# The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*

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**Abstract The *HXK2* gene plays an important role in glucose repression in the yeast *Saccharomyces cerevisiae*. Recently we have described that the *HXK2* gene product, isoenzyme 2 of hexokinase, is located both in the nucleus and in the cytoplasm of *S. cerevisiae* cells. In this work we used deletion analysis to identify the essential part of the protein-mediating nuclear localisation. Determinations of fructose-kinase activity and immunoblot analysis using anti-Hxk2 antibodies in isolated nuclei, together with observations of the fluorescence distribution of Hxk2-GFP fusion protein in cells transformed with an *HXK2::gfp* mutant gene, indicated that the decapeptide KKPQARKGSM, located between amino acid residues 7 and 16 of hexokinase 2, is important for nuclear localisation of the protein. Further experimental evidence, measuring invertase activity in wild-type and mutant cells expressing a truncated version of the Hxk2 protein unable to enter the nucleus, shows that a nuclear localisation of Hxk2 is necessary for glucose repression signalling of the *SUC2* gene. Furthermore, we demonstrate using gel mobility shift analysis that Hxk2 participates in DNA-protein complexes with *cis*-acting regulatory elements of the *SUC2* gene promoter.**

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*Key words:* HXK2; SUC2; Glucose repression; *Saccharomyces cerevisiae*

## 1. Introduction

Genetic analysis of *Saccharomyces cerevisiae* has led to the identification of several genes necessary for glucose repression and for derepression of enzyme synthesis after depletion of glucose. Several gene products (Hxk2, Grr1, Reg1, Glc7, Mig1, Ssn6, Tup1) act negatively in glucose repression of disaccharide utilising enzymes (for reviews see [1–3]). The *SUC2* gene of *S. cerevisiae* encodes the secreted enzyme invertase which hydrolyses sucrose and raffinose. Like other genes required for utilisation of alternative carbon sources, expression of the *SUC2* gene is repressed by glucose. A central component and also one of the first gene products acting in the *SUC2* glucose repression cascade seems to be *HXK2* [4], but it is not known how glucose modulates Hxk2 function through these proteins. It has been proposed that the sugar-kinase activity of hexokinase 2 is correlated with glucose repression [5,6]. However, if the glucokinase gene (*GLK1*) is overexpressed in a hexokinase 1/hexokinase 2 double-null mutant genetic background no effect on glucose repression is observed, even in strains with a threefold increase of sugar-phosphorylating activity [6]. This indicates that glucose repression is not only associated with the sugar-kinase activity of hexokinase 2 but that the presence of the Hxk2 protein is also necessary to give the signal for glucose repression. Recently, it has been described that an *HXK2* mutation encoding a serine to alanine change at position 15 produces a Hxk2 protein unable to undergo phosphorylation [7] and that the transformed cells with the *HXK2*($S^{15}A$) mutant gene could not provide glucose repression of invertase, suggesting that the phosphorylation of Hxk2 is essential in vivo for glucose signal transduction [8].

On the other hand, it has also been described that the Hxk2 is located in both the nucleus and the cytoplasm of *S. cerevisiae* cells [9] and this observation opens new possibilities toward explaining the role of Hxk2 in glucose repression signalling.

Here we report that the nuclear localisation of Hxk2 protein is mediated by an internal decapeptide sequence identified as a nuclear localisation sequence (NLS) that is necessary to direct the protein to the nucleus. We also show that a nuclear localisation of the Hxk2 protein is necessary for glucose repression signalling of the *SUC2* gene. Furthermore, we show that the nuclear Hxk2 is involved in the formation of specific DNA-protein complexes during glucose-induced repression of the *SUC2* gene. These results suggest that the Hxk2 protein could participate in the transduction of the glucose repression signal by interacting with transcriptional factors related to this regulatory mechanism.

## 2. Materials and methods

### 2.1. Strains and plasmids

*S. cerevisiae* strains DBY1315 (*MATα ura3-52 leu2-3,2-112 lys2-801 gal2*) and DBY2052 (*MATα hxk1::LEU2 hxk2-202 ura3-52 leu2-3,2-112 lys2-801 gal2*) were donated by D. Botstein, and were used as recipients in transformation experiments. Bacterial transformation and large-scale preparation of plasmid DNA were performed in *Escherichia coli* MC1061.

