doi:10.1016/S0022-2836(02)00377-7 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2002) 319, 703–714

JMB

Mediator Factor Med8p Interacts with the Hexokinase 2: Implication in the Glucose Signalling Pathway of *Saccharomyces cerevisiae*

T. de la Cera[1]†, P. Herrero[1]†, F. Moreno-Herrero[2], R.S. Chaves[1] and F. Moreno[1]*

[1]*Departamento de Bioquímica y Biología Molecular, Inst. Univ. de Biotoecn. de Asturias Universidad de Oviedo, 33006 Oviedo, Spain*

[2]*Laboratorio de Nuevas Microscopías, Departamento de Física de la Materia Condensada, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

**Corresponding author*

In the presence of glucose the protein hexokinase 2 (Hxk2p), normally resident in the cytosol, is translocated to the nucleus where it impairs the activation of transcription of the glucose-repressed genes *HXK*1, *GLK*1 and *SUC*2, and promotes the activation of transcription of the glucose-induced genes *HXK*2 and *HXT*1. Here, we demonstrate the involvement of an heptameric motif, named the MED8 site, in the direct binding of the mediator protein Med8p, either as a monomer or as a homodimer. Because this site was previously involved in the Hxk2p-dependent glucose-induced regulation of gene transcription, we tested whether Hxk2p interacts with Med8p. Our results show that Hxk2 and Med8 proteins are physically associated and that this Hxk2p–Med8p interaction is of physiological significance because both proteins have been found interacting together in a cluster with DNA fragments containing the MED8 site. We conclude that Hxk2p operates through the MED8 site, by interacting with Med8p, in the glucose signal transduction pathway of *Saccharomyces cerevisiae*.

Keywords: Saccharomyces cerevisiae; Med8p; Hxk2p; protein interactions; glucose repression

Introduction

Glucose regulates carbon utilization in *Saccharomyces cerevisiae* mainly by inhibiting or activating the transcription of numerous genes. The genes affected include among others those involved in gluconeogenesis, the glyoxylate and Krebs cycle, respiration, and in the utilization of alternative carbon sources.[1–3] This mechanism, called glucose repression, ensures the use of glucose in preference to other carbon sources. Glucose also induces expression of genes required for its own utilization, like the genes encoding several glycolytic enzymes and glucose transporters.[4] Although several of the genes implicated in the pathways that control glucose repression and induction have been identified a complete mechanistic picture of the phenomenon is not yet available. In particular the position of each factor in the signalling cascade and the interactions among them are still not well known.

One central component of the glucose sensing pathway seems to be the hexokinase 2 protein (Hxk2p), that in addition to its classical metabolic role in glucose phosphorylation at C6, plays a role in glucose repression and activation signalling as a transcriptional regulator.[5,6] If indeed Hxk2p plays a role as a transcriptional regulator, one should expect that, at least under certain conditions, the enzyme will be present in the nucleus. Several results with different approaches support such a localization.[7,8] In addition, it has been shown that a nuclear localization of the Hxk2 protein is required for glucose repression of *SUC*2, *HXK*1 and *GLK*1, and that the nuclear Hxk2p is involved in the formation of specific DNA–protein complexes during glucose-dependent repression of these genes.[6,8]

Recently, looking for new factors required for expression of *SUC*2 in *S. cerevisiae*, a protein called Med8p was identified. Med8p specifically binds to

† These authors made an equal contribution to this work.

Abbreviations used: GST, glutathione-*S*-transferase; AFM, atomic force microscopy; HA, haemagglutinin; GBD, Gal4p binding domain; GAD, Gal4p activation domain.

E-mail address of the corresponding author: fmoreno@correo.uniovi.es

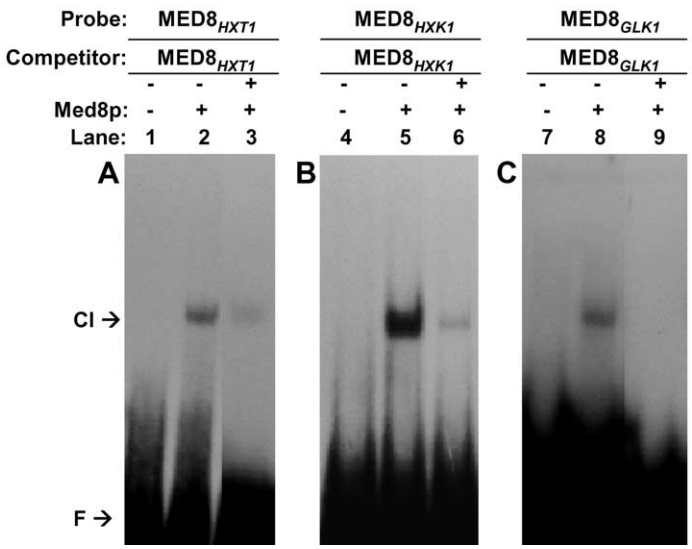


Figure 1. Gel-mobility shift analysis of Med8p binding to the regulatory regions of the *HXT*1, *HXK*1 and *GLK*1 promoters at sequences containing the MED8 site. Each reaction included 0.5 ng of [^{32}P]MED8$_{HXT1}$ ((a), lanes 1 to 3), [^{32}P]- MED8$_{HXK1}$ ((b), lanes 4 to 6) or [^{32}P]MED8$_{GLK1}$ ((c), lanes 7 to 9) as DNA probe and, except for lanes 1, 4 and 7, 6 μl (3 μg) of Med8p purified protein were added. Med8p was obtained from GST-Med8 fusion protein coupled to glutathione-Sepharose beads by site-specific separation of the GST affinity tag using 2.5 units of thrombin. The competitor for binding was 20 ng (lanes 3, 6 and 9) of the corresponding unlabelled MED8 annealed oligonucleotides. Nucleoprotein complexes were resolved from free DNA by non-denaturing PAGE. F, Unbound fragment; CI, position of shifted band observed with purified Med8p.

the downstream repressing sequences (DRSs) of the *HXK*2 gene and to the upstream activating sequences (UASs) of the *SUC*2 gene.[9,10] Med8p was shown to constitute a component of a large multiprotein complex called Srb/mediator complex. The latter enhances basal transcription and facilitates activated transcription by interacting with the C-terminal domain (CTD) of RNA polymerase II.[11–13] The transcription cofactors, as Med8p, mediate access to genes in chromatin and they help to establish, maintain or activate regulatory networks. Thus, a possible role of Med8p could be to affect the formation and activity of basal initiation complexes by linking specific DNA–protein regulatory complexes to the RNA polymerase II holoenzyme transcriptional machinery.

Here, we demonstrate that Med8p binds to DNA both as a monomer and as a homodimer through a heptameric sequence designated "MED8 site". We also demonstrate that Hxk2p interacts specifically with Med8p both *in vivo* and *in vitro*. Furthermore, we map the interacting domains of both proteins and describe a role for the carboxy-terminal domains of Hxk2p and Med8p in this interaction. These results suggest a possible model of how Hxk2p is involved in glucose signalling.

