); ng, amount, in ng, of competitor DNA added. The complexes isolated were designated CI and CII and are marked with arrows.

ditions described above for the *lacZ* experiments, revealed the formation of two specific DNA–protein complexes (Figure 4). Non-specific competition with calf-thymus DNA did not reduce the formation of these complexes (Figure 4A, lanes 4 and 7),

Figure 5 Competition in band-shift assays of the *GLK1*_{ERA} double-stranded oligonucleotide, which contains the URS element from the *GLK1* gene promoter

Each reaction included 0.5 ng of [³²P]*GLK1*_{ERA} double-stranded oligonucleotide (see Table 3) and, except for lane 1, 6 µl of protein extract from repressed or de-repressed cells, prepared as described in the Materials and methods section. The competitor for binding was 50 ng of unlabelled *GLK1*_{ERA} (lanes 3 and 6) and 50 ng of unlabelled calf-thymus DNA (CT) (lanes 4 and 7). The complexes isolated were designated CI, CII and CIII and are marked with arrows.

whereas a decrease was apparent with specific competition (Figure 4A; lanes 3 and 6). Figure 4(A) (lanes 2 and 5) demonstrates that the formation of the STRE–protein complex is subjected to nutrient regulation; in the presence of glucose only the one complex (CI) was detected, whereas two complexes, CI and CII, were observed in medium where ethanol was the sole carbon source. The fact that the double-stranded oligonucleotide used in the band-shift assays contained the consensus Msn2/Msn4 binding site suggested that this transcription factor was responsible for the band shift observed. To test this hypothesis, a protein extract prepared from a *msn2 msn4* double mutant strain, grown under the conditions described above, was used in band-shift assays (Figure 4B). When protein extracts from cells grown on YEPD medium were used, a similar DNA–protein complex was formed in wild-type and *msn2 msn4* mutant strains (Figure 4B; lanes 2 and 3). However, when the protein extract prepared from the *msn2 msn4* strain grown with ethanol as the sole carbon source was used in band-shift assays, a weak band with different mobility was clearly observed (Figure 4B, lane 5) which was different to that obtained when the extract was prepared from the wild-type strain (Figure 4B, lane 4). This observation confirms that the *MSN2* and *MSN4* genes products bind in the *GLK1* controlling region, designated UAS2.

Functional elements of the URS region

The functional region of *GLK1* promoter designated URS was analysed with the promoter database of *S. cerevisiae* (SCPD), and one feature that could be involved in transcriptional regu-

lation was revealed. At –530 bp (relative to the ATG translation start codon) resides a putative ethanol repression autoregulation (ERA) element [23], followed by a twelve-fold TA repeat (TAB) around –493 and –517. To determine the functional role of these elements they were deleted from construct 1 (Figure 1) by PCR using suitable inside-out oligonucleotides, and the effects of internal deletions spanning the consensus sequence of each element were examined. The results are shown in Figure 1 (lines 12 and 13). Removal of the TAB element at –493 (line 12) had a moderate effect, increasing by 39 % the β-galactosidase activity in cells growing in YEPE medium. However, the deletion spanning the region from –488 to –545, containing the ERA and TAB elements, results in an important increase in expression (line 13), indicating that the ERA element is essential in all cases for physiological expression of the gene and thus appears to be the repressor element of the *GLK1* promoter.

Gel-shift assays using the *GLK1*_{ERA} element (Table 3) and total proteins extracted from yeast cells grown under the conditions described for the *lacZ* experiments (see above), revealed the formation of two specific DNA–protein complexes (Figure 5). Non-specific competition with calf-thymus DNA did not reduce the formation of the three complexes (Figure 5, lanes 4 and 7), whereas specific competition produced a decrease in the CI and CII complexes (Figure 5, lanes 3 and 6). Figure 5 (lanes 2 and 5) demonstrates that the formation of the ERA–protein complexes is not subject to nutrient regulation, the three complexes CI, CII and CIII were detected in protein extracts from cells grown in media containing glucose or ethanol as the sole carbon source. These experiments show that the CAAGAAATGCACGCG sequence binds proteins specifically but, at this moment, the identity of the proteins that modulate ERA/TAB function is not known and further studies will be necessary to elucidate their nature.

