A 27 kDa protein binds to a positive and a negative regulatory sequence in the promoter of the *ICL1* gene from *Saccharomyces cerevisiae*

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Isocitrate lyase, encoded by *ICL1*, is one of the key enzymes of the glyoxylate pathway, which operates as an anaplerotic route for replenishing the tricarboxylic acid cycle; it is required for growth of *Saccharomyces cerevisiae* on carbon sources such as ethanol, but is dispensable when fermentable carbon sources are available. The positive regulation of the *ICL1* gene by an upstream activating sequence (UAS) element located between −397 and −388 has been previously reported. In this paper we show that the *ICL1* promoter sequence 5′-AGTCCGGACT-AGCATCCCAG-3′ located between −261 and −242 contains an upstream repressing sequence (URS) element. We have identified and partially purified a 27 kDa protein that binds specifically to both the UAS and URS sequences of the *ICL1* promoter. For both UAS and URS, binding requires the protein Snf1 (Cat1), a protein kinase essential for the derepression of genes repressed by glucose. Binding does not take place with extracts from glucose-grown strains, unless they lack Mig1, a negative regulatory protein involved in glucose repression.

INTRODUCTION

Control of gene expression is often mediated by the combined action of positive and negative *cis*-acting regulatory sequences in the corresponding promoters [1]. These control elements usually reinforce each other, expanding the range of expression available to the cell. A representative case of such a complex system is the regulation of the *GAL* genes in *Saccharomyces cerevisiae*. Glucose represses the expression of the *GAL* genes both by decreasing the level of the transcriptional activator Gal4 and by facilitating the operation of the inhibitor of transcription Mig1 [2].

Although there are many genes in yeast, whose expression is controlled by the available carbon source, the mechanisms underlying the regulation vary from case to case [3]. Nevertheless, two regulatory proteins appear to play a very general role in the control of these genes. One of them is the protein kinase Snf1(Cat1), which is required for derepression to occur [4,5], and the other is the zinc-finger protein Mig1, which is able to bind to the promoters of a large number of genes and which represses their transcription in the presence of glucose [6]. For isocitrate lyase, a key enzyme of the glyoxylate pathway, dispensable when fermentable carbon sources are present, but required during growth on sources such as ethanol, as an anaplerotic enzyme to replenish the tricarboxylic acid cycle [7,8], regulation occurs at different levels. Ethanol induces and glucose represses isocitrate lyase biosynthesis [9], glucose elicits reversible inactivation of the derepressed enzyme by phosphorylation [10] and also its proteolytic degradation [11–13].

As the *ICL1* gene was cloned [14,15] and its promoter sequence subjected to deletion analysis [16,17], an upstream activating sequence (UAS) element was identified in the promoter region [16–18]. Since this UAS element activates transcription only in the absence of glucose, it has been called a carbon-source-responsive element [17]; it is very similar to UASs from the gluconeogenic genes *FBP1* [19,20] and *PCK1* [21]. Deletion of this UAS element abolished expression of the *ICL1* gene [17,22], and band-shift experiments showed the formation of carbon-source-dependent protein complexes [18,19]. On the other hand, analysis of the *ICL1* promoter suggested also the existence of a second regulatory element in the region −295 to −169 [16]. This element would play a negative role, as its removal increased the expression of a reporter gene more than 10-fold.

We undertook therefore the study of the putative upstream repressing sequence (URS) element present in this region of the *ICL1* promoter. We identified a DNA sequence that acts as a URS in a reporter gene and interacts specifically with DNA-binding proteins. Interestingly, this URS competes with the formerly described UAS for protein binding and we have identified and partially purified a 27 kDa protein able to bind to both the URS and UAS elements. Moreover, the positive and negative regulatory elements appear to be regulated in parallel: both bind proteins only when the extracts derive from derepressed cells, and both respond in the same way to the absence of the regulatory proteins Snf1 and Mig1.