Plasmids YEp356 [10] and pRS306 [11] are yeast-*E. coli* shuttle vectors.

### 2.2. Media, growth conditions and enzymatic analysis

Yeasts were grown on 1% yeast extract and 2% peptone supplemented with 4% glucose (YEPD). The cells were grown in this medium until the optical density at 600 nm reached 1.0. To select for transformants, synthetic medium with yeast nitrogen base, 2% glucose and the adequate supplements was used.

Invertase and hexokinase were assayed as described by Moreno et al. [12]. The protein concentrations were determined according to [13], using bovine serum albumin as the standard. Specific activities are expressed as nmol substrate consumed/min/mg protein.

### 2.3. Preparation of crude protein extracts

Yeast protein extracts were prepared as follows: yeast was grown

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on 10–20 ml of rich medium (YEPD) at 28°C until an optical density at 600 nm of 1.0. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl 50 mM Tris-HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.42 M NaCl and 1.5 mM $MgCl_2$. The cells were broken by vortexing (6×20 s) in the presence of glass beads (0.5 g), and 400 μl of the same buffer was added to the suspension. After centrifugation at 19 000×*g* (14 000 rpm) for 15 min at 4°C, the supernatant was used as crude protein extract.

### 2.4. General DNA techniques

Restriction enzymes and T4 DNA ligase were from Boehringer, Sequenase V2.0 from USB. Radioactively labelled isotopes were from Amersham International. The dideoxyribonucleotide chain termination procedure was used for DNA sequencing analysis [14]. All other DNA manipulations were as previously described [15].

### 2.5. Construction of yeast Y strains with HXK2, HXK2($S^{15}A$), HXK2Δ$K^7M^{16}$, HXK2::gfp or HXK2Δ$K^7M^{16}$::gfp genes

A DNA fragment containing the complete *HXK2* promoter was isolated from the vector pRS-HXK2 [16] as a 0.88 kb *Sph*I-*Nco*I fragment and subcloned into a *Sph*I-*Nco*I previously cleaved vector pSP73-HG (this plasmid contains in a 2.75 kb fragment the complete coding region of *HXK2* gene and 254 bp of the 5′ non-coding region). The resulting plasmid pSP73-HXK2 contains in a 3.35 kb *Sph*I-*Eco*RI fragment the complete *HXK2* gene under the control of its own promoter. This fragment was cloned into YEp356. The resulting plasmid was called YEp356-HXK2.

In vitro mutagenesis of *HXK2* was done using the Sculptor mutagenesis system (Amersham). The template used for mutagenesis was bacteriophage M13mp18, with an inserted 750 bp *Asp*718 fragment of the *HXK2* gene and promoter obtained from pSP73-HXK2. The nucleotide 5′-AAAGGGTgccATGGCCG-3′ was used in the mutagenesis, the changed codon is shown in lower case. The 750 bp *Asp*718 mutated fragment from a positive phage was subcloned in YEp356-HXK2 cleaved with the same enzymes. The resulting plasmid YEp356-HXK2($S^{15}A$) carries a *HXK2* allele codifying a hexokinase 2 with $Ser^{15}$ changed to Ala.

Oligonucleotides 5′-ATGAACCATTTTATT-3′ (primer 1) and 5′-GGTTCCATGGCCGATGTG-3′ (primer 2) were used to generate construction pSP73-HXK2Δ$K^7M^{16}$ by PCR. The construct was obtained by using 0.1 μg of the primer pair 1+2, 1 μg of pSP73-HXK2 as template, 2.5 U of *Taq* Polymerase (Promega), 0.2 mM dNTPs (Pharmacia) in a total reaction volume of 25 μl 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% agarose gels and ligated. The resulting plasmid (pSP73-HXK2Δ$K^7M^{16}$) was cleaved by *Sph*I and *Eco*RI to obtain an approximately 3.35 kb fragment which was subcloned into a *Sph*I-*Eco*RI previously cleaved vector YEp356. The plasmid obtained, YEp356-HXK2Δ$K^7M^{16}$, has a 30 nucleotides deletion between nucleotides +19 and +48 of the *HXK2* gene. Expression of this mutant gene generates a truncated Hxk2 protein with similar specific activity as the wild-type hexokinase 2 but lacking amino acids from $K^7$ to $M^{16}$.

