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Identification and characterisation of two transcriptional repressor elements within the coding sequence of the *Saccharomyces cerevisiae HXK2* gene

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ABSTRACT

A well-defined set of isogenic yeast strains has been constructed whereby each strain contains a different *HXK2::lacZ* gene fusion integrated at the *URA3* locus. These *HXK2::lacZ* fusions differ in the amount of the *HXK2* gene (encoding hexokinase 2 isoenzyme) that is fused to the lacZ reporter gene. Comparison of the β-galactosidase activities of each strain during growth on glucose or ethanol revealed that some part of the coding region between +39 and +404 bp is involved in repressing gene expression in a carbon source dependent manner. A series of deletions of this *HXK2* coding region were constructed and fused upstream of a minimal *CYC1::lacZ* promoter. β-Galactosidase activities on glucose or ethanol growth yeast cells revealed that two different regulatory elements are present in this DNA region. Gel mobility shift analysis and *in vitro* DNase I footprinting have shown that proteins bind specifically to two downstream repressor sequences (DRS1 located from +140 to +163 and DRS2 located between +231 and +251) that influence the rate of *HXK2* transcription when ethanol is used as carbon source by *Saccharomyces cerevisiae*. We identified and partially purified a 18 kDa protein that binds specifically to synthetic double-stranded oligonucleotides containing the (A/C)(A/G)GAAAT box sequence. Our data suggest that p18 synthesis is under the control of genes involved in glucose repression (*MIG1* = *CAT4*) and glucose derepression (*SNF1* = *CAT1*).

INTRODUCTION

In *Saccharomyces cerevisiae*, glucose exerts at least four different effects: (i) glucose repression, (ii) glucose inactivation, (iii) induction and (iv) modifications of enzyme activity. The most important of these could be glucose repression, in which the synthesis of enzymes necessary for disaccharide or galactose utilisation and also those for growth on non-fermentable carbon sources is repressed during growth on glucose. With very few exceptions, glucose control is exerted at the level of transcription (1). Genetic analysis of *S.cerevisiae* has led to the identification of several genes necessary for glucose repression and for derepression of enzyme synthesis after depletion of glucose (2). A first set of genes is involved in glucose repression of disaccharide and galactose utilising enzymes and these genes act negatively on the expression of glucose-repressible genes. A second set of genes is involved in the derepression process when glucose is removed from the medium and these exert a positive effect. The large number of genes implicated and the existence of at least two different but interacting regulatory circuits shows that glucose repression is a very complex regulatory system which is far from being understood at present. In addition to the interaction of regulatory genes in glucose repression, the mechanism which triggers the repression reaction is of considerable interest. One of the first genes acting in the glucose repression cascade seems to be *HXK2* (3), a gene encoding hexokinase 2, one of the three enzymes that can phosphorylate glucose. Recently, it has been proposed that the phosphorylation activity of hexokinase 2 is correlated with glucose repression (4). However, if the glucokinase gene (*GLK1*) is overexpressed in a hexokinase 1/hexokinase 2 double-null mutant no effect on glucose repression is observed, even in strains with a 3-fold increase of phosphorylating activity (5). This indicates that glucose repression is not only associated with the phosphorylation activity of hexokinase 2 but that the presence of the hexokinase 2 protein is also necessary to give the signal for glucose repression, perhaps by acting as the initial sensor for glucose levels.

Due to the interest in discovering the mechanism that controls the expression of the hexokinase 2 gene, we have recently analysed the *HXK2* promoter and identified a strong *cis*-acting regulatory element within the coding region of this gene (6). This element modulates the rate of transcription when ethanol is the carbon source present in the culture medium.

Transcriptional control of the expression of most genes requires *cis*-acting sequences which lie upstream of the TATA box. The mechanism by which these sequences and the cognate transcription factors influence the rate of transcription are beginning to be discovered (7,8). Less understood are the regulatory sequences of a number of bacterial, viral and mammalian genes that are located

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within transcribed sequences, that is, within either the 5′ untranslated leader sequence, introns, or translated sequences.

Though well documented in higher eukaryotes, intragenic transcriptional regulation of *S.cerevisiae* remains largely speculative. There are only a few well documented examples from yeast, some of them are the *LPD1* gene and the retrotransposons Ty1 and Ty2 which have been shown to contain multiple downstream elements that either activate (DASs) or repress (DRSs) transcription (9–12). Moreover, the expression of heterologous sequences by using upstream promoters from yeast genes frequently requires a minimal length of the coding sequence (13–15). These results could suggest that downstream elements are necessary for transcription to take place in a physiological way.

In this study, we report the identification and localisation of two downstream repressing sequences (DRS1 and DRS2) within the coding region of the *HXK2* gene and show that they interact specifically with DNA-binding proteins. In addition, we have identified and partially purified a 18 kDa protein that binds specifically to both DRS1 and DRS2. Gel retardation assays using p18 partially purified from yeast regulatory mutants indicate that the regulation of 18 kDa protein synthesis is under the control of a complex interplay of both positively and negatively acting genes.

MATERIALS AND METHODS

Strains

S.cerevisiae strain DBY1315 (*MATα ura3-52 leu2-3,2-112 lys2-801 gal2*) donated by D. Botstein, was used as a recipient in transformation experiments. Bacterial transformation and large scale propagation of plasmid DNA were performed in *Escherichia coli* MC1061.