MATERIALS AND METHODS

Strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Bacterial transformation and large-scale preparation of plasmid

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

Strain	Genotype	Reference
AMW-13C^{+}	*MATa trp1(fs) ura3(fs) leu2-3,112 his3-11,15 can1*	14
H250	*MATα SUC2 ade2-1 can1-100 his2-11, 15 leu2-3, 112 trp1-1 ura3-1 mig1-δ2::LEU2*	6
MYC1595	*MATα snf1-Δ3 his4-539 lys2-801 ura3-52 his3-Δ200 SUC2*	23

Abbreviations used: DTT, dithiothreitol; UAS, upstream activating sequence; URS, upstream repressing sequence; YEPD, yeast extract–peptone–dextrose; YEPE, yeast extract–peptone–ethanol.

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This work is dedicated to the memory of Santiago Gascón, a good friend and colleague and an excellent scientist, who died on 7 July 1997.

DNA were performed in *Escherichia coli* MC1061 [*hsdR mcrB araD139*Δ(*araABC-leu*)*7679*Δ*lacx74 galU galK rpsL thi*].

Plasmid pNI9 is a derivative, lacking *TRP1* and autonomous replicating sequences, 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* [24].

Media, growth conditions and enzymic analysis

Yeasts were grown on 1% yeast extract and 2% peptone supplemented with 2% glucose (YEPD) or 3% ethanol (YEPE). The cells were grown on YEPD or YEPE until the A_{600} reached 2.0 (6.5 mg wet weight/ml). *snf1* mutant cells, which are unable to grow on ethanol, were grown on YEPD until the A_{600} reached 2.0 and were then transferred to YEPE for 8 h.

To select for transformants, synthetic medium with yeast nitrogen base, 2% glucose and the required supplements was used. β-Galactosidase activity was assayed as described previously [25].

General DNA techniques

Restriction enzymes and T4 DNA ligase were from Boehringer, and Sequenase Version 2.0 was from USB. Radioactively labelled compounds were from Amersham International. The dideoxy-ribonucleotide-chain-termination procedure was used for DNA sequencing analysis [26]. All other DNA manipulations were as previously described [27].

Construction of yeast strains with derivatives of a *CYC1::lacZ* fusion gene

A DNA fragment located between −331 and −164 in the promoter region of the *ICL1* gene was amplified from pICL-1.2 [14] by PCR, with primers OL1 (5′-ATCCTCGAGAAGCC-AATCACC-3′) and OL2 (5′-ATCCTCGAGAATTCCGAT-GTG-3′), which generate *Xho*I sites (underlined). The 167 bp fragment generated was cut with *Xho*I and inserted into the *Sal*I site of the polylinker region of plasmid pNI9. The resulting plasmid was digested with *Stu*I and integrated into the *URA3* locus of yeast strain AMW-13C$^+$, and the transformed yeasts were selected as uracil prototrophs. As controls, we constructed strains transformed with the original vector (pNI9) containing the *CYC1* activating sequences, a plasmid where the latter had been deleted (pNI17) or a plasmid (pNI-F141) containing a 141 bp fragment from the coding region (+263 to +404) of the *HXK2* gene [27]. For further experiments, plasmid pNI-OL20 was constructed, in which the double-stranded oligonucleotide $OL20_{ICL1}$ (see below) was inserted into the *Sal*I site of plasmid pNI9. The constructions were verified by sequence analysis. Single-copy integration of the different plasmids at the *URA3* locus was confirmed by Southern-blot analysis of genomic DNA digested with *Bgl*II using as probe a 1.1 kb *Hin*dIII–*Hin*dIII fragment containing the *URA3* gene.

DNA probes

Oligonucleotides, corresponding to both strands of the URS or the UAS element of the *ICL1* gene, were synthesized with an added TCGA nucleotide overhang at the 5′-terminus: $OL20_{ICL1sense}$, 5′-tcgAGTCCGGACTAGCATCCCAG-3′; $OL20_{ICL1antisense}$, 5′-tcgACTGGGATGCTAGTCCGGAC-3′; $OL18_{ICL1sense}$, 5′-tcgaGTTTCCATTCATCCGAGC-3′; $OL18_{ICL1antisense}$, 5′-tcgaGCTCGGATGAATGGAAAC-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. The labelled double-stranded $OL20_{ICL1}$ and $OL18_{ICL1}$ probes were also used for Southwestern-blot analysis.