DISCUSSION

Using systematic deletion analysis of an *GLK1::lacZ* fusion we have demonstrated that the transcription of *GLK1* is regulated by a region of 739 bp which contain three functional elements: UAS1, UAS2 and URS (illustrated in Scheme 1). As nutrients regulate the transcription of the *GLK1* gene, we investigated the role of the different *cis*-acting regulatory elements identified in the *GLK1* promoter under growth conditions where glucose and ethanol were the carbon source. First, we demonstrated that the transcriptional activators Msn2 and Msn4 are required both for the formation of a specific DNA–protein complex with the three

Scheme 1 Schematic structure of *GLK1* 5' untranslated region

The *cis*- and *trans*-acting elements that respond to the nutritional signals glucose (Glc) or ethanol (EtOH) are illustrated. A positive role is marked + and a negative role is marked –. ? = unknown transcription factor(s) that act through the ERA/TAB element.

STRE of the *GLK1* promoter, and for the expression of the *GLK1* gene in YEPE medium. Accordingly, it has been reported that the nuclear localization of the Msn2 protein is modulated by cAMP-dependent protein kinase (cAPK); in the presence of high cAPK activity in cells growing exponentially in glucose medium, or in non-stressed conditions, Msn2 is confined to the cytoplasm, whereas upon stress it is translocated to the nucleus [11]. Down-regulation of the cAMP signalling pathway seems to be an important controlling factor in this transition. A decrease in the level of intracellular cAMP during the consumption of glucose has been reported [24,25], and it is required for subsequent growth on ethanol after the diauxic transition [25]. Our results are also in agreement with the observation, previously reported [8], that the *GLK1* gene product is dependent upon Msn2 and Msn4 proteins for their induction at the diauxic transition.

Repression of *GLK1* expression through the ERA/TAB element can be partially relieved only upon full activation of both STRE and GCR1 elements during ethanol growth. Although, in the presence of glucose, activation of the GCR1 element apparently takes place, it is not sufficient to overcome ERA/TAB repression because the STRE are inactivated by the high activity of cAPK and, in these metabolic conditions, *S. cerevisiae* cells do not express the *GLK1* gene. We therefore propose that the Msn2/Msn4-STRE system functions as a positive signal that is absolutely required for the transcription of the *GLK1* gene. The fact that the *GLK1* gene is expressed under nutritional-stress conditions suggests that it would have a large role in cell physiology, considering that this enzyme could have a critical role in environmental adaptation to relieve nutritional-stress conditions when a preferential carbon source, such as glucose, fructose or mannose, was available in the culture medium. Taking this into account, an interesting function for glucokinase, which came to our attention recently, was a possible role in cell-cycle progression. Yeast cells accumulate reserve carbohydrates, trehalose and glycogen, under nutritional-stress conditions so that they are well prepared to survive long periods under poor conditions [26–28], and can go quickly through a cell-division cycle when conditions improve [29] by degrading reserve carbohydrates and hence increasing the glycolytic flux [30]. Since trehalose degradation, but not glycogen degradation, generates intracellular glucose, which requires phosphorylation at C6 for further utilization, a direct link between *GLK1* expression and trehalose metabolism can be considered. Thus our results lead us to suggest that trehalase and glucokinase activities might be required for maintenance when extracellular carbohydrates become exhausted, or for a rapid cell-division cycle by increasing the glucose flux when nutritional conditions improve.

We are very grateful to Dr J. M. Gancedo and Dr C. Martínez-Campa for a critical reading of the manuscript. We would also like to thank Dr F. Estruch and Dr H. V. Baker for providing the yeast strains Wmsn2msn4 and HBY4 respectively. This work was supported by grant PB97-1213-C02-02 from the DGESIC.