Media, growth conditions and enzymatic analysis

Rich media were based on 1% yeast extract and 2% peptone (YEP); 2% glucose (YEPD) or 3% 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 media was utilized to select for transformants when plasmids carrying *URA3* were used. β-galactosidase activity was assayed according to (16).

General DNA techniques

Restriction enzymes and T4 DNA ligase were obtained from Boehringer, sequenase V2.0 from USB. Radioactively labelled isotopes were obtained from Amersham International. The dideoxyribonucleotide chain termination procedure was used for DNA sequencing analysis (17). All other DNA manipulations were as previously described (18).

Subcloning deletions with different lengths of the *HXK2* coding sequence into an heterologous *CYC1* promoter

pNI9, a derivative of plasmid pNG22, a yeast–*E.coli* shuttle vector containing the 5′ regulatory region of the *CYC1* gene and the translation start site fused in frame to *lacZ* (19), lacking *TRP1/ARS* sequences, was used to study the function of a series of 5′ nested deletions located between +39 and +404 bp of the coding region of the *HXK2* gene. Several plasmids were constructed by subcloning fragments of different lengths (blunt-end by filling) of the *HXK2* gene coding region in the *Sal*I site (blunt-end by filling) of the polycloning region of plasmid pNI9. The resulting plasmids were integrated into the *URA3* locus by digestion with *Stu*I prior to transformation of the yeast strain and single copy integration was confirmed by Southern analysis of genomic DNA digested with *Bgl*II and probing with a 1.1 kb *Hin*dIII fragment containing the *URA3* gene. As controls, the original vector (pNI9) containing the *CYC1* activating sequences and a plasmid where the latter had been deleted (pNI17) were also tested.

DNA probes and competitors

Probe DNAs were *Eco*RI–*Hin*dIII fragments of plasmids pUK268, pUK211, pUK184 and pUK144 (20). Oligonucleotides corresponding to both strands of the DRS1 or DRS2 were synthesised with an added TCGA nucleotide overhang at the 5′-terminal end of each oligonucleotide. The complementary strands were annealed and either end labelled with [α-^{32}P]dCTP by the Klenow fragment of DNA polymerase I at the two ends or used as unlabeled competitors in protein binding experiments. The sequences of these oligonucleotides were as follows:

DRS1 sense, 5′-TCGACTTCATTTCCGAATTGGAAAAGGG-3′;
DRS1 antisense,5′-TCGACCCTTTTCCAATTCGGAAATGAAG-3′;
DRS2 sense, 5′-TCGAGAATCCGGTGATTTCTTGGC-3′;
DRS2 antisense, 5′-TCGAGCCAAGAAATCACCGGATTC-3′.

For use in Southwestern blot analysis, the DRS1 (OL28) and DRS2 (OL24) probes were end labelled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP. For biochemical isolation of the DRS-binding protein, biotin was incorporated into the ends by using the Klenow fragment of DNA polymerase I in the presence of biotin-14-dATP (Gibco-BRL).

Preparation of protein extracts

Protein extracts were prepared as follows: 10–20 ml rich medium (YEPD or YEPE) was inoculated with yeast cells and allowed to grow at 28°C until the optical density at 600 nm reached 2.0. Cells were collected, washed twice with 1 ml 1 M sorbitol and suspended in 400 µl 20 mM HEPES (pH 7.9) buffer containing 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.42 M NaCl, 1.5 mM $MgCl_2$ and 25% glycerol. The cells were broken by hard vortex (6× 20 s) in the presence of glass beads. After centrifugation at 14 000 r.p.m. for 15 min at 4°C, the supernatant was transferred to a clean tube. This fraction was considered the protein extract.

Gel retardation assay

The typical binding reactions contained 10 mM HEPES (pH 7.5), 1 mM dithiothreitol, 1–5 µg poly(dI-dC) and 0.5 ng end-labelled probe DNA in a 25 µl volume. Sixty µg (6 µl) of protein extract was added. The amount of unlabeled competitor DNA added is indicated in the figure legends. After 30 min of incubation at room temperature the binding reaction mixtures were loaded onto a 4% non-denaturing polyacrylamide gel. Electrophoresis was allowed to proceed at 10 V/cm of gel for 45 min to 1 h in 0.5× TBE buffer. Gels were dried and autoradiographed at –70°C with an intensifying screen.

DNase I footprint analysis

The binding reaction was the same as in the electrophoretic retardation assays, except that the final volume was 50 µl and the probes were labelled at one end only. After 30 min incubation, 5 µl of 10× DNase I buffer (1× DNase I buffer was 10 mM $MgCl_2$ plus 5 mM $CaCl_2$) and 1–5 U DNase I were added. After 1 min at room temperature, an equal volume of stop buffer (200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulphate (SDS), 2 µg/ml yeast tRNA) was added. The mixture was phenol extracted, ethanol precipitated and electrophoresed on an 8% denaturing polyacrylamide gel.