For isolation of the $OL20_{ICL1}$- and $OL18_{ICL1}$-binding proteins, the oligonucleotides were biotinylated with biotin-14-dATP (Gibco/BRL) using the Klenow fragment.

Preparation of protein extracts

Yeast protein extracts were prepared as follows: yeast was grown on 10–20 ml of rich medium (YEPD or YEPE) at 28 °C to an A_{600} of 2.0 (6.5 mg wet weight/ml). Cells were collected, washed twice with 1 ml of 1 M sorbitol and suspended in 100 μl of buffer C [20 mM Hepes, pH 7.9, containing 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.42 M NaCl, 1.5 mM $MgCl_2$ and 25% glycerol]. The cells were broken by vortex-mixing (6 × 20 s) in the presence of glass beads (0.5 g), and 400 μl of buffer C was added to the suspension. After centrifugation at 19000 ***g*** (14000 rev./min) for 15 min at 4 °C, the supernatant was used as crude protein extract.

Gel retardation assays

Binding reaction mixtures contained 10 mM Hepes, pH 7.5, 1 mM DTT, 1–5 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 60 μg (6 μl) of protein extract and after 30 min of incubation at room temperature they were loaded on to a 4% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 10 V/cm of gel for 45 min 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.

Southwestern-blot analysis

The protocol is based on that described in [28] with the following modifications: protein extract (approx. 100 μg of protein in 10 μl) was mixed with 3.3 μl of buffer (250 mM Tris/HCl, pH 6.8, 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% 2-mercaptoethanol and 0.1% Bromophenol Blue) and incubated in a boiling-water bath for 4 min. Proteins were resolved by SDS/PAGE. After 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 (Bio-Rad) at 120 mA in transfer buffer (25 mM Tris/glycine, pH 8.8, 20% methanol). 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 DTT) supplemented with 6 M guanidinium chloride and gently rocked for 10 min at 4 °C. The nitrocellulose paper was transferred to binding buffer containing 3 M guanidinium chloride and gently rocked for 10 min at 4 °C. This procedure was repeated eight times, with each subsequent wash containing a twofold lower concentration of guanidinium chloride than in the previous one. The final wash step lacked guanidinium chloride. 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 (5 ng; about 10000 c.p.m.) was added to 0.25% gelatin/BG buffer and incubated with the nitrocellulose filter overnight at room temperature. The filter was then washed four times with binding

buffer for 7 min at room temperature, air-dried and autoradiographed at −70 °C with an intensifying screen.

Isolation of $OL20_{ICL1}$ (URS)- and $OL18_{ICL1}$ (UAS)-binding factors

The double-stranded DNA fragments $OL20_{ICL1}$ and $OL18_{ICL1}$ were ligated to form homopolymers of between 400 and 800 bp. The concatenamers 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 in 100 μl of the buffer used in the gel retardation assay. Streptavidin/agarose (0.5 ml; Sigma) was equilibrated with buffer 1 [10 mM Hepes, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM DTT, 0.2% Nonidet P40, 0.1 mM PMSF, 30 μg of poly(dI-dC)/ml] and added to the mixture of DNA and protein extract. The mixture was incubated with gentle rolling overnight at room temperature and then poured into a 1 ml column. The column was washed five times with 1 ml of buffer 2 (10 mM Hepes, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, 0.1 mM PMSF, 10% glycerol). The URS- and UAS-binding proteins were eluted stepwise using successively buffer 3 (10 mM Tris/HCl, pH 6.8, 50 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 0.2% Nonidet P40, 0.1 mM PMSF, 10% glycerol), buffer 4 (buffer 3 with 250 mM KCl), buffer 5 (buffer 3 with 500 mM KCl) and buffer 6 (buffer 3 with 750 mM KCl). In all cases 0.5 ml of buffer was added and 50 μl fractions were collected. The eluted proteins were loaded on to an SDS/polyacrylamide gel. The proteins were visualized by silver staining.