Results

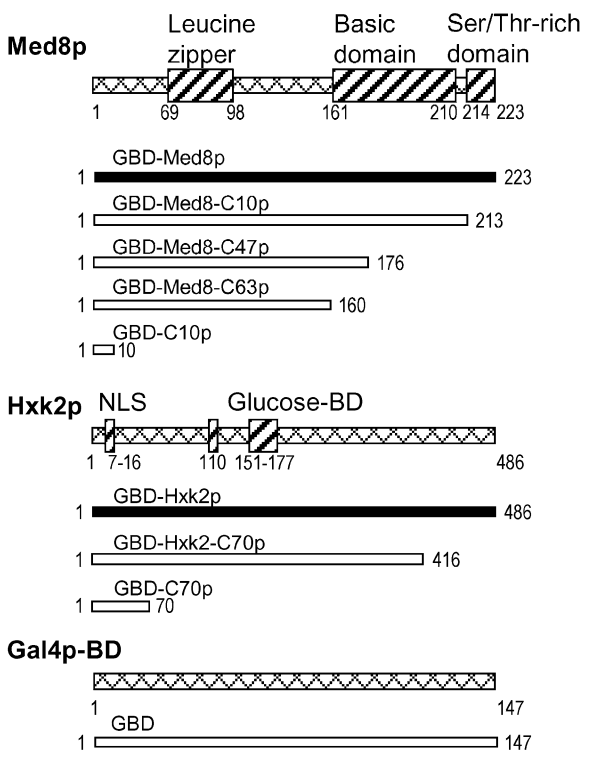
Med8p binds to an heptameric motif present in several glucose-regulated genes

Med8p binds to *cis*-acting elements present in regulatory regions of many glucose-repressed and induced genes. Previous results with different approaches support the notion that Med8p binds to the *SUC*2 and *HXK*2 regulatory region through the heptameric motif, (C/A)(G/A)GAAAT.[9,10] Interestingly, as can be seen in Figure 1, gel-mobility shift analysis has demonstrated that Med8p also binds to regulatory regions of the *HXT*1, *GLK*1 and *HXK*1 promoters through sequences containing a UAS$_{SUC}$-like heptameric motif with the consensus sequence: (C/A)(G/A)(G/A) AAAT. Thus, we designate this sequence as the "MED8 site". Among all these genes, *HXT*1 and *HXK*2, are induced,[4,14] whereas *GLK*1, *HXK*1 and *SUC*2 are repressed by high glucose levels.[14,15]

Hxk2p participates in DNA–protein complexes with *cis*-acting regulatory elements of the *SUC*2 gene, which contain the heptameric motif (C/A)(G/A)GAAAT.[8] The sequences of both DRSs in the *HXK*2 gene includes the DNA motives, CGGAAAT and AAGAAAT,[16] which show a perfect match with those in the UAS element of *SUC*2 gene.[15] Moreover, gel-mobility shift analysis demonstrated that Hxk2p-mediated regulation of *HXK*1 and *GLK*1 is exerted through MED8 regulatory sequences present in the promoters of these genes.[6] These results suggest that both Hxk2p and Med8p function through the MED8 site. To gain further insight into Hxk2p and Med8p function, we have investigated the interactions between these proteins.

Interaction of Hxk2p with Med8p

Although Hxk2 and Med8 proteins form part of the same DNA–protein complex with a regulatory element of the *SUC*2 promoter,[8–10] an interaction between both proteins has not yet been demonstrated. To study this possibility, we have used a yeast two-hybrid system to detect if Hxk2p interacts with a Gal4p DNA-binding domain (GBD)



β-galactosidase (m-units/mg of protein)		
Interaction with GAD-Hxk2p	Interaction with GAD-Med8p	Interaction with GAD $_{(768-881)}$
2250	200	4
8	5	-
20	22	-
7	4	-
29	12	-
2125	2160	20
16	9	-
23	25	-
27	6	17

Figure 2. Two-hybrid interaction of Hxk2p with Med8p and analysis of dimer formation of Hxk2p and Med8p. Yeast two-hybrid assay of full-length and fragmented Med8 protein interactions with Hxk2p and Med8p. Partials (Med8p-C10, aa 1–213; Med8p-C47, aa 1–176 and Med8p-C63, aa 1–160) and full-length (Med8p, aa 1–223) Med8p fused to the Gal4p binding domain (GBD) were individually co-transformed into yeast strain Y187 with constructs encoding the Gal4p activation domain alone (GAD), GAD fused to full-length (Hxk2p, aa 1–485) Hxk2p or full-length (Med8p, aa 1–223) Med8p, as indicated. Yeast two-hybrid assay of full-length and partial Hxk2p interactions with Hxk2p and Med8p. Partial (Hxk2-C70, aa 1–416) and full-length (Hxk2p, aa 1–485) Hxk2p fused to the GBD were individually co-transformed into yeast strain Y187 with constructs encoding the Gal4p activation domain alone (GAD), GAD fused to full-length (Hxk2p, aa 1–485) Hxk2p or full-length (Med8p, aa 1–223) Med8p, as indicated. Protein–protein interactions were examined in each transformant by the qualitative and quantitative assay methods for β-galactosidase activity. Closed bars, blue colony colour; open bars, white colony colour. The values are the averages of β-galactosidase activity for three transformants. Each measured value was within 30% of the average.

fusion encoding the Med8 protein. The ORFs encoding the full-length Med8 protein or truncated versions of this protein were fused to *GAL*4 DNA-binding domains. Each of the pGBKT7-MED8 fusion plasmids was co-transformed with a plasmid expressing a fusion of *GAL*4 activation domain with Hxk2p or a plasmid expressing a fusion of the *GAL*4 activation domain with Med8p into an appropriate reporter strain. The interaction between Hxk2–Med8, Med8–Med8 and Hxk2–Hxk2 proteins was monitored by β-galactosidase expression levels, with a filter assay and a liquid-culture assay.

As shown in Figure 2, only the full-length Med8p gave a strong interaction with Hxk2p. The strength of the interaction obtained with two-hybrid approaches could be affected by factors others than the binding affinity of the protein pairs in question. This is exemplified by the variation in the strength of the signal obtained when a given pair of interacting proteins is exchanged between GAD and GBD. However, in our study such variation in β-galactosidase activity was not detected when Hxk2p and Med8p were exchanged between GAD and GBD. This result suggests that the strength of the signals obtained is mainly due to binding affinity of the Hxk2p–Med8p pair.

Med8 protein has a leucine zipper motif between amino acid residues 69 and 90 which can work as a protein interaction domain that allows proteins to either hetero- or homodimerize.[17] To attempt to resolve whether this protein is part of a monomer or a homodimer we have used the two-hybrid assay approach. When paired with itself, full-length Med8p gave a weak interaction (Figure 2). To provide a positive control assay for these results, we extended the analysis to the Hxk2p–Hxk2p pair, for which homodimerization has been reported.[18–20] As can be seen in Figure 2, the results obtained confirmed the ability of Hxk2p to interact with itself to form a homodimer.

Identification of Med8p and Hxk2p domains involved in the interaction

Med8p contains various motifs such as a C-terminal serine/threonine-rich motif, a basic motif and a leucine zipper motif as indicated in Figure 2.[21] Subfragments of Med8p were fused to the Gal4p DNA-binding domain and tested for interaction with Hxk2p and Med8p by two-hybrid assays to identify the domains of Med8p that are important for interaction with these proteins. We detected no interaction between Hxk2p and any Med8p truncated derivative (Figure 2). Identical results were obtained when we analysed the interaction between Med8p and any other Med8p truncated derivative. This lack of interaction cannot be attributed to a lack of expression, as all GBD-Med8p fusion proteins were detected by Western blotting using anti-GBD antibody (Figure 3(a)). As shown in Figure 2, GBD-Med8-C10p did not interact with either Hxk2p or Med8p. The deleted region of GBD-Med8-C10p contains the serine/threonine-rich domain with several potential phosphorylation sites. These results indicate that the interacting domain of Med8p includes the C-terminal decapeptide and suggests that phosphorylation of this region could be involved in the modulation of binding affinity of the protein pairs.