Southwestern blot analysis

The protocol is based on that of Bassel-Duby *et al.* (21) with the following modifications. Protein extract (~100 µg protein) was mixed with electrophoresis sample buffer and boiled for 4 min. Proteins were resolved by SDS–PAGE. Following electrophoresis at 15 V/cm for 2 h in 12% gels, the proteins were transferred to nitrocellulose paper overnight at 4°C, using the mini Trans-Blot Cell (BioRad) at 120 mA in transfer buffer. The nitrocellulose paper was air dried at room temperature, immersed in binding buffer (25 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) supplemented with 6 M guanidine hydrochloride and gently rocked for 10 min at 4°C. The nitrocellulose paper was transferred to binding buffer containing 3 M guanidine hydrochloride and gently rocked for 10 min at 4°C. This procedure was repeated eight times, with each subsequent wash containing a concentration of guanidine hydrochloride 2-fold less than that in the previous wash. The final wash step consisted of binding buffer without guanidine hydrochloride. The nitrocellulose was incubated in BG buffer (binding buffer containing 5% gelatin and 5 µg/ml sonicated salmon sperm DNA) for 1 h at room temperature. Next, the nitrocellulose filter was immersed in BG buffer containing 0.25% gelatin and incubated for 30 min. ^{32}P-labelled DNA probe was added to 0.25% gelatin BG buffer and incubated with nitrocellulose filter overnight at room temperature. The filter was then washed four times with binding buffer for 7 min at room temperature. The filter was air dried and autoradiographed at –70°C with an intensifying screen.

Isolation of DRS1 and DRS2 binding factor

Chemically synthesised oligonucleotides containing the DRS1 and DRS2 sites were used to produce double-stranded DNA fragments OL28 and OL24 by annealing. The DNA fragments were ligated into a 400–800 bp polymer. The concatamers were phenol extracted, precipitated, resuspended in TE buffer and biotinylated. Biotinylated DNA (250 µg) was incubated with protein extract (300 µg) for 30 min at room temperature. One-half millilitre of streptavidin–agarose (Sigma) was equilibrated with buffer 1 [10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.2% Nonidet P-40, 0.1 mM PMSF, 30 µg/ml poly(dI-dC)] and added to the mixture of DNA and protein extract. The mixture was incubated overnight at room temperature and then poured into a 1 ml column. The column was washed five times with 1 ml buffer 2 (10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM PMSF, 10% glycerol). The DRS1 and DRS2 binding proteins were eluted stepwise: first by adding 0.5 ml buffer 3 (10 mM Tris–HCl, pH 6.8, 150 mM KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol, 0.2% Nonidet P-40, 0.1 mM PMSF, 10% glycerol) and collecting 50 µl fractions. Second by adding 0.5 ml buffer 4 (buffer 3 containing 250 mM KCl) and collecting 50 µl fractions and then by adding 0.5 ml buffer 5 (buffer 3 containing 500 mM KCl) and collecting 50 µl fractions. The eluted proteins were loaded onto an SDS–PAGE. The proteins were visualised by silver staining.

RESULTS

A *HXK2* coding sequence can repress gene expression in a carbon source dependent manner

The *HXK2* coding sequence (between +39 and +404) can repress gene expression when ethanol is the carbon source present in the culture medium. Deletion of this region from the wild-type *HXK2::lacZ* fusion resulted in a 90-fold increase of β-galactosidase specific activity when cell-free extracts were obtained from ethanol-grown cells, maintaining an identical specific activity in cell-free extracts from glucose-grown cells (data not shown). Because there exists a correlation between variations in β-galactosidase specific activity and the level of transcriptional expression of *lacZ* gene, it was proposed that the *HXK2* gene contains a downstream repressor sequence (DRS) between +39 and +404 bp (6).

To more accurately determine the location of the elements responsible for downstream repression, a series of 5′ nested deletions of the *HXK2* coding sequence were constructed. These deletions were generated from the +39 bp end of the fragment and extended 3′ toward the downstream region of the *HXK2* (to +263 relative to the start of the coding sequence). All deletion end points were verified by sequence analysis. To test the promoter activity of the *HXK2* downstream region in an upstream context, the downstream deletions were fused upstream of a minimal *CYC1::lacZ* promoter. Each deletion construct was integrated at *URA3*, and all transformants were screened by Southern analysis for the presence of a single copy of the construct.

The level of β-galactosidase expressed by each of the deletion constructs was then determined in response to growth of the cells on glucose or ethanol as carbon sources (Fig. 1). On glucose medium, none of the fragments affect gene expression producing a basal level of β-galactosidase; on ethanol medium, the fragments of 365 and 268 bp contained in plasmids pNI365 and pNI268 respectively repressed gene expression ~99-fold below the induced level. This may be taken as evidence for downstream repressing sequence being located between +136 and +404 bp. When sequences prior to +193 and +220 bp are removed (pNI211 and pNI184) the expression on ethanol medium for both is increased 50-fold, suggesting that a repressing element (DRS1) located between +136 and +193 has been removed. An ~2-fold increase in β-galactosidase activity resulted after deletion of the sequence beyond +263 bp which removed a second regulatory element (DRS2) located between +220 and +263 bp. Thus, the expression from the *HXK2* DRS is regulated in a carbon-source dependent manner (6) and is position independent, functioning both downstream and upstream of the RNA initiation site in promoters.