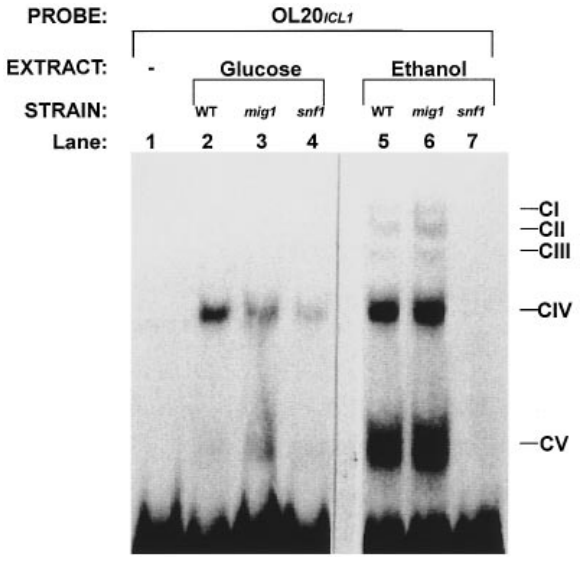

Figure 2 Influence of regulatory mutations on protein binding to the *ICL1* promoter negative element

Gel mobility-shift assays were performed with ^{32}P-labelled $OL20_{ICL1}$ and protein extracts from repressed or derepressed cells of the indicated strains, prepared as described in the Materials and methods section. Lane 1, no protein added; lanes 2–4, protein from repressed cells; lanes 5–7, protein from derepressed cells. WT, wild-type.

RESULTS

As a first step to characterizing the putative repressor element from the *ICL1* promoter, its capacity to decrease the expression of a reporter gene was tested. The *CYC1-lacZ* gene, present in plasmid pNI9, is highly expressed in yeast grown on ethanol. A significant decrease in β-galactosidase specific activity was observed after insertion into pNI9 of the fragment $F167_{ICL1}$ which contains the sequence −331 to −164 of the *ICL1* promoter (Figure 1). Insertion of 141 bp of the coding region of the *HXK2* gene, a sequence apparently lacking any regulatory element [27], has no effect in the *lacZ* gene expression. This might be taken as evidence for a URS located between −331 and −164 bp of *ICL1* promoter. However, since insertion of yeast DNA fragments of around 160 bp in a reporter gene can block β-galactosidase expression in an unspecific manner [29], it was imperative to test a much shorter element.

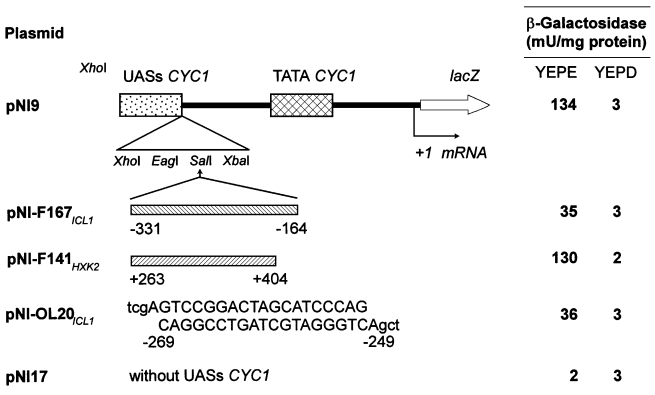

Figure 1 Effect of the insertion of a 167 bp fragment and a 20 bp double-stranded oligonucleotide from the *ICL1* promoter on the expression of β-galactosidase from transformants containing a *CYC1::lacZ* fusion gene

Plasmids pNI-F141, pNI-F167 and pNI-OL20 were constructed and integrated into the *URA3* gene of yeast strain AMW-13C$^+$, as described in the Materials and methods section. β-Galactosidase activity was determined in transformants grown on YEPE or YEPD as described in the Materials and methods section.

We chose a sequence between −261 and −250 in the $F167_{ICL1}$ fragment which appears to be protected against DNase I digestion in a footprint assay (results not shown) and which could be considered as a degenerate palindrome. When an oligonucleotide including this sequence ($OL20_{ICL1}$) was inserted into the heterologous *CYC1* promoter, we found that it caused a 70–75% decrease in β-galactosidase specific activity in ethanol-grown cells (Figure 1). These results indicated that the sequence responsible for the regulated repression of transcription was included in the $OL20_{ICL1}$ oligonucleotide.