A 969 bp *Pst*I-*Bgl*II fragment containing the *gfp* gene was subcloned into the pSP73-HXK2 and the pSP73-HXK2Δ$K^7M^{16}$ vectors, first cleaved with *Pst*I-*Bgl*II. The resulting plasmids pSP73-HXK2::gfp and pSP73-HXK2Δ$K^7M^{16}$::gfp were used to obtain *Xho*I-*Bgl*II fragments containing respectively the *HXK2* gene and the *HXK2Δ$K^7M^{16}$* mutant gene fused in frame with the *gfp* gene. These fragments were subcloned in a pRS306 plasmid first cleaved with *Xho*I-*Bam*HI revealing plasmids pRS306-HXK2::gfp and pRS306-HXK2Δ$K^7M^{16}$::gfp.

All the clones used were verified by sequencing analysis of fusion points.

Plasmids YEp356-HXK2, YEp356-HXK2($S^{15}A$) and YEp356-HXK2Δ$K^7M^{16}$ were used to transform the yeast strain DBY2052 and plasmids pRS306-HXK2::gfp and pRS306-HXK2Δ$K^7M^{16}$::gfp were used to transform yeast strain DBY1315.

### 2.6. Preparation of yeast nuclei and nuclear extracts

Nuclei were prepared from strains DBY1315; DBY2052; DBY1315 transformed with plasmids pRS306-HXK2::gfp or pRS306-HXK2Δ$K^7M^{16}$::gfp; and DBY2052 transformed with plasmids YEp356-HXK2, YEp356-HXK2($S^{15}A$) or YEp356-HXK2Δ$K^7M^{16}$, by the method described previously [9]. Nuclear extracts were prepared as follows: the band, consisting of clean nuclei [9], collected from near the top of the Percoll gradient was diluted three times with lysis buffer (50 mM Tris-HCl, pH 7.5; 10 mM $Mg_2SO_4$; 1 mM EDTA; 10 mM potassium acetate; 1 mM DTT and 1 mM PMSF), and centrifuged at 19 000×*g* for 10 min at 4°C. The supernatant was carefully removed by aspiration and the nuclei pellet was resuspended in 100 μl of lysis buffer. The nuclei were broken by vortexing (6×20 s) in the presence of glass beads (0.5 g), and 200 μl of the same buffer was added to the suspension. After centrifugation at 19 000×*g* for 15 min at 4°C, the supernatant was used as nuclear extract.

### 2.7. DNA probes

Oligonucleotides, corresponding to both strands of the UAS elements (underlined) of the *SUC2* gene [17,18], were synthesised with an added TCGA nucleotide overhang at the 5′-terminal end. $OL34_{SUC2}$ sense: 5′-tcgaGTTTAGGAAATTATCCGGGGGCGAAGAAATACGC-3′; $OL34_{SUC2}$ antisense: 5′-tcgaGCGTATTTCTTCGCCCCCGGATAATTTCCTAAAC-3′.

The complementary strands were annealed and either end-labelled with [α-$^{32}$P]dCTP using the Klenow fragment of DNA polymerase I or used as unlabelled competitors in protein-binding experiments.

### 2.8. Gel retardation assays

Binding reactions mixtures contained 10 mM HEPES (pH 7.5), 1 mM DTT, 1–5 μg of poly(dI-dC) and 0.5 ng of end-labelled DNA in a volume of 25 μl. When unlabelled competitor DNA was added, its amount is indicated in the figure legends. The binding reaction mixtures included 12 μg (6 μl) of protein from a nuclear extract and after 30 min of incubation at room temperature they were loaded onto a 4% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 10 V/cm of gel for 45 min to 1 h in 0.5×TBE buffer (45 mM Tris-borate, 1 mM EDTA). Gels were dried and autoradiographed at −70°C with an intensifying screen.