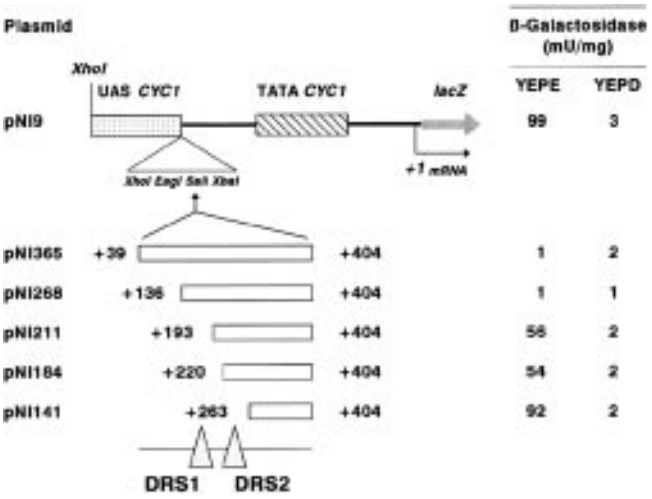

Figure 1. Effect of several deletions in the *HXK2* coding sequence (+39/+404) on the expression of β-galactosidase from transformants containing a *CYC1::lacZ* fusion. A series of 5′ deletions located between +39 and +404 bp of the coding region of the *HXK2* gene were cloned into the polylinker of expression vector pNI9. The resulting plasmids were transformed into yeast strain DBY1315, selecting for uracil prototrophy. Only strains with single copy integrations were used to obtain the data presented. Transformants were assayed for β-galactosidase activity as described in Materials and Methods. YEPD: cells grown in medium containing 1% yeast extract, 2% peptone, 2% glucose; YEPE: cells grown in medium containing 1% yeast extract, 2% peptone, 3% ethanol.

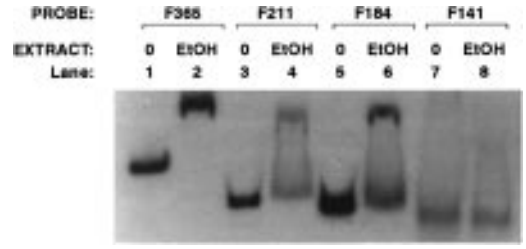

Figure 2. DNA band shift analysis of the *HXK2* controlling region. Four DNA fragments with 268, 211, 184 and 141 bp were isolated from deletion constructs described in Figure 1. The DNA fragments were ^{32}P-labelled and used in band shift experiments. The fragments were incubated with protein extract from ethanol-grown cells. The odd numbered lanes serve as controls; the respective fragments were incubated in binding buffer without the addition of protein extract. In the even numbered lanes the respective fragments were incubated in binding buffer with the addition of protein extract (60 µg). Lane 2, 365 bp fragment; lane 4, 211 bp fragment; lane 6, 184 bp fragment; lane 8, 141 bp fragment.

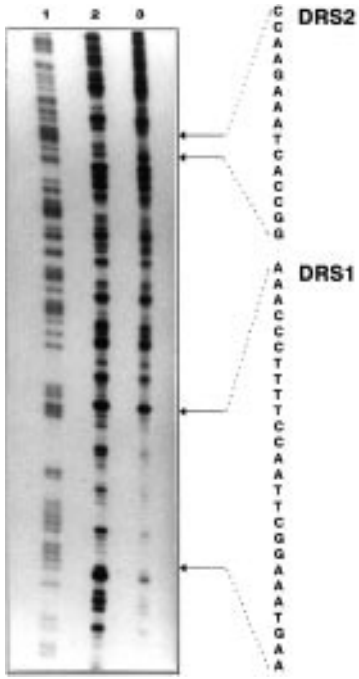

Figure 3. DNase I protection experiment of DRS probe with a protein extract. Lane 1, AG-specific sequencing reaction; lane 2, DNase I digestions in the absence of protein extract; lane 3, DNase I digestions in the presence of 60 µg protein. Each reaction contained 1 ng probe. The protein extract was used at 10 µg/µl. The protected sequences are shown.

The DRS1 and DRS2 elements of *HXK2* gene forms complexes with yeast proteins

To characterise the binding motifs and associated proteins, 'filled-in' end-labelled deletion fragments encompassing the downstream region were used in mobility shift assays with protein extracts from YEPE-grown cells (Fig. 2). These assays indicated that the shifted complex (Fig. 2, lane 2) associated with the 365 bp fragment was similar to the ones obtained with the 211 and 184 bp fragments (Fig. 2, lanes 4 and 6 respectively). Furthermore, specific binding of proteins to the 365 bp fragment was demonstrated by competition assays with a 365 bp non-labelled fragment (data not shown). The 211 and 184 bp fragments were less efficient in forming DNA–protein complexes. The 141 bp deletion fragment does not form any DNA–protein complexes (Fig. 2, lane 8).

Determination of the binding sites of proteins to DRS1 and DRS2

To locate *cis*-acting sequences, in coding region of *HXK2* gene which are recognised by transcription factors synthesised under ethanol growing conditions, we employed DNase I footprint analysis. As shown in Figure 3 (lane 3), two regions from +140 to +163 (DRS1) and from +231 to +251 (DRS2), were protected against DNase I digestion.

To confirm this result two kinds of double stranded oligonucleotides were synthesised. One corresponds to the 24 bp region (5′-CTTCATTTCCGAATTGGAAAAGGG-3′) identified by the DNase I footprinting experiment as DRS1 and the other corresponds to the 20 bp region (5′-GAATCCGGTGATTTCTTGGC-3′) identified by the DNase I footprinting experiment as DRS2. Both double-stranded oligonucleotides have added *Xho*I compatible ends which were used for subsequent subcloning at the *Sal*I site of the promoter test plasmid pNI9 (Fig. 4). After oligonucleotide insertion, a 56 and 82% decrease of the β-galactosidase specific activity in YEPE-grown cells can be observed when OL28 (DRS1) and OL24 (DRS2) oligonucleotides were respectively used.