This $OL20_{ICL1}$ double-stranded oligonucleotide was subjected to gel mobility-shift analysis using protein extracts obtained from wild-type and regulatory mutant strains (*snf1* and *mig1*) in a repressed or a derepressed state (Figure 2). We observed up to five different protein–DNA complexes (CI–CV) with protein extracts prepared from derepressed wild-type cells (Figure 2, lane 5). The CI, CII, CIII and CV complexes were absent when protein extracts from glucose-grown cells were used (Figure 2, lane 2). Competition assays with an unlabelled oligonucleotide indicated that the binding was specific in all cases (not shown). Regarding the effects of *mig1* and *snf1* deletion mutations on the formation of the complexes, it can be seen in Figure 2 (lanes 3 and 6) that complex CV could be formed when protein extracts from a repressed *mig1* mutant were used. The weak CI, CII and CIII complexes were only seen with derepressed extracts. On the other hand extracts from a *snf1* mutant gave only weak retardation signals in both repression and derepression conditions.

An inspection of the $OL20_{ICL1}$ sequence revealed that the 3′ end was similar to that of UAS_{ICL1} (Figure 3). As the retardation

URS$_{ICL1}$	C T A G C A T C C C A G
UAS$_{ICL1}$	C A T T C A T C C G A G C
UAS$_{TDH3}$	C T G G C A T C C A C T A T T C
UAS$_{TDH3}$	C T G G C A T C C A G A A A A A
URS$_{TDH3}$	A A A G A A T C C C A G C A C C
Consensus	C t N G C A T C C N a g

Figure 3 Comparison of sequences in UAS and URS regions of the *ICL1* and *TDH3* genes

Bases forming a degenerated palindrome in URS$_{ICL1}$ are marked with a dot. The *TDH3* gene encodes yeast glyceraldehyde-3-phosphate dehydrogenase.

pattern observed with OL20$_{ICL1}$ is also reminiscent of that reported for UAS$_{ICL1}$ [17,18], we explored the possibility that the same proteins could bind OL20$_{ICL1}$ and OL18$_{ICL1}$, an oligonucleotide containing the UAS sequence.

To look for proteins that bind to the regulatory sequences, we used the Southwestern-blotting technique. Protein extracts from cells exponentially growing on ethanol were subjected to SDS/PAGE, electroblotted on to a nitrocellulose sheet, renatured and probed with ^{32}P-labelled OL20$_{ICL1}$ and OL18$_{ICL1}$ oligonucleotides. Both of these probes bound to a protein of around 27 kDa (Figure 4A), which we called p27. The UAS probe (OL18$_{ICL1}$) also bound an approx. 18 kDa protein (Figure 4A, lane 2), which we called p18.

Partial purification of p27 was achieved by DNA-affinity chromatography using either OL20$_{ICL1}$ (Figure 4B) or OL18$_{ICL1}$ (Figure 4C) as ligand. Protein extracts from exponentially growing YEPE cells were incubated with biotinylated OL20$_{ICL1}$ or OL18$_{ICL1}$ 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 0.05 M KCl (Figure 4B, lane 3) or 0.5 M KCl (Figure 4C, lane 5). The protein bands at 27 kDa in both gels were able to bind to either of the oligonucleotides as demonstrated by Southwestern blotting (results not shown). On the other hand, no binding of other retained proteins was observed to either of the oligonucleotides. After SDS/PAGE of the two preparations, the p27 proteins were subjected to N-terminal Edman degradation in an automated protein sequencer, with negative results. As the proteins appeared to have a blocked N-terminus, internal peptides would be required to identify the genes coding for the proteins.