A truncated Hxk2p (Hxk2-C70p), without 70 amino acid residues from the C-terminal end, was fused to the Gal4p DNA-binding domain and tested for interaction with Hxk2p and Med8p by the two-hybrid assay in order to identify the domain of Hxk2p that is important for interaction with these proteins. We detected no interaction between Hxk2−C70p and full-length Hxk2p or Med8p (Figure 2). These results indicate that the interacting domain of Hxk2p might include a region contained in the 70 amino acid residues of its C-terminal end. This lack of interaction cannot be attributed to any lack of expression, as the GBD-Hxk2-C70p fusion protein was detected by Western blotting using anti-Hxk2p antibody (Figure 3(b)).

In our efforts to understand the mode of action of both the C-terminal decapeptide of Med8p and the 70 amino acid residues of the C-terminal region of Hxk2p we also tested for its direct interaction with the full-length Med8p and Hxk2p. Our results indicate that this is not the only region of interaction within Med8p and Hxk2p. The isolated peptides no longer interacted in the two-hybrid system (Figure 2). In this context it is puzzling that two-hybrid GBD-constructs with smaller fragments carrying only the C-terminal peptides of Med8p and Hxk2p failed to interact with the full-length proteins. This apparent discrepancy could be caused by the artificial nature of the two-hybrid system itself. Testing a fusion protein with only the C-terminal part of Med8p or Hxk2p could result in a misfolding of the Med8p and Hxk2p interacting domains that destroys the protein surface necessary for interaction.

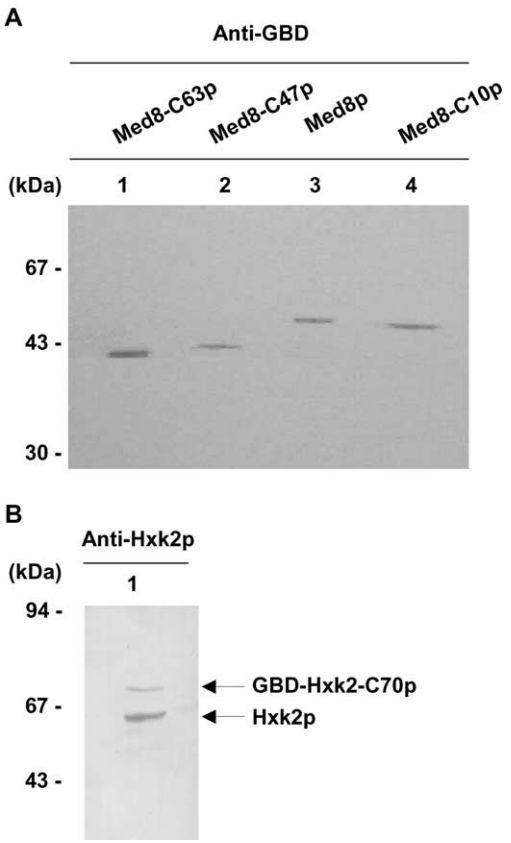


Figure 3. Western blot analysis of Med8 and Hxk2 mutant proteins. (a) Expression of Med8 and truncated versions of Med8p proteins fused with the GBD. Monoclonal antibody against Gal4p DNA-BD was used as primary antibody, as described in Materials and Methods. (b) Expression of Hxk2 and mutant Hxk2 proteins. The mutant Hxk2p, lacking 70 amino acid residues from the C-terminal end, was fused to the GBD and detected by using anti-Hxk2 polyclonal antibody. The positions of molecular mass markers are indicated at the left.

AFM analysis of Med8p dimerization

Our results suggest that Med8p may form part of a homodimer but these results do not show whether the dimerization of the protein is required for DNA binding. To provide an independent assay we have used atomic force microscopy (AFM) to image and map Med8p binding sites within the *HXT*1 promoter.

A 538 bp DNA fragment of the *HXT*1 promoter containing the consensus sequence of MED8 site at 174 bp from one of the ends of the fragment was used. The AFM images show most of the Med8 protein free of any interaction with the DNA but some of them appeared as Med8-*HXT*1 promoter complexes. Visualization of the 538 bp DNA fragment complexed with Med8p by AFM has demonstrated that Med8p coats a region of the DNA molecules (Figure 4(b) and (c)) at a mean position of 170 bp from one end. This result is consistent with the protein binding to the DNA

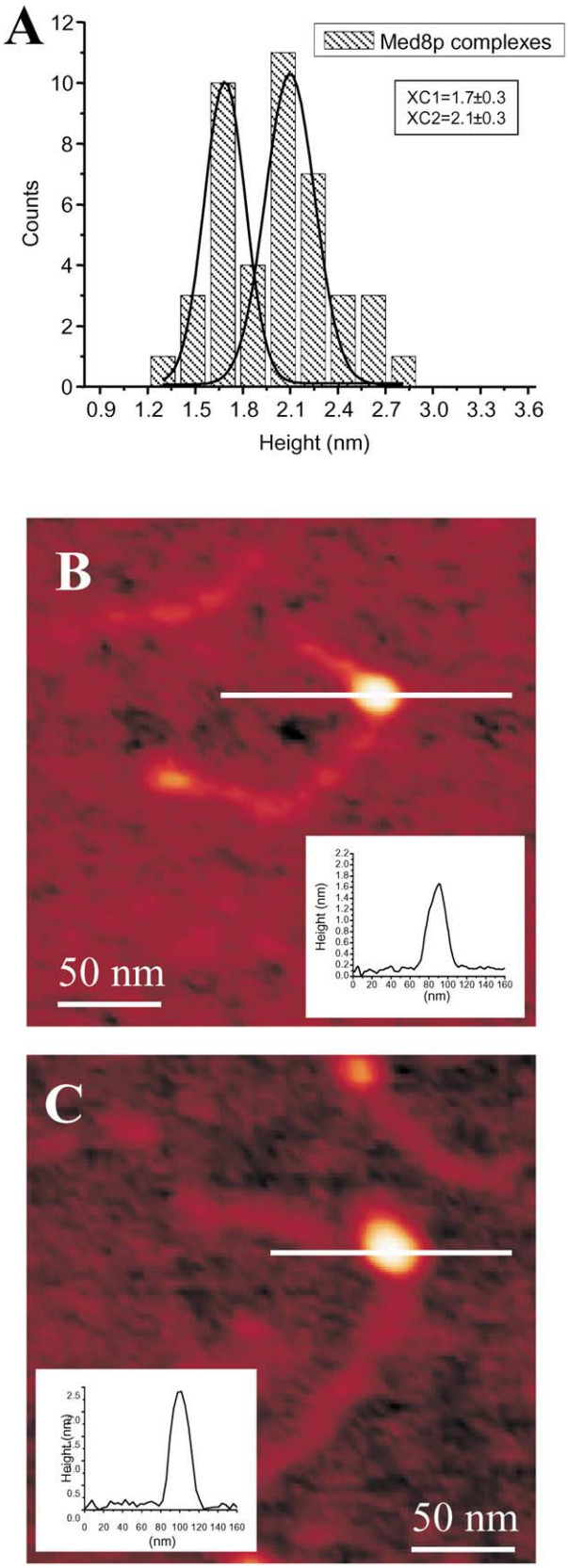


Figure 4. AFM analysis of Med8p binding to *HXT*1 promoter. The heights of Med8p complexed with *HXT*1 are analysed and plotted as a histogram in (a). The measured height of Med8p interacting with the DNA is a bimodal distribution with the most probable heights sited at 1.7(±0.3) nm and 2.1(±0.3) nm. (b) and (c) Two nucleoprotein complexes. The profile over the protein is shown in the inset. The measured height of Med8p in (b) is compatible with Med8p binding as a monomer and the height measured in (c) with Med8 binding as a homodimer.