The OL28 and OL24 double-stranded oligonucleotides used for the insertion study described were also examined in gel mobility shift assays with protein extracts from YEPE-grown

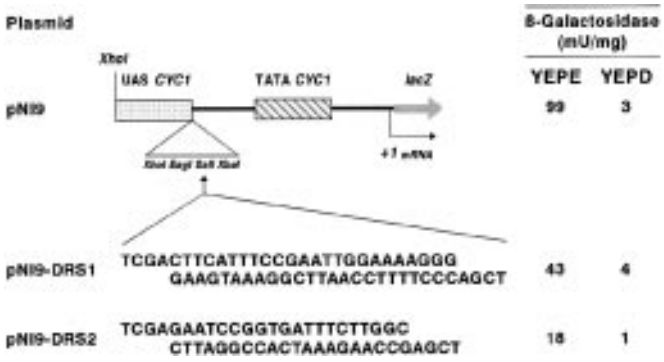

Figure 4. Transcriptional repression of the *lacZ* gene under control of the *CYC1* promoter by DRS1 and DRS2 regulatory elements. The synthetic oligonucleotides indicated were cloned in the plasmid pNI9. The resulting plasmids were then used to transform yeast strain DBY1315. The transformants were grown in media with glucose (YEPD) or ethanol (YEPE) as carbon sources and assayed for β-galactosidase activity as described in Materials and Methods.

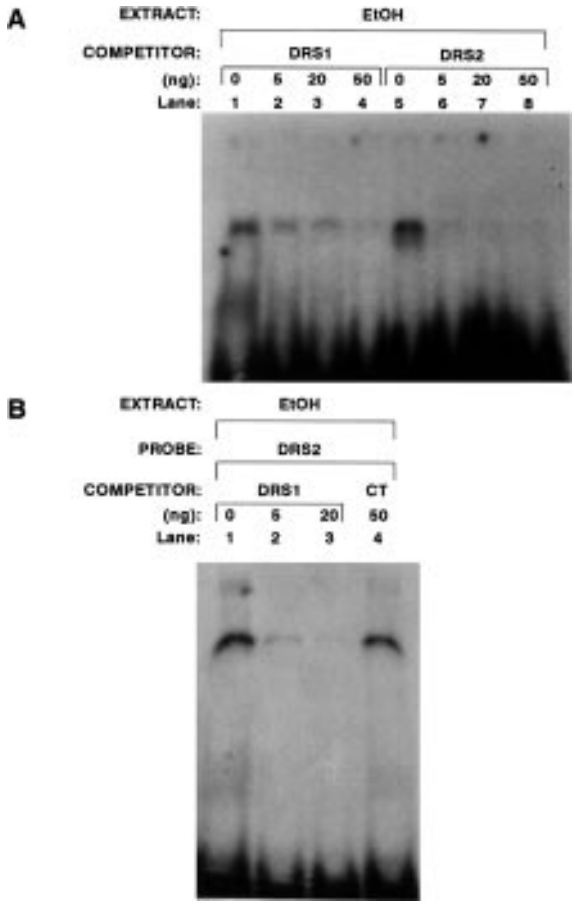

Figure 5. Competition of band shift patterns with oligonucleotides which contain sequence from the DRS1 and DRS2 controlling regions of *HXK2* gene. (**A**) Competition experiments were carried out with radiolabelled DRS1 (lanes 1–4) and DRS2 (lanes 5–8) elements and protein extract from ethanol growing yeast cells. Each reaction had 0.5 ng ^{32}P-DRS DNA probe and 6 µl (2 mg/ml) protein extract (lanes 1–8). The specific competitor for binding, unlabeled DRS DNA, was present at 0 (lanes 1 and 4), 5 (lanes 2 and 6), 20 (lanes 3 and 7) and 50 ng (lanes 4 and 8). (**B**) Cross competition assays were carried out with radiolabelled DRS2 (lane 1). The competitor for binding was unlabeled DRS1 at 5 (lane 2) and 20 ng (lane 3), or calf thymus DNA at 50 ng (lane 4).

cells (Fig. 5A). These assays indicated that the shifted complex (Fig. 5A, lane 1) associated with the OL28 oligonucleotide was similar to the one obtained with the OL24 oligonucleotide (Fig. 5A, lane 5). Furthermore, specific binding of proteins to both double-stranded oligonucleotides was demonstrated by competition assays with non-labelled oligonucleotides. Cross competition assays (Fig. 5B) show that the complex shifted associated with the OL28 and OL24 oligonucleotides can be removed respectively by OL24 and OL28 no-labelled oligonucleotides, suggesting that the same proteins and the same DNA sequence are involved in both complexes.

Analysis of OL28 and OL24 double-stranded oligonucleotides for the presence of reported eukaryotic transcriptional control sequences, showed that both DRS1 and DRS2 contain a heptamer motif (A/C)(A/G)GAAAT thought to be essential for derepression and glucose regulation of the *SUC2* gene (22).