The partially purified proteins contained in the 0.05 M KCl and the 0.5 M KCl fractions obtained respectively from the OL20$_{ICL1}$ and OL18$_{ICL1}$ affinity-chromatography columns were also examined in gel mobility-shift assays with ^{32}P-labelled OL20$_{ICL1}$ and OL18$_{ICL1}$ oligonucleotides (Figure 5). These assays indicated that the CIV and CV complexes associated with the OL20$_{ICL1}$ oligonucleotide (Figure 5, lane 2) are similar to those obtained with the OL18$_{ICL1}$ oligonucleotide (Figure 5, lane 5). Specific binding of proteins to the OL20$_{ICL1}$ and OL18$_{ICL1}$ oligonucleotides was demonstrated by competition assays with the corresponding unlabelled oligonucleotides (Figure 5, lanes 3 and 6). Cross-competition assays (Figure 5, lanes 4 and 7) with the OL20$_{ICL1}$ and OL18$_{ICL1}$ oligonucleotides suggest that the same proteins are involved in both complexes.

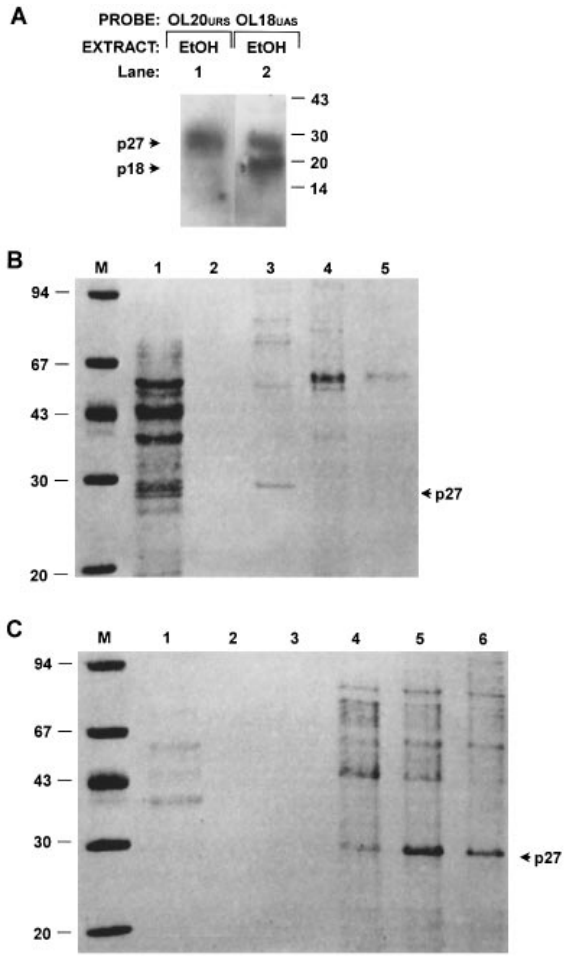

Figure 4 Binding of ^{32}P-labelled oligonucleotides to renatured protein blots (A) and partial purification of OL20$_{ICL1}$ (B) and OL18$_{ICL1}$ (UAS) (C) binding factors

(**A**) A protein extract from cells growing exponentially on YEPE was subjected to SDS/PAGE (12% polyacrylamide gel) and electrophoretically transferred to nitrocellulose. Proteins bound to nitrocellulose were renatured using guanidinium chloride. The blot was probed with ^{32}P-labelled OL20$_{ICL1}$ (lane 1) or ^{32}P-labelled OL18$_{ICL1}$ (lane 2). (**B**) and (**C**) Silver stain of proteins separated by SDS/PAGE (12% polyacrylamide gel). The gel was loaded with 10 μl of the pooled fractions from the OL20$_{ICL1}$ affinity-chromatography column (**B**) or the OL18$_{ICL1}$ affinity-chromatography column (**C**). Proteins were eluted from streptavidin/agarose columns with 10 mM KCl buffer (lane 1) until no proteins were detected (lane 2), then KCl concentration was increased step-wise at 50 mM (lane 3), 250 mM (lane 4), 500 mM (lane 5) and 750 mM (lane 6). The arrow points to the 27 kDa protein corresponding in size to the protein binding to OL20$_{ICL1}$ and OL18$_{ICL1}$ (UAS) in renatured protein blots.