through the MED8 site of the *HXT*1 promoter. To detect unspecific Med8p binding to DNA, two DNA fragments of the *HXT*1 promoter without the Med8 site were used in control experiments. Our results confirmed that the Med8p–*HXT*1 promoter interaction is specific. The height of Med8 interacting with the DNA was also measured with AFM. Figure 4(a) shows the histogram of the height of the nucleoprotein complexes. The measured height of Med8p interacting with the DNA is a bimodal distribution with the most probable heights sited at 1.7(±0.3) nm and 2.1(±0.3) nm. This result supports the idea that this protein can bind to the Med8p binding sequences present in the *HXT*1 promoter either as a monomer or as a homodimer. It can be argued that some of the complexes found are composed of two unassociated monomers of Med8p binding to the DNA. In that case the height measured would coincide with the height measured for a single molecule of Med8p binding. AFM does not have high spatial resolution and in that case two unassociated monomers or a just a single monomer cannot be resolved if both heights are equal. On the other hand, AFM is characterized by a high resolution in the *Z* dimension. Our experiments show two clear populations of complexes in terms of height. Thus, we believe that Med8p interacts with itself resulting in a homodimer with an effective height of 2.1(±0.3) nm. In Figure 4(b) and (c) two AFM images of Med8–*HXT*1 complexes are shown. In (b) the measured height (see inset) is compatible with the protein binding as a monomer and in (c) the height of the protein suggests that Med8p is binding as a homodimer.

An alternative study with the DNA-unassociated Med8 proteins was performed. In this case we wanted to know whether Med8p homodimerizes without interacting with the DNA. The AFM study of the heights of the free Med8p revealed a wide distribution of heights ranging from 1.0–1.9 nm with two most probable heights at 1.3(±0.3) and 1.6(±0.3) nm (data not shown). These values are compatible with the data from the Med8p–DNA analysis if we add the measured height of the DNA, which is 0.6(±0.1) nm. The wide distribution indicates that structures are more distorted when they are free of interaction with the DNA than when they are interacting with the DNA. However, the data of the protein free of interaction with the DNA indicates that this protein can also homodimerize.

Hxk2p interacts with Med8p *in vivo* and *in vitro*

We sought to examine further the differential binding activities observed in the yeast two-hybrid system and to determine whether full-length Med8p interacts with Hxk2p. To accomplish this we performed glutathione-*S*-transferase (GST) pull-down experiments with crude protein extracts and a purified GST-Med8 fusion protein. As shown in Figure 5(a), a strong retention of Hxk2 protein was observed for samples containing GST-Med8p and crude extracts from the wild-type yeast strain DBY1315. When a control using crude extracts from the double mutant strain DBY2052 (*hxk*1 *hxk*2) was performed to detect unspecific protein binding to anti-Hxk2p antibody no signal was observed.

To confirm the interaction of Hxk2p with Med8p under physiological conditions, we constructed a yeast strain expressing an N-terminal HA-tagged version of full-length Med8p. Whole-cell extracts were prepared and immunoprecipitated with the antibodies indicated in Figure 5(b). The resulting immunoprecipitates were assayed for the presence of Med8p by immunoblot analysis with anti-HA antibodies. As shown in Figure 5(b), a strong and specific signal of Med8p was observed for samples immunoprecipitated with anti-Hxk2 antibody. When an anti-Pho4 antibody or no antibody was used to detect unspecific immunoprecipitation and unspecific protein binding to anti-HA-Med8p antibody, respectively, no signals were observed. Thus, this interaction is dependent on the production of exogenous HA-tagged Med8p as well as anti-Hxk2p antibody.

We cannot exclude the possibility that some unknown proteins might be present and could mediate the interaction between Hxk2p and Med8p. Therefore, we tried to show direct interaction of Med8p with Hxk2p by GST pull-down assays with purified Hxk2 and Hxk2-C70 proteins and the bacterially produced GST-Med8 fusion protein. As shown in Figure 5(c), a strong and specific retention of full-length Hxk2p was observed for the sample containing GST-Med8p. No retention was observed for the sample containing C-terminal truncated Hxk2-C70p.

Taken together, these results demonstrate that full-length Hxk2p interacts directly with the full-length Med8p *in vivo* and *in vitro*. In addition, a 70 amino acid residue C-terminal region of Hxk2p and the Med8p C-terminal decapeptide are required for Hxk2p–Med8p interaction.

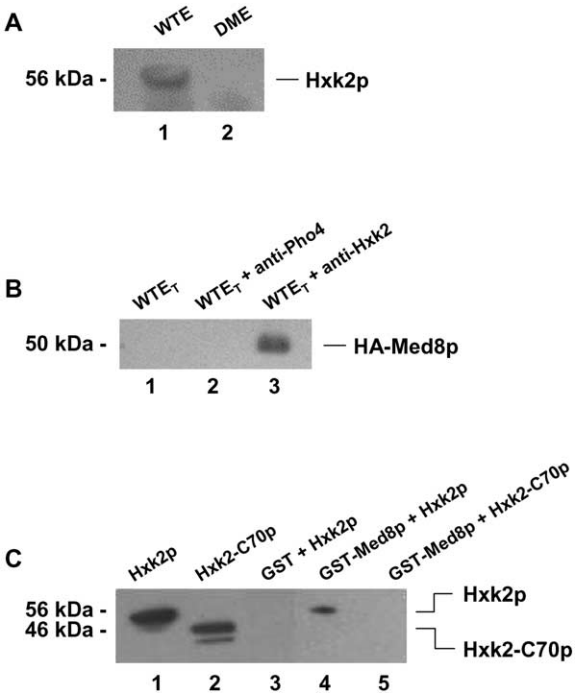


Figure 5. GST pull-down and immunoprecipitation assays of the interaction Med8p–Hxk2p. (a) Med8p co-precipitates together with Hxk2p from cell extracts. Extracts from wild-type (WTE) (DBY1315, *HXK*1 *HXK*2) (lane 1) and double mutant (DME) (DBY2050, Δ*hxk*1 Δ*hxk*2) strains (lane 2) were incubated with GST-Med8p purified from *E. coli* on glutathione beads. Co-precipitated proteins were separated by SDS-PAGE and visualized on a Western blot with anti-Hxk2p antibody. (b) *In vivo* co-immunoprecipitation of Med8p with Hxk2p. *S. cerevisiae* Y187 cells were transformed with HA-tagged GAD-Med8p. Cell extracts from the transformed Y187 wild-type strain (lane 1) were immunoprecipitated with a polyclonal antibody to Pho4p (lane 2) or a polyclonal antibody to Hxk2p (lane 3). Immunoprecipitates were separated by SDS-10% PAGE and co-precipitated GAD-Med8p was visualized on a Western blot with an anti-HA antibody with the help of an HA tag present in the GAD moiety. (c) Purified Hxk2p interacts with purified Med8p. A GST-Hxk2 and a GST-Hxk2-C70 fusion protein were purified on glutathione columns and incubated with thrombin to isolate the native Hxk2 (lane 1) and the Hxk2-C70 (lane 2) protein, respectively. Hxk2p was incubated with purified GST protein on glutathione beads (lane 3) and washed extensively. Both proteins, Hxk2p (lane 4) or Hxk2-C70p (lane 5), were also incubated with purified GST-Med8p on glutathione beads and washed extensively. Co-precipitated proteins were resolved on SDS-10% PAGE. The Hxk2 and Hxk2-C70 proteins were visualized on a Western blot with anti-Hxk2 antibody.