Characterisation and partial purification of DRS1 and DRS2 *trans*-acting binding factors

To identify proteins that bind to the (A/C)(A/G)GAAAT box sequence, we used a protein-blotting technique (Southwestern blotting). Protein extracts from YEPE exponentially growing cells were subjected to SDS–PAGE, electroblotted onto nitrocellulose paper, renatured, and probed with ^{32}P-labelled OL28 or OL24 oligonucleotides. Both of these probes identified 27 and 18 kDa proteins in renatured protein blots (Fig. 6A, lane 1) that we termed p27 and p18 respectively.

Partial purification of p18 was achieved by DNA affinity chromatography. Protein extracts from YEPE exponentially growing cells were incubated with biotinylated OL28 or OL24 oligonucleotides. Protein–DNA complexes were bound to streptavidin–agarose beads, and proteins were eluted stepwise with buffer containing increasing concentrations of KCl. After separation by SDS–PAGE, a limited number of protein bands was observed by silver staining in fractions eluted with 0.5 M KCl (Fig. 6B, lane 6). The protein band at 18 kDa corresponds with the result of oligonucleotide binding to renatured protein blots as was demonstrated by Southwestern blotting using the partially purified proteins of 0.5 M KCl fraction and probing with ^{32}P-labelled OL28 or OL24 oligonucleotides (Fig. 6A, lane 2).

The p18 protein was transferred to an immobilon membrane and subjected to N-terminal Edman degradation in an automated protein sequenator. After 10 cycles no sequence was obtained. This suggests that the protein contains a blocked N-terminus. Studies are in progress to determine the nature of the N-terminal modification.

The partially purified proteins of the 0.5 M KCl fraction were also examined in gel mobility shift assays with ^{32}P-labelled OL28 and OL24 double-stranded oligonucleotides (Fig. 7). These assays indicated that the CI and CII complexes (Fig. 7, lane 2) associated with the OL28 oligonucleotide are similar to those obtained with the OL24 oligonucleotide. Furthermore, specific binding of proteins to the OL28 and OL24 double-stranded oligonucleotides was demonstrated by competition assays with

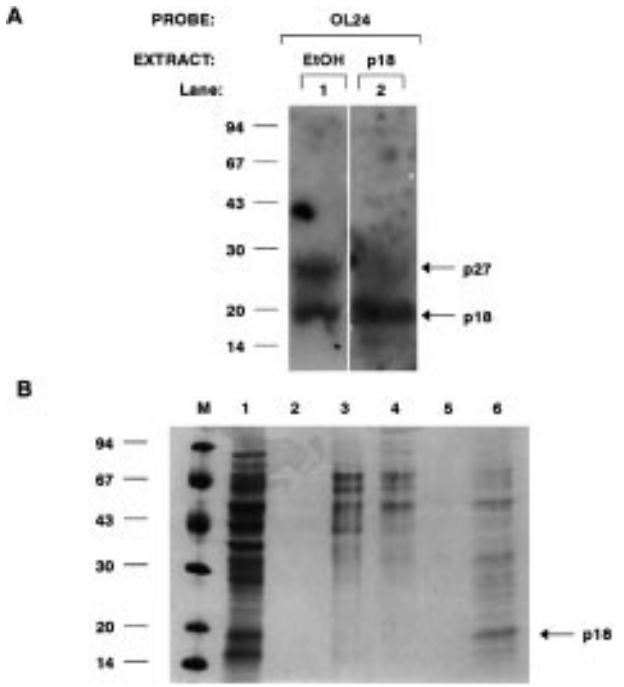

Figure 6. Binding of ^{32}P-labelled oligonucleotides to renatured protein blots (A) and partial purification of a binding factor (B). (**A**) Protein extracts from YEPE exponentially growing cells (lane 1) or partially purified proteins (lane 2) from the 0.5 M KCl fraction of B were subjected to SDS–PAGE (12% polyacrylamide gel) and electrophoretically transferred to nitrocellulose. Proteins bound to nitrocellulose were renatured as described in Materials and Methods. The blots were probed with ^{32}P-labelled OL24. Similar results were obtained with the OL28 probe (data not shown). (**B**) Silver stain of proteins separated by SDS–PAGE (12% polyacrylamide gel) loaded with 10 µl of affinity chromatography purified fractions. Proteins were eluted from streptavidin–agarose column with 10 mM KCl buffer (lane 1) until no proteins were detected (lane 2), 150 mM KCl buffer (lane 3), 250 mM KCl buffer (lane 4) until no proteins were detected (lane 5) and 500 mM KCl buffer (lane 6). The arrow points to the 18 kDa protein corresponding in size to the protein binding to DRS1 and DRS2 in renatured protein blots.

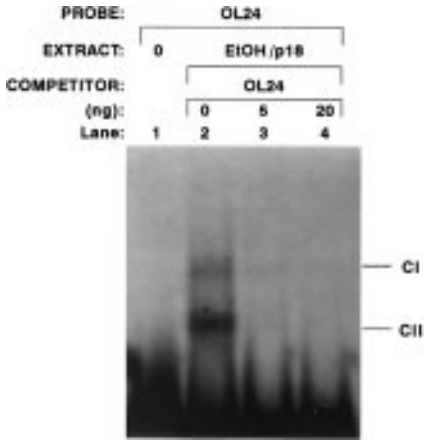

Figure 7. Competition of band shift patterns with the DRS1 and DRS2 controlling regions of *HXK2* gene and purified p18 protein. Gel mobility shift assay with ^{32}P-labelled OL24 (lanes 1–4). Binding reactions were performed with p18 protein eluted at 0.5 M KCl from the DNA-affinity chromatography column. The specific competitor for binding, unlabelled OL24, was present at 10 (lane 3) and 50 ng (lane 4). Lane 1, radiolabelled OL24 incubated in binding buffer without protein extract. Similar results were obtained with the OL28 probe (data not shown).