Extracts from wild-type, *mig1* and *snf1* yeasts, in either a repressed or a derepressed state, were subject to affinity chromatography, as described above, using the OL20$_{ICL1}$ oligonucleotide as ligand. The fractions eluted at 0.05 M KCl gave a strong band at 27 kDa when derepressed extracts from a wild-type or a *mig1* mutant were used. The band was weaker for repressed extracts, from any background, and it was absent when the extract came from an *snf1* strain incubated under derepressing conditions. The different fractions were tested in a gel mobility-shift assay with the results shown in Figure 6. These results are similar to those observed using crude extracts (Figure 2). Complex CV formation requires an operational Snf1 and occurs only in derepression,

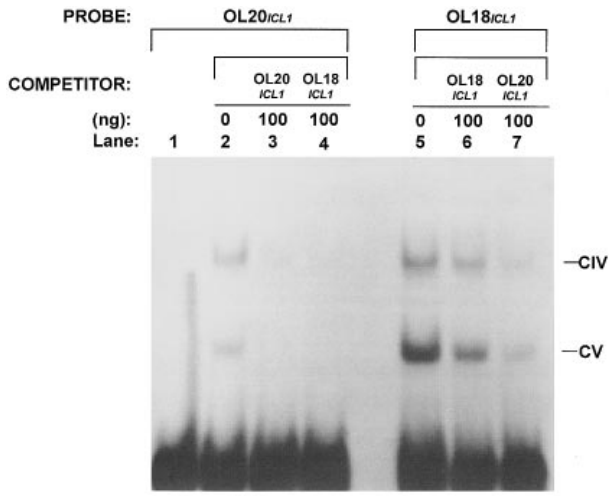

Figure 5 Competition in band-shift assays of the $OL20_{ICL1}$ and $OL18_{ICL1}$ oligonucleotides which contain regulatory sequences from the *ICL1* gene promoter

Each reaction mixture included 0.5 ng of ^{32}P-labelled $OL20_{ICL1}$ (lanes 1–4) or ^{32}P-labelled $OL18_{ICL1}$ DNA probe (lanes 5–7) and, except for lane 1, 6 μl of p27 protein partially purified from protein extracts from derepressed cells as described in the Materials and methods section. For lanes 2–4 the protein had been eluted at 0.05 M KCl from an $OL20_{ICL1}$ DNA-affinity chromatography column while for lanes 5–7 the protein was eluted at 0.5 M KCl from an $OL18_{ICL1}$ DNA-affinity chromatography column. The competitor for binding was 100 ng of unlabelled $OL20_{ICL1}$ (lanes 3 and 7) and 100 ng of unlabelled $OL18_{ICL1}$ (lanes 4 and 6).

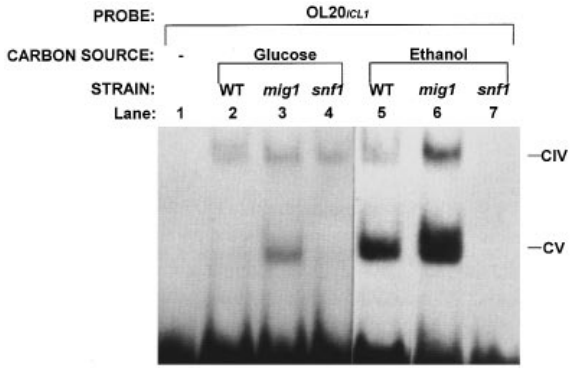

Figure 6 Influence of regulatory mutations on p27 protein binding to $OL20_{ICL1}$

Gel mobility-shift assays were performed with ^{32}P-labelled $OL20_{ICL1}$ and the protein eluted at 0.05 M KCl from an $OL20_{ICL1}$ DNA-affinity chromatography column. Lane 1, no protein added; lanes 2–4, protein purified from extracts obtained from repressed cells; lanes 5–7, protein purified from extracts obtained from derepressed cells. Similar results were obtained with the $OL18_{ICL1}$ probe (results not shown). WT, wild-type.

although a *mig1* mutation can counteract the repressive effect of glucose. Complex CIV is weaker and less sensitive to the repressing conditions.