Co-localization of Hxk2p and Med8p

To determine the biological significance of the interaction between Hxk2p and Med8p, we investigated whether these two proteins co-localize in DNA–protein complexes by gel-retardation analyses. Therefore we tried to detect a differential pattern of band shifts between Med8p and Med8p together with Hxk2p with a fragment carrying the MED8 site. The purified Med8 protein was examined in gel-mobility shift assays with ^{32}P-labelled MED8$_{SUC2}$. As can be seen in Figure 6 (lane 3), a protein–DNA complex (CI) was detected. A specific binding of Med8p to the MED8$_{SUC2}$ oligonucleotide was demonstrated by competition assays with the corresponding non-labelled oligonucleotide and with calf thymus DNA (data not

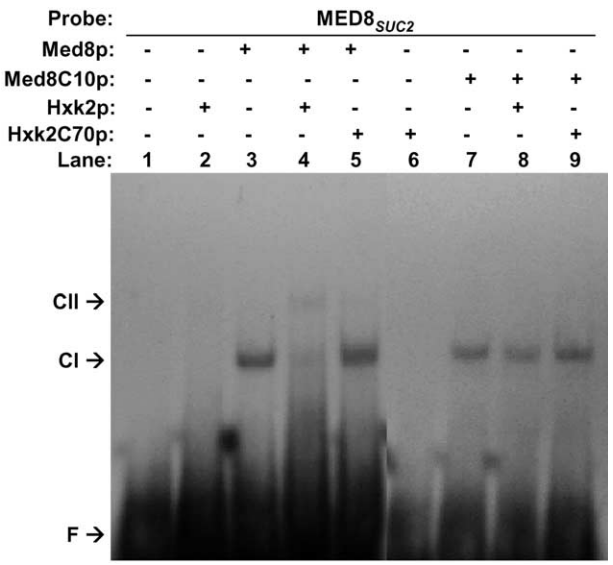


Figure 6. Gel-mobility shift analysis of Med8p binding to the $MED8_{SUC2}$ regulatory region. Band shift experiments were carried out as described in the legend to Figure 1. Hxk2p and Hxk2-C70p were obtained, respectively, from GST-Hxk2p and GST-Hxk2-C70p fusion proteins coupled to glutathione-Sepharose beads by site-specific separation of the GST affinity tag using 2.5 units of thrombin. For the control lane 1 the radiolabelled DNA fragment was added alone. F, Unbound fragment; CI, position of shifted bands observed with purified Med8p or Med8-C10p; CII, position of shifted bands observed with purified Med8p and Hxk2p.

shown). A similar result for GST-Med8 fusion protein binding has previously been observed.[9] Regarding the effect of Hxk2p on the formation of the Med8p–$MED8_{SUC2}$ complex, incorporation of Hxk2p to the band shift reaction mixture shifted the position of the CI Med8p–DNA complex towards the top of the gel, to a complex (CII) migrating more slowly (Figure 6, lane 4). A similar result was observed when purified Hxk2p was added to the band shift reaction mixture after the Med8p–DNA complex was allowed to form (data not shown). To confirm that the CII complex was caused by Hxk2p interaction with Med8p and not by directly binding to DNA, we performed two different band shift assays in order to: (i) directly analyze DNA-binding activities of purified Hxk2p and (ii) to study the effect of Hxk2-C70p (a truncated derivative of Hxk2p not interacting with Med8p) on the formation of the Med8p–$MED8_{SUC2}$ complex. The Hxk2 protein did not bind the DNA fragment and the Hxk2-C70p had no effect on the pattern of band shifting observed with Med8p (Figure 6, lanes 2 and 5). Moreover, the Hxk2-C70p does not bind the DNA fragment (lane 6). These results suggest that Hxk2p confers slow mobility to CII by interaction with the Med8p component of the nucleoprotein complex.

To analyse the role of the Med8p C-terminal decapeptide on Med8p binding to the MED8 site, we utilized the Med8-C10p truncated version of Med8p to measure its affinity for a DNA fragment containing the optimal MED8 binding site. As can be seen in Figure 6 (lane 7), the Med8-C10p binds as efficiently as the wild-type Med8p. Regarding the effect of Hxk2p and Hxk2-C70p on the formation of the Med8-C10p-$MED8_{SUC2}$ complex, the incorporation of Hxk2p or Hxk2-C70p to the band shift reaction mixture had no effect on the pattern of band shifting observed with Med8-C10p alone (Figure 6, lanes 8 and 9). This evidence suggests that the Med8p C-terminal deletion does not eliminate the DNA-binding activity but eliminates the interaction with Hxk2p in the mutant Med8-C10 protein.

Discussion

Here, we have carried out a molecular analysis of the *MED*8 and *HXK*2 gene products. Several results suggest that many genes controlled by glucose induction and repression have Med8p binding sites, with a consensus sequence of (C/A)(G/A)(G/A)AAAT. The Med8p binds to the MED8 site both as a monomer and in its homodimeric state. A simple model considers Med8p as a sphere. From Figure 4(a) the radius of the monomer binding to the DNA is 1.7 nm. If the protein appears as a dimer then the volume doubles and the new radius of the sphere-shaped protein will be 2.14 nm, which is in good agreement with the value measured. Note that dehydration phenomena, which are always present in AFM samples of biological material when imaging in air, would affect both monomer and dimer in the same proportion, so the ratio between radii (dimer/monomer) will not be affected.

We report evidence that Hxk2 and Med8 proteins are physically associated. The most important findings with regard to the mechanism of Hxk2p action is that two-hybrid assays, co-precipitation experiments, and gel-mobility shift analyses with purified proteins involved in the glucose signalling pathway of *S. cerevisiae*, demonstrated that full-length Hxk2p interacts at its C-terminal region directly with the amino acid region from 213 to 223 of Med8p. The association represents relatively tight binding, as the complex survived co-precipitation experiments under conditions of moderate salt and detergent concentrations. Moreover, gel-mobility shift assays suggest that these Hxk2p–Med8p interactions are of physiological significance because they interact in a cluster with DNA fragments containing the MED8 site.

These findings provide a molecular basis for understanding the effect of Hxk2p on the glucose signal transduction pathway of *S. cerevisiae*. Taking into account that a Hxk2p–Med8p–DNA complex seems to be required for inhibition of Med8p activating function by high levels of glucose, the simple model consistent with both genetic and biochemical evidences is that the nuclear Hxk2p[6] negatively affects the function of Med8p at glucose

repressible genes in virtue of its presence in this complex with the mediator protein. This idea is consistent with evidence suggesting that deletion of the C-terminal decapeptide of Med8p results in a truncated protein with residual function (DNA binding) that is independent of Hxk2p interaction. More complicated mechanisms of Hxk2p function can also be imagined; for example, we cannot exclude the possibility that Hxk2p activates or interacts with a negative effector of Med8p.

In the presence of glucose the regulatory protein Reg1p targets the protein phosphatase 1 (Glc7p), to dephosphorylate serine 14 in Hxk2p[22] and the equilibrium between the two isoforms of Hxk2p, a monomer and a dimer[18] is shifted to the homodimeric form.[19,20] In yeast cells grown in the absence of glucose Hxk2p is phosphorylated at serine 14[23] and displaces the equilibrium towards the monomeric form. How the new conformation of the phosphoenzyme may affect the fate of the nuclear Hxk2p is unknown at the moment. A plausible hypothesis could be that, in derepressed cells the phosphorylated Hxk2p is translocated to the cytosol, as happens with Mig1p,[24] and the absence of nuclear Hxk2p causes Med8p to function as a transcriptional activator by correctly positioning the RNA polymerase II holoenzyme transcriptional machinery.