### 2.9. Electrophoretic analysis, immunoblotting and antibodies

Electrophoresis of proteins (SDS-PAGE) was performed on 10%

Table 1
Sequence present within the amino-terminal 16 residues of the Hxk2 protein and in the other known or presumed nuclear proteins

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | V | H | L | G | P | K | K 8 | P | Q | A | R 42 | K | G | S | M | Hxk2 (485 aa) |
| | | | | M | N | K | I | P | I | K 7 | D | L | L | N | Matα2 (210 aa) |
| | | | M | F | T | S | K 5 | P | A | F | K 8 | I | K | N | K | Matα1 (175 aa) |
| M | S | A | K | A | E | K | K 8 | P | A | S | K 12 | A | P | A | E | H2B.1 (131 aa) |
| | | | L | K | C | S | K 23 | E | K | P | K 27 | C | A | K | C | Gal4 (881 aa) |
| | | | Q | G | I | T | K 32 | P | A | I | R 36 | R | L | A | R | H4 (103 aa) |
| | | | N | K | K | T | R 79 | I | I | P | R 83 | H | L | Q | L | H2A.1 (132 aa) |
| | | | H | A | P | L | K 112 | P | V | V | R 116 | K | K | P | E | Spt2 (333 aa) |
| | | | A | A | A | P | K 277 | P | I | I | K 281 | K | R | T | V | Hex2 (1026 aa)* |
| | | | I | K | Y | F | K 311 | K | F | P | K 315 | D | P | L | A | Ski3 (1432 aa) |
| | | | S | V | Y | E | K 411 | F | A | P | K 415 | G | K | Q | L | Rad52 (504 aa) |
| | | | V | V | I | K | R 645 | S | P | R | K 649 | R | G | R | P | Swi5 (709 aa) |

Matα1 and Matα2 [32], H2B.1 [33], Gal4 [34], H4 [35], H2A.1 [36], Spt2 [37], Hex2 [38], Ski3 [39], Rad52 [40] and Swi5 [41]. Numbers below an amino acid indicate the position of the amino acid within the respective protein. Numbers in parentheses indicate the total number of amino acids in the designated proteins.
*Consensus sequence also present at positions 595 and 875.

polyacrylamide gels using the buffer system described in [19]. Western transfer of proteins to a nitrocellulose membrane was carried out as described in [20]. Hxk2 protein was detected by sequential incubation with crude polyclonal antibody (1:1500 dilution) and protein A-peroxidase (1:4000 dilution). Specific anti-Hxk2 serum was raised in rabbits by sequential immunisation with a purified fraction of hexokinase 2 [21].

## 3. Results

### 3.1. Identification of a NLS in the Hxk2 protein

Previous results obtained by measuring hexokinase activity and by detecting Hxk2 protein with specific antibodies in isolated nuclei indicate a double cytosolic-nuclear localisation of the Hxk2 protein in yeast growing in glucose-containing media. The localisation of a fraction of the Hxk2 protein in the nucleus was further confirmed by expressing a Hxk2-GFP fusion protein in yeast ruling out a possible cross-contamination during subcellular fractionation [9]. Therefore we further investigated the presence in Hxk2 of signal sequences described previously as nuclear-targeting signals.

A large number of NLSs have been characterised to date and that allows analysis of their general features. All contain a number of basic residues, but they do not conform to the consensus bipartite sequence proposed previously [22]. However, comparison of amino acids sequences of other known or presumed yeast nuclear proteins with the amino-terminal 16 residues of Hxk2 reveals a sequence that might be important for nuclear targeting, $Lys^{8}$-Pro-Gln-Ala-$Arg^{12}$ (Table 1). A similar sequence of two positively charged amino acids flanking three residues, one of which is proline, is present in several other yeast nuclear proteins but this sequence is not present in any yeast cytoplasmic proteins currently known [22,23].

The nuclear targeting ability of this putative NLS identified in Hxk2 was determined by deletion analysis. A *HXK2* mutant gene (*HXK2ΔK⁷M¹⁶*) was constructed by PCR, and this gene has a 30 bp deletion between nucleotides +19 and +48. The expression of this mutant gene generates a truncated Hxk2 protein with similar specific activity as the wild-type hexokinase 2 but without the amino acids from $Lys^{7}$ to $Met^{16}$.