DISCUSSION

We have observed that two elements from the *ICL1* promoter, which act as UAS and URS respectively in an heterologous system, compete for the same protein(s) in band-shift experiments. Analysis of these regulatory elements shows that they share an 8 bp sequence with a single change (Figure 3); this near-identity is probably sufficient to account for the competition observed. Such competition is unusual but not unique: in the promoter of *ENO1*, encoding enolase, UAS and URS elements have been described that are both able to bind to the regulatory protein Reb1 and that compete with each other for the binding of another protein unrelated to Reb1 [30]. A similar situation has been reported for the promoter of the *TDH3* gene, encoding triose-phosphate dehydrogenase [31]. It was shown that a 20 kDa nuclear protein, present in yeast cells grown on a non-fermentable carbon source, was able to bind to three 13 bp elements, two of them included in a UAS1 sequence, and the third one in a URS sequence from the *TDH3* promoter. Interestingly, when the sequence of these elements is compared with that of UAS_{ICL1} and URS_{ICL1} (Figure 3), a conserved sequence ATCC is found within a larger consensus sequence C(A/T)NGCATCCNAG, which looks like a degenerated palindrome. The UAS and URS elements from *ENO1*, on the other hand, show no homology to this consensus sequence [30].

For *TDH3*, it has been proposed that, during growth on non-fermentable carbon sources, the 20 kDa protein may compete with a positive regulatory factor binding UAS1, thus preventing UAS1-enhanced transcription. This same protein would compete with a negative regulatory factor binding URS, thereby relieving the repression of UAS2 by URS [31].

With respect to the *ICL1* promoter, a 27 kDa protein appears to bind both the UAS and URS sequences, although the affinity seems to be greater for the UAS, as 500 mM KCl was required to elute the protein during affinity chromatography on agarose gels ligated to oligomers derived from UAS_{ICL1} compared with 50 mM KCl when the purification was performed using oligomers derived from URS_{ICL1}. The fact that two complexes, CIV and CV, are observed in band-shift experiments with partially purified p27 protein suggests that p27 can bind, both to UAS_{ICL1} and URS_{ICL1}, as a monomer or a dimer. It appears likely that *in vivo* the regulatory elements bind more than one kind of protein molecule; specifically, the UAS_{ICL1} would bind at least an 18 kDa protein, in addition to the 27 kDa protein. At this stage it is not possible to establish whether any of these proteins are identical with the 20 kDa protein reported by Kuroda et al. [31] to bind the *TDH3* promoter.

The results presented indicate that in a wild-type background, p27 is only operative in derepressed cells. For a *mig1* mutant, formation of a DNA–protein complex can still be observed when extracts from repressed cells are used. On the other hand, formation of the complex is absolutely dependent on a functional Snf1 protein. A reasonable interpretation of these observations is that the transcription of the gene encoding p27 is itself subject to glucose repression and that Mig1 and Snf1 are involved in the repression and derepression of the p27 gene. Although we cannot be certain whether *mig1* and *snf1* mutations affect p27 synthesis or play a role in its capacity to bind DNA, our results suggest that the transcription factors Mig1 and Snf1 are involved in the regulation of p27 synthesis, as deduced from SDS/PAGE analysis of fractions eluted from the DNA-affinity chromatography columns. Alternative explanations are possible; for instance, p27 could be expressed constitutively and some other protein(s) regulating p27 activity would be the targets for glucose repression mediated by Mig1 and Snf1.

What would be the physiological meaning of having a UAS and a URS element controlled in parallel and not opposite to each other, as is generally the case? A working hypothesis could be as follows: as a yeast cell is starved for fermentable carbon sources, p27 is either synthesized *de novo* or converted into an active form. At an early time, the level of active p27 is low,

sufficient to activate UAS, but not to bind to URS, as this sequence has less affinity for the protein. In these conditions isocitrate lyase mRNA would be synthesized at a high rate; later on, the level of active p27 increases, the URS site becomes occupied and a lower steady-state rate of synthesis for isocitrate lyase mRNA is achieved. This would parallel the mode of regulation of some glycolytic enzymes, which are controlled by a delicate balance between positive and negative acting sequences in their promoters [31,32].

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