Materials and Methods

Strains and growth media

Yeast two-hybrid experiments employed strain Y187 (*MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ gal80Δ URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*). Co-precipitation experiments utilized yeast 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*). Bacterial transformation and preparation of recombinant plasmid DNA were performed in *Escherichia coli* MC1061 (*hsdR mcrB araD 139Δ(araABC-leu)7679Δlacx74 galU galK rpsL thi*). Fusion protein expression was performed in *E. coli* BL21(DE3)pLysS (Promega).

Yeast cells were grown in the following media: YEPD (2% (w/v) glucose, 2% (w/v) Peptone, and 1% (w/v) yeast extract), YEPE (3% (v/v) ethanol, 2% Peptone, and 1% yeast extract) and SD/ − Leu,Trp (2% glucose and 0.67% yeast nitrogen base without amino acids, supplemented with all essential amino acids and nucleotides except leucine and tryptophan). Amino acids and other growth requirements were added at a final concentration of 20–150 μg/ml. The solid media contained 2% (w/v) agar in addition to the components described above.

General DNA techniques

Routine DNA manipulations were essentially performed as described.[25] The dideoxyribonucleotide chain termination procedure was used for DNA sequencing analysis.[26]

Construction of plasmid DNAs

The plasmids used here are described in Table 1. Hxk2p is numbered from residues 1 to 485[27,28] and the Med8p is numbered from residues 1 to 223.[29] In both cases, residue 1 is the initiator methionine in the primary translation products. The plasmids carrying partial segments of a gene and the encoded products are named according to the codons/amino acids that are retained in the constructs. Plasmids pGBKT7-HXK2 and pACT2-HXK2 carry, respectively, a 1455 bp *Eco*RI-*Bam*HI and a *Nco*I/*Bam*HI fragment with the complete coding region of the *HXK*2 gene in pGBKT7 (Clontech) and pACT2.[30] The *HXK*2 insert was synthesised by PCR using plasmid pSP73-HXK2[8] as the template with the primer pairs OL1 + OL3 and OL2 + OL3 (Table 2). Plasmids pGBKT7-MED8 and pGADT7-MED8 carry a 669 bp *Eco*RI-*Bam*HI fragment with the complete coding region of the *MED*8 gene in pGBKT7 and pGADT7 (Clontech). The *MED*8 insert was synthesised by PCR using genomic DNA as the template with the primer pairs OL4 + OL5 (Table 2). To make plasmid pGBKT7-HXK2C70 a 1245 bp *Eco*RI-*Pst*I fragment, obtained from pGBKT7-HXK2, and carrying a 210 bp deletion in the 3′ end of *HXK*2 DNA was first subcloned into the *Eco*RI and *Pst*I sites of pUK21 and then isolated as an *Eco*RI-*Sal*I fragment and ligated into the *Eco*RI and *Sal*I sites of pGBKT7. To make plasmid pGBKT7-C70$_{HXK2}$ a 210 bp *Pst* I fragment, obtained from pGBKT7-HXK2, was subcloned in the *Pst*I site of pGBKT7. Plasmids

Table 1. Recombinant plasmids and encoded proteins

Plasmid	Encoded product	Source
pGBKT7/HXK2	Hxk2p[a]	This study
pGBKT7/MED8	Med8p[a]	This study
pGBKT7/HXK2-C70	Hxk2p without the C-terminal fragment (D^{416}-A^{485})[a]	This study
pGBKT7-C70$_{HXK2}$	C-terminal fragment (D^{416}-A^{485}) of Hxk2p[a]	This study
pGBKT7/MED8-C10	Med8p without the C-terminal fragment (T^{214}-N^{223})[a]	This study
pGBKT7/MED8-C47	Med8p without the C-terminal fragment (S^{177}-N^{223})[a]	This study
pGBKT7/MED8-C63	Med8p without the C-terminal fragment (T^{161}-N^{223})[a]	This study
pGBKT7-C10$_{MED8}$	C-terminal fragment (T^{214}-N^{223}) of Med8p[a]	This study
pACT2/HXK2	Hxk2p[b]	This study
pGADT7/MED8	Med8p[b]	This study
pGEX/HXK2	GST-Hxk2 fusion protein	This study
pGEX/HXK2-C70	GST-Hxk2-C70 (D^{416}-A^{485}) fusion protein	This study
PGEX/MED8	GST-Med8 fusion protein	This study

[a] Fusion protein with the DNA-binding domain of Gal4p.
[b] Fusion protein with the activation domain of Gal4p.

Table 2. Oligonucleotide primers for PCR

Primer	Sequence (5′ → 3′)	Gene location	Restriction sites for cloning
OL1	TAGAATTCATGGTTCATTTAGGTCCAAAA	*HXK2*; +1 to +21	*Eco*RI
OL2	TACCATGGTTCATTTAGGTCCAAAAA	*HXK2*; +1 to +22	*Nco*I
OL3	ATGGATCCAGCACCGATGATACCAAC	*HXK2*; +1437 to +1455[a]	*Bam*HI
OL4	GCGGAATTCATGTCACAATCTACTGCATC	*MED8*; +1 to +20	*Eco*RI
OL5	AAGGATCCGCATTACTAGATGATGTTGA	*MED8*; +648 to +669[a]	*Bam*HI
OL6	ATGGATCCTCATGTCACAATCTACTGC	*MED8*; +1 to +17	*Bam*HI
OL7	GCGAATTCAATTACTAGATGATGTTGA	*MED8*; +648 to +669[a]	*Eco*RI
OL8	ATAGTCGACTCAAATAATGGGCTTTTCCC	*MED8*; +622 to +639[a]	*Sal*I
OL9	ATAGTCGACTCATTCTTCATTTTTGAAATC	*MED8*; +510 to +528[a]	*Sal*I
OL10	ATAGTCGACTCAGCGCGCCCAATTCGTTA	*MED8*; +463 to +480[a]	*Sal*I
OL11	GATCATTACTAGATGATGTTGAAGTAGATCCCGT	*MED8*; +638 to +669	
OL12	GATCACGGGATCTACTTCAACATCATCTAGTAAT	*MED8*; +638 to +669[a]	

[a] Complementary strand.

pGBKT7-MED8C10, pGBKT7-MED8C47 and pGBKT7-MED8C63 carry respectively 639 bp, 528 bp and 480 bp *Bam*HI-*Sal*I fragments, with 30 bp, 141 bp and 189 bp deletions at the 3′ end of the *MED8* gene, in pGBKT7. The deleted *MED8* DNA inserts were synthesised by PCR using pGBKT7-MED8 plasmid as the template with the primer pairs OL6 + OL8, OL6 + OL9 and OL6 + OL10 (Table 2). To make plasmid pGBKT7-$C10_{MED8}$, we reconstituted a 30 bp fragment in the 3′ end of the *MED8* gene from two complementary oligonucleotides OL11 + OL12 (Table 2), with an added GATC nucleotide overhang at the 5′ end. The complementary strands were annealed and subcloned in the *Bam*HI site of pGBKT7.