As is shown in Table 2, the wild-type phenotype was partially restored after transformation of a *hxk1/hxk2* double mutant strain with a *HXK2ΔK⁷M¹⁶*-containing plasmid. The mutant strain produced about 97% of the enzyme activity compared to that of wild-type cells, but no hexokinase activity was detected in isolated nuclei from this strain. However, the wild-type phenotype was completely restored after transformation of *hxk1 hxk2* double mutant strain with the YEP356-HXK2 or the YEP356-HXK2($S^{15}$A) plasmids. Moreover, laser scanning confocal microscopy (Fig. 1) demonstrated that Hxk2::GFP and Hxk2Δ$K^{7}M^{16}$::GFP fusion proteins were distributed in the cell in a manner consistent with a single cytosolic localisation, such that each cell had an uniform distribution of green fluorescence except the vacuole which was virtually free (Fig. 1a,d). Furthermore, nuclei purified by Percoll gradients from wild-type cells transformed with a plasmid containing the *HXK2::gfp* gene showed a clear associated fluorescence (Fig. 1c). However, nuclei purified by Percoll gradients from wild-type cells transformed with a plasmid containing the *HXK2ΔK⁷M¹⁶::gfp* gene do not show any associated green fluorescence (Fig. 1f).

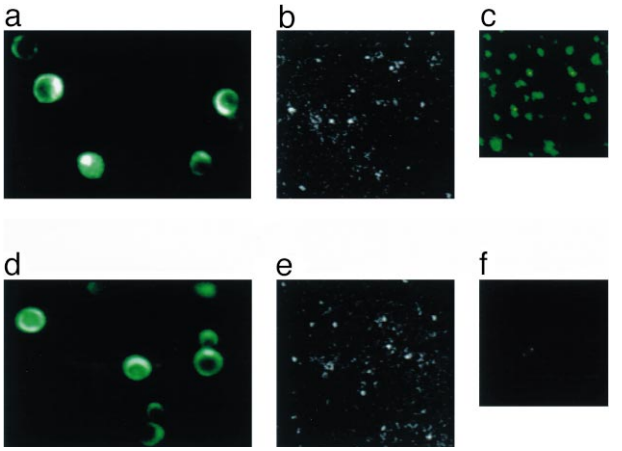


Fig. 1. Detection of Hxk2-GFP fusion protein in living yeast cells. Yeast strain DBY1315 was transformed with the integrative expression plasmids pRS306-HXK2::gfp or pRS306-HXK2Δ$K^{7}M^{16}$::gfp. The resulting single copy transformed strains were grown exponentially in YEPD liquid media and cell-associated fluorescence was analysed in whole cells transformed with the pRS306-HXK2::gfp plasmid (a) or the pRS306-HXK2Δ$K^{7}M^{16}$::gfp plasmid (d) by confocal microscopy. Panels b and e are corresponding nuclei photographed by phase contrast. The fluorescence associated with the nuclei isolated from cells transformed with the pRS306-HXK2::gfp plasmid (c) or the pRS306-HXK2Δ$K^{7}M^{16}$::gfp plasmid (f) was also analysed by confocal microscopy. Confocal images of Hxk2-GFP fusion protein expression were obtained on a Bio-Ras MRC 600 inverted laser confocal microscope using a standard fluorescein isothiocyanate filter providing excitation at 490 nm and emission at 527 nm. The image files were processed using a computer-based graphic system (COMOS) where they were arranged and annotated. (a, d: ×150; b, e: ×1000; c, f: ×2000).

These results were confirmed by immunoblot analysis using polyclonal antibody against Hxk2 protein. As we can see in Fig. 2, Hxk2 protein was detected in the crude extracts ob-

Table 2
Specific hexokinase activity in crude extracts and nuclear extracts from different yeast strains

| Strain | Plasmid | Hexokinase (mU/mg protein) | |
|---|---|---|---|
| | | Crude extract | Nuclear extract |
| DBY1315 | – | 1150 | 161 |
| DBY2052 | – | n.d. | n.d. |
| DBY2052 | YEp356 | n.d. | n.d. |
| DBY2052 | YEp356-HXK2 | 1108 | 153 |
| DBY2052 | YEp356-HXK2Δ$K^{7}M^{16}$ | 1120 | n.d. |
| DBY2052 | YEp356-HXK2($S^{15}$A) | 1109 | 166 |

Plasmids YEp356-HXK2, YEp356-HXK2($S^{15}$A) and YEp356-HXK2Δ$K^{7}M^{16}$ are described in Section 2. Transformed cells with plasmid YEp356 were used as reference. Cells were grown in YEPD medium, harvested at the mid-log phase ($A_{600}$, 1.0) and fractionated as described in Section 2. The hexose-phosphorylating activity was measured using fructose as substrate. n.d., not detectable.