REFERENCES

- Herrero, P., Galíndez, J., Ruiz, N., Martínez-Campa, C. and Moreno, F. (1995) *Yeast* **11**, 137–144
- DeRisi, J. L., Iyer, V. R. and Brown, P. O. (1997) *Science* **278**, 680–686
- Scherens, B., Dubois, E. and Messenguy, F. (1991) *Yeast* **7**, 185–193
- LaMantia, M. and Lennarz, W. J. (1993) *Cell* **74**, 899–908
- Martínez-Pastor, M. T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1996) *EMBO J.* **15**, 2227–2235
- Schmitt, A. P. and McEntee, K. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 5777–5782
- Mager, W. H. and Kruijff, A. J. (1995) *Microbiol. Rev.* **59**, 506–531
- Boy-Marcotte, E., Perrot, M., Bussereau, F., Boucherie, H. and Jacquet, M. (1998) *J. Bacteriol.* **180**, 1044–1052
- Marchler, G., Schüller, C., Adam, G. and Ruis, H. (1993) *EMBO J.* **12**, 1997–2003
- Varela, J. C., Praekelt, U. M., Meacock, P. A., Planta, R. J. and Mager, W. H. (1995) *Mol. Cell. Biol.* **15**, 6232–6245
- Görner, W., Durchschlag, E., Martínez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H. and Schüller, C. (1998) *Genes Dev.* **12**, 586–597
- Clifton, D. and Fraenkel, D. G. (1981) *J. Biol. Chem.* **256**, 13074–13078
- Baker, H. V. (1986) *Mol. Cell. Biol.* **6**, 3774–3784
- Chambers, A., Packham, E. A. and Graham, I. R. (1995) *Curr. Genet.* **29**, 1–9
- Uemura, H., Koshio, M., Inoue, Y., López, M. C. and Baker, H. V. (1997) *Genetics* **147**, 521–532
- Turkel, S., Liao, X. B. and Farabaugh, P. J. (1997) *Yeast* **13**, 917–930
- Fernández, E., Moreno, F. and Rodicio, R. (1992) *Eur. J. Biochem.* **204**, 983–990
- Scott, E. W., Allison, H. E. and Baker, H. V. (1990) *Nucleic Acids Res.* **18**, 7099–7107
- Myers, A. M., Tzagoloff, A., Kinney, D. M. and Lusty, C. J. (1986) *Gene* **45**, 299–310
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Sanger, F., Nicklen, S. and Coulson, S. A. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Herrero, P., Ramírez, M., Martínez-Campa, C. and Moreno, F. (1996) *Nucleic Acids Res.* **24**, 1822–1828
- Liesen, T., Hollenberg, C. P. and Heinisch, J. J. (1996) *Mol. Microbiol.* **21**, 621–632
- Francois, J. M., Eraso, P. and Gancedo, C. (1987) *Eur. J. Biochem.* **164**, 369–373
- Russel, M., Bradshaw-Rouse, J., Markwardt, D. and Heideman, W. (1993) *Mol. Biol. Cell* **4**, 757–765
- Crowe, J. H., Crowe, L. M. and Chapman, D. (1984) *Science* **223**, 701–703
- Hottiger, T., de Virgilio, C., Hall, M. N., Boller, T. and Wiemken, A. (1994) *Eur. J. Biochem.* **219**, 187–193
- Singer, M. A. and Lindquist, S. (1998) *Mol. Cell.* **1**, 639–648
- Silljé, H. H. W., ter Schore, E. G., Rommens, A. J. M., Huls, P. G., Woldringh, C. L., Verkleij, A. J., Boonstra, J. and Verrips, C. T. (1997) *J. Bacteriol.* **179**, 6560–6565
- Silljé, H. H. W., Paalman, J. W. G., ter Schore, E. G., Olsthoorn, S. Q. B., Verkleij, A. J., Boonstra, J. and Verrips, C. T. (1999) *J. Bacteriol.* **181**, 396–400