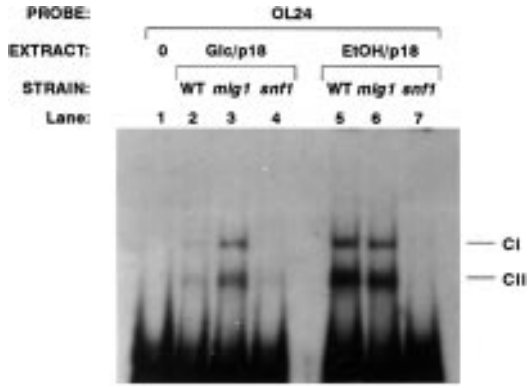

Figure 8. Influence of regulatory mutations on p18 protein binding to DRS1 and DRS2 elements. Gel mobility shift assay with ^{32}P-labelled OL24. Binding reactions were performed with p18 protein eluted at 0.5 M KCl from the DNA-affinity chromatography columns. Lane 1, no protein added; lanes 2–4, p18 purified from protein extracts obtained from repressed wild-type (lane 2), *mig1* (lane 3) and *snf1* (lane 4) strains; lanes 5–7, p18 purified from protein extracts obtained from derepressed wild-type (lane 5), *mig1* (lane 6) and *snf1* (lane 7) strains. Similar results were obtained with the OL28 probe (data not shown).

the corresponding non-labelled oligonucleotides (Fig. 7, lanes 3 and 4).

Interaction of *mig1* and *snf1* mutations in p18 synthesis

As shown in Figure 8 two different protein–DNA complexes (CI and CII) were observed with p18 partially purified protein prepared from derepressed wild-type cells using synthetic double-stranded oligonucleotides OL28 or OL24, representing DRS1 and DRS2 respectively. Interestingly, CI and CII exclusively occurred with p18 preparations from derepressed cells (Fig. 8, lane 5), the complexes are absent when p18 partially purified protein from glucose-growth cells is used (Fig. 8, lane 2). We tested the effects of *mig1* and *snf1* deletion mutations on the formation of these complexes by using p18 partially purified protein from *mig1* and *snf1* yeast strains grown either with glucose or ethanol as carbon sources. As can be seen in Figure 8 (lanes 3 and 6) both CI and CII complexes are present when we use p18 protein purified from repressed as well as derepressed *mig1* mutants. However, complexes are absent when we use p18 protein purified from repressed as well as derepressed *snf1* mutants.

DISCUSSION

We have demonstrated that two sequences (DRS1 and DRS2) in the coding region of the *HXK2* gene block the expression of *HXK2::lacZ* fusions when the yeasts are grown with ethanol as the carbon source, and that the regulation of the *HXK2* gene in response to carbon source is mainly mediated by these repressors. This carbon-source dependent regulation may be a direct effect of the downstream repressors, but the differences in expression seen on glucose and ethanol may arise from additive or synergistic interactions between the repressors in the coding region and other

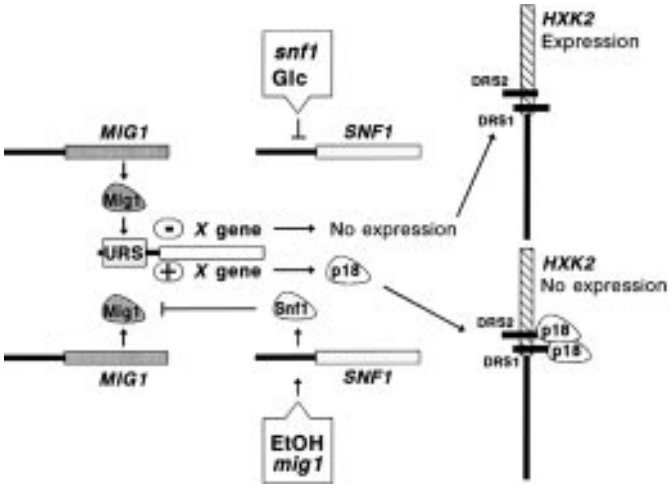

Figure 9. Model for the regulatory pathway controlling the *HXK2* expression.

upstream elements (6). The yeast *PGK1* and *PYK1* genes appear to have transcriptional activators within their coding regions (9,23,24) and the *LPD1* gene appears to have both activation and repression sites within the coding region (10,11). However, no common sequence motifs which mediate the transcriptional control have been identified.

The sequence of DRS1 and DRS2 of the *HXK2* gene includes a DNA motif which shows a perfect match to the yeast UAS element of *SUC2* gene (21). This element is necessary for derepression of *SUC2* gene and for repression of *HXK2* gene when ethanol is used as carbon source by *S.cerevisiae*. DNA–protein gel retardation experiments show protein binding to both DRS1 and DRS2 elements, and footprint analyses demonstrate protein protection of this heptamer motif.