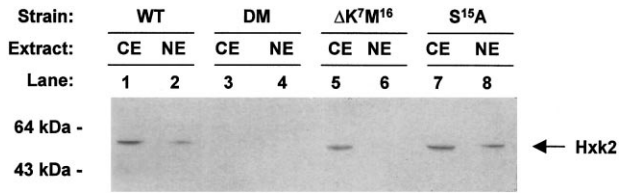


Fig. 2. Immunoblot analysis of the nuclear fraction from different strains. Crude and nuclear extracts from glucose growing yeast cells of the indicated strains, prepared as described in Section 2, were fractionated by SDS-PAGE, the proteins were transferred to nitrocellulose membrane and Hxk2 protein was detected with polyclonal anti-Hxk2 antibodies. Strains: WT, wild-type (DBY1315); DM, double mutant *hxk1 hxk2* (DBY2052); $\Delta K^7M^{16}$, DBY2052 transformed with plasmid YEp356-HXK2$\Delta K^7M^{16}$; $S^{15}A$, DBY2052 transformed with plasmid YEp356-HXK2($S^{15}A$). CE, crude extract; NE, nuclear extract.

tained from all the yeast strains analysed, except from the *hxk1 hxk2* double mutant strain as expected. However, Hxk2 was found in the nuclear extract obtained from the wild-type strain and the double mutant strain transformed with YEP356-HXK2($S^{15}A$) plasmid, but not in the nuclear extract obtained from the double mutant strain or the double mutant strain transformed with a *HXK2*$\Delta K^7M^{16}$-containing plasmid.

Therefore, we can conclude that the accumulation of hexokinase 2 in the nuclear fraction strongly depends on the presence of a NLS, which was localised between amino acids $Lys^7$ and $Met^{16}$ of the Hxk2 protein.

### 3.2. The nuclear localisation of Hxk2 is required for glucose repression of SUC2 gene

A number of authors have proposed signalling pathway models in order to integrate positively and negatively acting factors into one comprehensive scheme [1–3,24,25]. These models account for the following details: the presence of glucose must be sensed in the cells; this information is transduced by intracellular messengers and specific target genes are ultimately turned on or off. Assuming that Hxk2 is a negative regulatory element and that a fraction of this protein is localised inside the nucleus during the repression cycle, it should be possible that the Hxk2 protein may interact with positively or negatively acting factors involved in the glucose repression cascade. Thus, our hypothesis assumes that: first, the expression of the *HXK2* gene acts as sensor of the glucose concentration in the culture medium [26], second, a nuclear localisation of the Hxk2 protein is required for glucose repression signalling.

Data presented in Table 3 clearly show that the wild-type phenotype (low invertase activity in cells growing in repressing medium) was restored after transformation of the *hxk1 hxk2* double mutant with the *HXK2* or the *HXK2*($S^{15}A$) genes. When the *hxk1 hxk2* double mutant was transformed with the NLS mutant gene (*HXK2*$\Delta K^7M^{16}$) the wild-type phenotype was not restored. Moreover, a two-fold increase of exocellular invertase activity, compared to that of *hxk1 hxk2* double mutant cells, was observed, indicating an overexpression of the *SUC2* gene in this genetic background. These results revealed that nuclear localisation of Hxk2 is essential for glucose repression signalling of the *SUC2* gene.

