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The Ssn6–Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes

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The response of yeast to osmotic stress has been proposed to rely on the HOG–MAP kinase signalling pathway and on transcriptional activation mediated by STRE promoter elements. However, the osmotic induction of *HAL1*, an important determinant of salt tolerance, is HOG independent and occurs through the release of transcriptional repression. We have identified an upstream repressing sequence in *HAL1* promoter (URS_{HAL1}) located between –231 and –156. This promoter region was able to repress transcription from a heterologous promoter and to bind proteins in non-stressed cells, but not in salt-treated cells. The repression conferred by URS_{HAL1} is mediated through the Ssn6–Tup1 protein complex and is abolished in the presence of osmotic stress. The Ssn6–Tup1 co-repressor is also involved in the regulation of HOG-dependent genes such as *GPD1*, *CTT1*, *ALD2*, *ENA1* and *SIP18*, and its deletion can suppress the osmotic sensitivity of *hog1* mutants. We propose that the Ssn6–Tup1 repressor complex might be a general component in the regulation of osmostress responses at the transcriptional level of both HOG-dependent and -independent genes.

Keywords: Hal1/HOG pathway/signal transduction/Ssn6/Tup1

Introduction

All living organisms can tolerate adverse conditions to a varying degree. Damaging agents, such as high levels of UV radiation, heat shock, oxidative stress etc., trigger metabolic responses in order to counteract the eventual damage. At the cellular level, these responses involve both modulation of enzymatic activities and changes in gene expression, and require the participation of sensor systems and signal transduction pathways (Neidhardt *et al*., 1990; Nover, 1993; Serrano, 1996; Hohmann and Mager, 1997). The study of these responses in model organisms is expanding our knowledge of basic cellular processes such as DNA damage repair, mechanisms of protein folding and sensing and signalling systems. At the same time, the studies on the mechanisms of salt tolerance in the yeast *Saccharomyces cerevisiae* are leading to the understanding of basic principles of osmotic regulation and ion homeostasis in the cell (Ferrando *et al*., 1995; Hohmann, 1997; Serrano *et al*., 1997). This knowledge may provide the tools for engineering salt tolerance in cultivated plants (Serrano and Gaxiola, 1994; Serrano, 1996).

Salt is highly toxic to yeast cells due to the combination of two different effects: the accumulation of toxic sodium ions inside the cell and the loss of turgor pressure produced by the decrease in the osmotic gradient across the plasma membrane. Hal1p is a part of the cellular machinery that regulates intracellular cation concentrations (Gaxiola *et al*., 1992). Although Hal1p is not a transporter itself, it co-operates together with the *PMR2/ENA1* gene, encoding a P-type ATPase involved in Na^+ efflux (Haro *et al*., 1993), and other transport systems in keeping low intracellular Na^+/K^+ ratios during salt stress, relieving in this way sodium toxicity (Ríos *et al*., 1997). On the other hand, osmotic adjustment relies mainly on the transcriptional activation of the *GPD1* gene. This gene codes for a glycerol phosphate dehydrogenase and its induction is required for glycerol accumulation and the recovery of turgor after salt shock (Larsson *et al*., 1993; Albertyn *et al*., 1994). Transcriptional activation of *GPD1* and *ENA1/PMR2* during salt stress is controlled by the high osmolarity glycerol (HOG) signal transduction pathway (Albertyn *et al*., 1994; Márquez and Serrano, 1996). This pathway consists of a MAP kinase cascade coupled to two different osmosensors and is essential for growth in high osmolarity media (Brewster *et al*., 1993; Maeda *et al*., 1994, 1995; Posas *et al*., 1996; Posas and Saito, 1997). Hog1p, which is the MAP kinase component of the pathway, is quickly phosphorylated during osmotic stress and is also required for the osmotic induction of other stress genes, such as *GPP2*, involved in glycerol synthesis, *CTT1*, coding for cytosolic catalase, *HSP12* and *SSA3*, coding for heat shock proteins, *DDR2*, encoding a DNA damage-induced protein, *ALD2*, coding for a cytosolic aldehyde dehydrogenase etc. (Schüller *et al*., 1994; Miralles and Serrano, 1995; Varela *et al*., 1995; Akhtar *et al*., 1997). The contribution of many of these genes to salt tolerance, if any, is not yet clear.