Using renatured protein blots 27 and 18 kDa proteins that bind to both DRS1 and DRS2 elements were identified. However, we were successful only in purifying the 18 kDa protein by DNA-affinity chromatography. Preparations of p18 obtained from affinity chromatography columns containing DRS1 or DRS2 elements give the same complexes in gel mobility shift assays and these complexes were shown to be specific. These results suggest that the heptameric motif common to both DRS elements is the target for the p18 factor. Moreover, p18 synthesis seems to be carbon source dependent because different complexes were obtained in gel mobility shift assays when p18 preparations from protein extracts from glucose or ethanol growth cells were used. Although we cannot ascertain whether *mig1* and *snf1* mutations affect p18 synthesis or play a role on its capacity to bind DNA. Our results suggest that the transcription factors Mig1 and Snf1 are involved in the regulation of p18 synthesis as deduced from SDS–PAGE analysis of fractions eluted with 0.5 M KCl from the DNA affinity chromatography columns. From these results a model for glucose repression and derepression can be derived.

As shown in Figure 9, the p18 protein is important for repression of *HXK2* gene expression. If we assume that the p18 protein is encoded by an unknown *X* gene, we can say that transcription of *X* gene is subject to glucose repression with Mig1p as its repressor. For derepression, the binding of Mig1p to *X* gene is prevented and consequently the gene is transcribed. This hypothesis is confirmed by *mig1* mutants which do not repress p18 and by *snf1* mutants which do not derepress p18. From the present data we conclude that under conditions of glucose repression, Mig1p binds as a repressor to *X* gene thus preventing its transcription. After glucose consumption, the Snf1/Snf4 protein kinase is activated and converts Mig1p to a non-binding conformation, possibly by its phosphorylation. Consequently, *X* gene is transcribed and the expression of *HXK2* gene is prevented. The model presented here is conclusive for the interpretation of the results obtained. However, we assume that other proteins in addition to p18 are involved in the repression conformation of the DRS's of *HXK2* gene.

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REFERENCES

1 Trumbly,R.J. (1992) *Mol. Microbiol.*, **6**, 15–21.
2 Gancedo,J.M. (1992) *Eur. J. Biochem.*, **206**, 297–313.
3 Entian,K.D., Kopetzki,E., Fröhlich,K.U.and Mecke,D. (1984) *Mol. Gen. Genet.*, **198**, 50–54.
4 Ma,H., Bloom,L.M., Walsh,C.T. and Botstein,D. (1989) *Mol. Cell. Biol.*, **9**, 5643–5649.
5 Rose,M., Albig,W. and Entian,K.D. (1991) *Eur. J. Biochem.*, **199**, 511–518.
6 Martínez-Campa,C., Herrero,P., Ramírez,M. and Moreno,F. (1996) *FEMS Lett.* (in press).
7 Cowell,I.G. (1994) *Trends Biochem. Sci.*, **19**, 38–42.
8 Johnson,P.F. and McKnight,S.L. (1989) *Annu. Rev. Biochem.*, **58**, 799–839.
9 Fulton,A.M., Rathjen,P.D., Kingsman,S.M. and Kingsman,A.J. (1988) *Nucleic Acids Res.*, **16**, 5439–5458.
10 Farabaugh,P., Liao,X.B., Belcourt,M., Zhao,H., Kapakos,J. and Clare,J. (1989) *Mol. Cell. Biol.*, **9**, 4824–4834.
11 Farabaugh,P., Vimaladithan,A., Turkel,S., Johnson,R. and Zhao,H. (1993) *Mol. Cell. Biol.*, **13**, 2081–2090.
12 Sinclair,D.A., Kornfeld,G.D. and Dawes,I.W. (1994) *Mol. Cell Biol.*, **14**, 214–225.
13 Heinisch,J., Vogelsang,K. and Hollenberg,C.P. (1991) *FEBS Lett.*, **289**, 77–82.
14 Mercado,J.J. and Gancedo,J.M. (1992) *FEBS Lett.*, **311**, 110–114.
15 Mellor,J., Dobson,M.J., Kingsman,A.J. and Kingsman,S.M. (1987) *Nucleic Acids Res.*, **15**, 6243–6259.
16 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor University Press, Cold Spring Harbor, NY.
17 Sanger,F., Nicklen,S. and Coulson,S.A. (1977) *Proc. Natl. Acad. Sci. USA.*, **74**, 5463–5467.
18 Fernández,E., Moreno,F. and Rodicio,R. (1992) *Eur. J. Biochem.*, **204**, 983–990.
19 Luche,R.M., Sumrada,R. and Cooper,T. (1990) *Mol. Cell. Biol.*, **10**, 3884–3895.
20 Viera,J. and Messing,J. (1991) *Gene*, **100**, 189–194.
21 Bassel-Duby,R., Hernández,M.D., González,M.A., Krueger,J.K. and Williams,R.S. (1992) *Mol. Cell Biol.* **12**, 5024–5032.
22 Sarokin,L. and Carlson,M. (1986) *Mol. Cell. Biol.* **6**, 2324–2336.
23 Kingsman,S.M., Kingsman,A.J., Dobson,M.J. and Mellor,J. (1985) *Biotech. Genet. Eng. Rev.*, **3**, 377–416.
24 Purvis,I.J., Loughlin,L., Bettany,A.J.E. and Brown,A.J.P. (1987) *Nucleic Acids Res.*, **15**, 7963–7973.