As a first step to characterise the putative role as repressor of Hxk2, its capacity to participate in DNA-protein complexes necessary for glucose repression of *SUC2* gene was tested. When a double stranded oligonucleotide ($OL34_{SUC2}$), including the UAS sequences of the *SUC2* gene [17,18], was subjected to gel mobility shift analysis using nuclear extracts obtained from wild-type and mutant strains as protein sources, we observed two different protein-DNA complexes (CI and CII) with nuclear extracts prepared from glucose-grown wild-type cells (Fig. 3, lane 2). Competition assays with a non-labelled oligonucleotide indicated that the binding was specific in all cases (complex I was only partially displaced by the non-labelled oligonucleotide concentrations used). Regarding the effects of *hxk1 hxk2* double mutations on the formation of the complexes, it can be seen in Fig. 3 (lane 6) that complex CI was not formed when nuclear extracts from this strain were

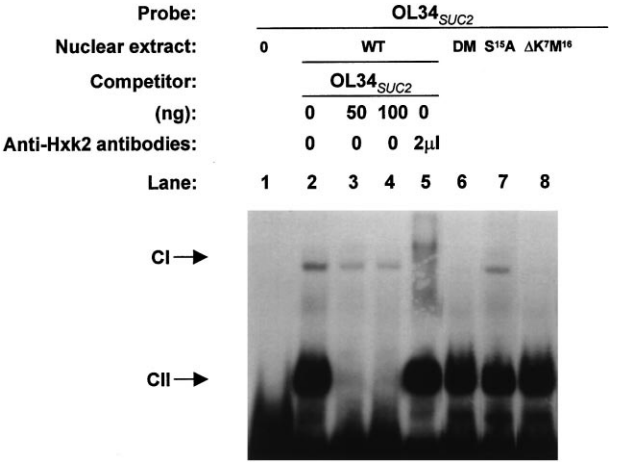


Fig. 3. Gel retardation assays with oligonucleotides which contain the sequence from the UAS controlling regions of *SUC2* gene. Gel mobility shift assays were performed with $^{32}$P-labelled $OL34_{SUC2}$ and nuclear extracts from glucose growing yeast cells of the indicated strains, prepared as described in Section 2. Lane 1, no protein added; lanes 2–5, protein from nuclear extracts obtained from wild-type repressed cells (lane 5: with 2 μl of anti-Hxk2 antibodies); lane 6, protein from nuclear extracts obtained from *hxk1 hxk2* double mutant repressed cells; lane 7, protein from nuclear extracts obtained from *hxk1 hxk2* double mutant repressed cells transformed with plasmid YEp356-HXK2($S^{15}A$); lane 8, protein from nuclear extracts obtained from *hxk1 hxk2* double mutant repressed cells transformed with plasmid YEp356-HXK2$\Delta K^7M^{16}$. Strains: WT. wild-type (DBY1315); DM, double mutant *hxk1 hxk2* (DBY2052); $\Delta K^7M^{16}$, DBY2052 transformed with plasmid YEp356-HXK2$\Delta K^7M^{16}$; $S^{15}A$, DBY2052 transformed with plasmid YEp356-HXK2($S^{15}A$).

Table 3
Specific activity of secreted invertase from different strains

| Strain | Plasmid | Invertase (mU/mg protein) |
|---|---|---|
| DBY1315 | – | 11 |
| DBY2052 | – | 2800 |
| DBY2052 | YEp356 | 2800 |
| DBY2052 | YEp356-HXK2 | 12 |
| DBY2052 | YEp356-HXK2$\Delta K^7M^{16}$ | 5700 |
| DBY2052 | YEp356-HXK2($S^{15}A$) | 13 |

Plasmids YEp356-HXK2, YEp356-HXK2($S^{15}A$) and YEp356-HXK2$\Delta K^7M^{16}$ are described in Section 2. Transformed cells with plasmid YEp356 were used as reference. The external invertase activity was assayed in whole cells grown in YEPD medium and harvested at the mid-log phase ($A_{600}$, 1.0).

used. The wild-type phenotype was restored (Fig. 3, lane 7) when we use nuclear extracts from repressed cells of a *hxk1 hxk2* double mutant strain transformed with a plasmid containing the mutant gene *HXK2($S^{15}A$)*. However, the wild-type phenotype was not restored when we used nuclear extracts from repressed cells of a *hxk1 hxk2* double mutant strain transformed with a plasmid containing the NLS mutant gene *HXK2Δ$K^{7}M^{16}$*, as can be seen in Fig. 3 (lane 8).