GST fusions vectors pGEX-HXK2, -HXK2C70 and -MED8 were constructed as follow. Plasmids pGEX-HXK2 and pGEX-HXK2C70 were constructed by subcloning *Eco*RI-*Sal*I fragments, obtained from plasmids pGBKT7-HXK2 and pGBKT7-HXK2C70, respectively, in-frame into the same sites of pGEX-4T (Amersham Biosciences). Plasmid pGEX-MED8 carries a 669 bp *Bam*HI-*Eco*RI fragment with the complete coding region of *MED8* gene in pGEX-2T (Amersham Biosciences). The *MED8* insert was synthesised by PCR using genomic DNA as the template with the primer pairs OL6 + OL7 (Table 2). The DNA sequence of all PCR-generated constructs was verified by sequencing.

The GAD fusion plasmids were constructed in the vectors pACT2 or pGADT7 and have an HA epitope tag at the 5′ end of the inserted DNA. The GBD fusion plasmids were constructed in the vector pGBDKT7 and have a c-Myc epitope tag at the 5′ end of the inserted DNA.

Yeast two-hybrid analysis

The yeast two-hybrid analysis[31] employed yeast vectors pGADT7, pACT2 and pGBDKT7 and host strain Y187 (described above) as supplied in the Matchmaker two-hybrid system 3 from Clontech. The transformed yeasts were grown in SD/ − Leu,Trp medium. Assays for β-galactosidase activity followed protocols described elsewhere.[25] Qualitative assessment of expression from the *lacZ* reporter gene was made using X-gal as a chromogenic substrate for β-galactosidase in a colony-lift filter assay. For quantitative determination of β-galactosidase activity, the absorbance at 420 nm of *o*-nitrophenol released from the substrate *o*-nitrophenyl β-D-galactopyranoside was normalized to total protein and is reported as m-units/mg of protein. Expression levels of the GAD and GBD fusion proteins were controlled by Western blot analysis. Experiments were performed a minimum of three times. Values shown are representative results from individual experiments.

AFM sample preparation and AFM imaging

Sample preparation for atomic force microscopy (AFM)[32] (also called scanning force microscopy) and the method of AFM imaging have been described.[33]

Antibodies and immunological methods

GBD and GAD fusion proteins were detected using Gal4p DNA-BD monoclonal antibodies and HA-tag polyclonal antibodies, respectively. Native and recombinant Hxk2p were detected using polyclonal antibodies raised against Hxk2p.[8]

Immunoprecipitation experiments were performed by using whole-cell extracts prepared as reported.[6] Extracts were incubated with anti-Hxk2p for one hour at 4 °C. Protein A-Sepharose beads (Amersham Biosciences) were then added and incubated for one hour at 4 °C. After extensive washes with Staph A buffer (150 mM NaCl, 101 mM Na_2HPO_4, 18 mM NaH_2PO_4 (pH 7.3), 20% (v/v) Triton X-100, 1% (w/v) SDS, 5% (w/v) deoxycholate), immunoprecipitated samples were boiled in SDS-loading buffer. The supernatant was subjected to SDS/12% polyacrylamide gel electrophoresis (SDS-PAGE) and detected by Western blot using anti-HA antibody and horseradish peroxidase-conjugated protein-A by the ECL system (Amersham Biosciences).

Western blot analysis was performed by using yeast transformed cells containing appropriate plasmids. Yeast crude extracts were prepared as follows: yeast was grown on 10 to 20 ml of rich medium (YEPD) at 28 °C until an absorbance at 600 nm of 1.0. Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl of 50 mM Tris–HCl (pH 7.5) buffer containing 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.42 M NaCl and 1.5 mM $MgCl_2$. The cells were broken by vortexing (6 × 20 seconds) in the presence of glass beads (0.5 g), and 400 μl of the same buffer were added to the suspension. After centrifugation at 19,000*g* (14,000 rpm) for 15 minutes at 4 °C, the supernatant was used as crude protein extract. Proteins were transferred to enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences), which was then incubated

Table 3. Complementary oligonucleotides carrying regulatory elements of *HXT*1, *HXK*1 and *GLK*1 promoters used in this study

DNA fragment	Sense	Antisense
$MED8_{HXT1}$	tcgaACTAGG***AAGAAAT***GCTGCAGGGCA (−369)	tcgaTGCCCTGCAGC***ATTTCTT***CCTAGT (−392)
$MED8_{HXK1}$	tcgaGACCTAAGAACTATGC***ATTTTTT***TTAAGGG (−334)	tcgaCCCTTAAA***AAAAAAT***GCATAGTTCTTAGGTC (−364)
$MED8_{GLK1}$	tcgaCA***AGAAAAT***GCACGCGTAACAAAATATATATATATATA (−526)	tcgaTATATATATATATATTTTGTTACGCGTGC***ATTTTCT***TG (−541)

with an anti-HA antibody or with anti-Hxk2p polyclonal antibody. Horseradish peroxidase-conjugated protein-A was used as secondary reactant. The complex was detected by the ECL detection system (Amersham Biosciences).

GST pull-down experiments

GST fusion protein expression vectors (pGEX-HXK2, -HXK2-C70 and -MED8) were transformed into *E. coli* strain BL21(DE3). Cells were grown to A_{600} 0.5–0.8, induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for three hours, and collected by centrifugation. Cell pellets were resuspended in PBS buffer (150 mM NaCl, 101 mM Na_2HPO_4, 18 mM NaH_2PO_4, pH 7.3) and sonicated. Insoluble material was removed by centrifugation (17,000*g* for 20 minutes at 4 °C). Soluble extracts were incubated with glutathione-Sepharose 4B (Amersham Biosciences) for one hour at 4 °C, washed extensively with PBS buffer and resuspended in the same buffer. The GST-Med8, GST-Hxk2 and GST-Hxk2C70 fusion proteins coupled to glutathione-Sepharose beads were incubated with 2.5 units of thrombin (two hours at 4 °C) for site-specific separation of the GST affinity tag from Med8, Hxk2 and Hxk2C70 proteins. Equal amounts of GST and GST-Med8p coupled to glutathione-Sepharose beads were incubated with Hxk2 and Hxk2C70 purified proteins or yeast whole-cell extracts for one hour at 4 °C in PBS buffer. Beads were gently washed five times with 2.5 ml of PBS buffer, boiled in 25 μl of sample-loading buffer (50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and analysed by SDS-PAGE followed by Western blot using anti-HA or anti-Hxk2p antibodies and horseradish peroxidase-conjugated protein-A. Bound antibodies were detected using the ECL system (Amersham Pharmacia Biotech).

DNA probes

To investigate interaction of Med8p with the sequences carrying regulatory elements in the *GLK*1, *HXK*1 and *SUC*2 promoter regions we reconstituted the fragment from two complementary oligonucleotides. The complementary strands were annealed and either end was labelled with [α-^{32}P]dCTP by fill-in, using the Klenow fragment of DNA polymerase I. The labelled double-stranded DNA was used as probe and the unlabelled was used as competitor in gel retardation assays. In all cases the oligonucleotides were synthesised with an added TCGA nucleotide overhang at the 5′-terminal end. The synthetic DNA fragments used in gel retardation experiments are listed in Table 3.

Gel retardation assays

Binding reactions contained 10 mM Hepes (pH 7.5), 1 mM DTT, 1 to 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 was 20 ng. The binding reaction mixtures included 3 μg (6 μl) of the corresponding purified protein and after 30 minutes of incubation at room temperature they were loaded onto non-denaturing 4% polyacrylamide gels. Electrophoresis was carried out at 10 V/cm of gel for 45 minutes to one hour in 0.5 × TBE buffer (45 mM Tris–borate, 1 mM EDTA). Gels were dried and autoradiographed at −70 °C with an intensifying screen.