To confirm that Hxk2 is present in the retarded complexes obtained with nuclear extracts from the wild-type strain, we use a polyclonal anti-Hxk2 serum [21]. As can be observed in Fig. 3 (lane 5) the anti-Hxk2 antiserum shifted the position of the CI protein-DNA complex towards the top of the gel, to a more slowly migrating complex. In the absence of protein extract the anti-Hxk2 antiserum did not produce any complex (data not shown).

Together, these results allow us to conclude that the Hxk2 is a component of at least one (CI) of the retarded complexes that form the UAS elements of the *SUC2* promoter with *trans*-acting regulatory factors involved in the signalling of the glucose repression cascade.

## 4. Discussion

These studies demonstrate that one region of yeast hexokinase 2 is important in determining its nuclear localisation and its function as mediator of glucose repression. Deletion of Hxk2 from residues $Lys^{7}$ to $Met^{16}$ abolished both nuclear localisation and glucose repression, thus indicating the importance in nuclear targeting of these amino acids of the $NH_2$-terminal region of the protein.

In this paper, we provide evidence supporting a novel idea. When there is glucose in the medium, the *HXK2* gene is expressed and a fraction of the Hxk2 protein, about 14% of total hexokinase 2 activity, is found in the nuclear compartment [9]. A clear correlation between nuclear location of the protein and glucose repression of the *SUC2* gene has been demonstrated. Deletion analysis identified the essential part of the protein-mediating nuclear localisation and glucose repression between amino acid residues 7 and 16 (Tables 2 and 3; Fig. 2). To gain insight into possible functions of the Hxk2 protein in the nucleus, we used gel mobility shift analysis. This analysis revealed that Hxk2 participates directly as part of a complex involved in glucose repression signalling of the *SUC2* gene (Fig. 3). At present, a DNA-binding function of the Hxk2 protein does not seem to be probable. As a preliminary hypothesis, we suppose nuclear Hxk2 to be a competitor for transcriptional factors, thus preventing transcription of the *SUC2* gene. This preliminary hypothesis raises the question of how Hxk2 protein localisation is regulated in response to glucose. One possibility is that Hxk2 localisation may be regulated by phosphorylation.

Although the targeting role of nuclear localisation signals has been known for some time, more recent results indicate that NLS-dependent nuclear protein import is precisely regulated. Phosphorylation appears to be the main mechanism controlling the nuclear transport of a number of proteins, including transcription factors such as NF-κB, c-rel, dorsal, and SWI5 from yeast [27]. Even nuclear localisation of the archetypal NLS-containing simian virus 40 large tumour antigen is regulated by phosphorylation of a 'CcN' motif close to the NLS [28]. The regulation of nuclear transport through phosphorylation appears to be common in eukaryotic cells from yeast and plants to higher mammals. Yeast hexokinase 2 is known to be a phosphoprotein in vitro [29] and in vivo [30]. The principal and perhaps sole site of phosphorylation was identified as $Ser^{15}$ by mutation to alanine, which prevents phosphorylation in vitro [31] and in vivo [8]. Because Hxk2 is phosphorylated at a site close to or inside NLS, it should be interesting to examine if $Ser^{15}$ phosphorylation specifically regulate NLS function. With this aim we constructed the mutant gene *HXK2($S^{15}A$)*. Expression of this gene generates a mutant Hxk2 protein with similar specific activity as the wild-type hexokinase 2 but with a change at residue 15 from serine to alanine. Transformation of a *hxk1 hxk2* double mutant strain with a *HXK2($S^{15}A$)*-containing plasmid restores both nuclear localisation of Hxk2 and glucose repression of *SUC2* gene (Tables 2 and 3; Fig. 2). These results suggest that phosphorylation of $Ser^{15}$ does not affect the nuclear targeting ability of the NLS. Thus, the Hxk2($S^{15}A$) protein can be transported to the nucleus (Fig. 2, Table 2) and in our hands, this nuclear localisation generates the glucose repression signalling of the *SUC2* gene (Fig. 3).

We have shown that yeast hexokinase 2 combines features which have been previously identified as important in nuclear protein targeting: (i) a specific localisation sequence, and (ii) specific binding to DNA *cis*-acting regulatory complexes involved in control of gene expression. Further elucidation of the mechanism controlling nuclear transport will be necessary to understand how these features function together.

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