Acknowledgments

This work was supported by grants PB97-1213-C02-02 and BMC2001-1690-C0202 from the DGI (Dirección General de Investigación). T.C. and F.M.-H. were supported, respectively, by fellowships from DGI and from the Community of Madrid.

References

1. Trumbly, R. J. (1992). Glucose repression in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **6**, 5–21.
2. Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microb. Mol. Biol. Rev.* **62**, 334–361.
3. Rolland, F., Winderickx, J. & Thevelein, J. M. (2001). Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem. Sci.* **26**, 310–317.
4. Özcan, S. & Johnston, M. (1999). Function and regulation of yeast hexose transporters. *Microb. Mol. Biol. Rev.* **63**, 554–569.
5. Petit, T., Diderich, J. A., Kruckeberg, A. L., Gancedo, C. & Van Dam, K. (2000). Hexokinase regulates kinetics of glucose transport and expression of genes encoding hexose transporters in *Saccharomyces cerevisiae*. *J. Bacteriol.* **182**, 6815–6818.
6. Rodríguez, A., De la Cera, T., Herrero, P. & Moreno, F. (2001). The hexokinase 2 protein regulates the expression of the *GLK*1, *HXK*1 and *HXK*2 genes of *Saccharomyces cerevisiae*. *Biochem. J.* **355**, 625–631.
7. Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J. A. & Moreno, F. (1998). Hexokinase PII has a double cytosolic-nuclear localisation in *Saccharomyces cerevisiae*. *FEBS Letters*, **425**, 475–478.
8. Herrero, P., Martínez-Campa, C. & Moreno, F. (1998). The hexokinase 2 protein participates in regulatory DNA–protein complexes necessary for glucose repression of the SUC2 gene in *Saccharomyces cerevisiae*. *FEBS Letters*, **434**, 71–76.
9. Chaves, R. S., Herrero, P. & Moreno, F. (1999). Med8, a subunit of the mediator CTD complex of RNA polymerase II, directly binds to regulatory elements of *SUC*2 and *HXK*2 genes. *Biochem. Biophys. Res. Commun.* **254**, 345–350.
10. Moreno-Herrero, F., Herrero, P., Colchero, J., Baro, A. M. & Moreno, F. (1999). Analysis by atomic force microscopy of Med8 binding to *cis*-acting regulatory elements of the *SUC*2 and *HXK*2 genes of *Saccharomyces cerevisiae*. *FEBS Letters*, **459**, 427–432.
11. Myers, L. C., Gustafsson, C. M., Bushnell, D. A., Lui, M., Erdjument-Bromage, H., Tempest, P. & Kornberg, R. D. (1998). The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. *Genes Dev.* **12**, 45–54.
12. Myers, L. C. & Kornberg, R. D. (2000). Mediator of transcriptional regulation. *Annu. Rev. Biochem.* **69**, 729–749.
13. Yudkovsky, N., Ranish, J. A. & Hahn, S. (2000). A transcription reinitiation intermediate that is stabilized by activator. *Nature*, **408**, 225–229.
14. Herrero, P., Galíndez, J., Ruiz, N., Martínez-Campa, C. & Moreno, F. (1995). Transcriptional regulation of the *Saccharomyces cerevisiae HXK*, *HXK*2 and *GLK*1 genes. *Yeast*, **11**, 137–144.
15. Bu, Y. & Schmidt, M. C. (1998). Identification of *cis*-acting elements in the *SUC*2 promoter of *Saccharomyces cerevisiae* required for activation of transcription. *Nucl. Acids Res.* **26**, 1002–1009.

16. Herrero, P., Ramírez, M., Martínez-Campa, C. & Moreno, F. (1996). Identification and characterisation of two transcriptional repressor elements within the coding sequence of the *Saccharomyces cerevisiae HXK2* gene. *Nucl. Acids Res.* **24**, 1822–1828.
17. Landschulz, W. H., Johnson, P. F. & McKnight, S. L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science*, **240**, 1759–1764.
18. Furman, T. C. & Neet, K. E. (1983). Association equilibria and reacting enzyme gel filtration of yeast hexokinase. *J. Biol. Chem.* **258**, 4930–4936.
19. Randez-Gil, F., Sanz, P., Entian, K. D. & Prieto, J. A. (1998). Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. *Mol. Cell. Biol.* **18**, 2940–2948.
20. Golbik, R., Naumann, M., Otto, A., Muller, E., Behlke, J., Reuter, R. *et al.* (2001). Regulation of phosphotransferase activity of hexokinase 2 from *Saccharomyces cerevisiae* by modification at serine-14. *Biochemistry*, **40**, 1083–1090.
21. Démolis, N., Mallet, L., Bussereau, F. & Jacquet, M. (1993). RIM2 MSI1 and PGI1 are located within an 8 kb segment of *Saccharomyces cerevisiae* chromosome II, which also contains the putative ribosomal gene L21 and a new putative essential gene with a leucine zipper motif. *Yeast*, **9**, 645–659.
22. Alms, G. R., Sanz, P., Carlson, M. & Haystead, T. A. J. (1999). Reg1p targets protein phosphatase 1 to dephosphorylate hexokinase II in *Saccharomyces cerevisiae*: characterizing the effects of a phosphatase subunit on the yeast proteome. *EMBO J.* **18**, 4157–4168.
23. Kriegel, T. M., Rush, J., Vojtek, A. B., Clifton, D. & Fraenkel, D. G. (1994). *In vivo* phosphorylation site of hexokinase 2 in *Saccharomyces cerevisiae*. *Biochemistry*, **33**, 148–152.
24. DeVit, M. J., Waddle, J. A. & Johnston, M. (1997). Regulated nuclear translocation of the Mig1 glucose repressor. *Mol. Biol. Cell*, **8**, 1603–1618.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edit., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. Sanger, F., Nicklen, S. & Coulson, S. A. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
27. Frohlich, K. U., Entian, K. D. & Mecke, D. (1985). The primary structure of the yeast hexokinase PII gene (*HXK2*) which is responsible for glucose repression. *Gene*, **36**, 105–111.
28. Walsh, R. B., Kawasaki, G. & Fraenkel, D. G. (1983). Cloning of genes that complement yeast hexokinase and glucokinase mutants. *J. Bacteriol.* **154**, 1002–1004.
29. Feldmann, H., Aigle, M., Aljinovic, G., Andre, B., Baclet, M. C., Barthe, C. *et al.* (1994). Complete DNA sequence of yeast chromosome II. *EMBO J.* **13**, 5795–5809.
30. Li, L., Elledge, S. J., Peterson, C. A., Bales, E. S. & Legerski, R. J. (1994). Specific association between the human DNA repair proteins XPA and ERCC1. *Proc. Natl Acad. Sci. USA*, **91**, 5012–5016.
31. Fields, S. & Song, O. (1989). A novel genetic system to detect protein–protein interactions. *Nature*, **340**, 245–246.
32. Binnig, G., Quate, C. F. & Gerber, C. (1986). Atomic force microscope. *Phys. Rev. Letters*, **56**, 930–933.
33. Moreno-Herrero, F., Herrero, P., Colchero, J., Baro, A. M. & Moreno, F. (2001). Imaging and mapping protein-binding sites on DNA regulatory regions with atomic force microscopy. *Biochem. Biophys. Res. Commun.* **280**, 151–177.

Edited by S. Reed

(Received 15 January 2002; received in revised form 15 April 2002; accepted 15 April 2002)