At the lower part of the HOG pathway, responsive genes contain a positive promoter element (STRE) with the consensus sequence CCCCT or AGGGG (Kobayashi and McEntee, 1993; Marchler *et al*., 1993; Schüller *et al*. 1994). This element activates transcription not only in response to osmotic stress, but also in response to other types of stresses which do not activate the HOG pathway, such as heat shock, low pH or oxidative stress, and is modulated negatively by high levels of activity of the cAMP-dependent protein kinase (protein kinase A; PKA) (Marchler *et al*., 1993). Two transcription factors, Msn2p and Msn4p, recently have been found to bind the STRE element (Martínez-Pastor *et al*., 1996; Schmitt and McEntee, 1996) and to be important for STRE-activated transcription (Martínez-Pastor *et al*., 1996).

There are indications, however, that the osmotic response of the yeast involves additional components. For example, while the STRE-mediated response to heat shock and low pH is completely abolished in a *msn2/msn4* double mutant, osmotic induction through the STRE element is still possible, suggesting that there might be other transcription factors involved (Martínez-Pastor *et al*., 1996). On the other hand, in addition to the STRE-activating sequences, the promoters of some stress genes, such as *CTT1* and *HSP12*, have been reported to contain negative elements (Belazzi *et al*., 1991; Varela, *et al*., 1995), although their participation in the induction by stress has not been studied. Moreover, salt induction of the *ENA1* gene is only partially dependent on the HOG pathway. We have reported recently that the calcineurin pathway, which was already reported to activate *ENA1* gene transcription (Nakamura *et al*., 1993; Mendoza *et al*., 1994; Ferrando *et al*., 1996), is triggered by high levels of Na^+ ions and not by osmotic stress. This pathway can induce *ENA1* gene expression in mutants lacking the HOG pathway (Márquez and Serrano, 1996). Finally, the DNA damage-induced gene *DDR48* recently has been found to be induced by osmotic stress in a HOG-independent manner (Miralles and Serrano, 1995). Thus, the response to salt stress seems to involve different signalling pathways.

In the present work, we find that the osmotic induction of *HAL1* is independent of the HOG MAP kinase pathway and does not require stress-activated STRE sequences. Instead, the regulation of *HAL1* occurs through a mechanism of transcriptional repression which is counteracted by osmotic stress. This mechanism requires the participation of the –231/–156 promoter region and the Ssn6–Tup1 co-repressor. We also demonstrate that the Ssn6–Tup1 complex is involved in the control of the expression of HOG-dependent genes, such as *CTT1*, *ENA1*, *ALD2* and *SIP18*, and provide evidence that at least part of the mechanism for HOG-dependent transcriptional activation could involve removal of repression by the Ssn6–Tup1 system.

Results

HAL1 expression is HOG independent and does not require an STRE promoter element

HAL1 is an osmotic stress-induced gene that modulates intracellular ion homeostasis and which confers tolerance to salt stress by overexpression (Gaxiola *et al*., 1992; Ríos *et al*., 1997). The *HAL1* promoter contains a putative STRE consensus sequence (CCCCT) located at –399 (see Figure 1). This element has been shown to be the target for the HOG MAP kinase cascade in several gene promoters (Schüller *et al*., 1994; Varela *et al*., 1995). In order to test whether *HAL1* osmotic induction was controlled through the HOG pathway, a plasmid carrying a *HAL1–LacZ* fusion (pRS909) (Gaxiola *et al*., 1992) was transformed into strains YPH499 (wild-type), JBY10 (*hog1*) and MAY1 (*pbs2*), corresponding to wild-type and mutants defective in the HOG pathway, respectively (Brewster *et al*., 1993), and the expression of the reporter gene determined under different growth conditions. As shown in Figure 2A, the salt induction of *HAL1* was not prevented by null mutations in either *HOG1* or *PBS2* genes, encoding the MAP kinase and MAP kinase kinase, respectively, of the HOG pathway (Brewster *et al*., 1993). Actually, the induction of *HAL1* is greater in the HOG mutants than in the wild-type strain. The same increase in *HAL1* gene response was observed by Northern blot, while, under the same conditions, the HOG-dependent *CTT1* gene showed no induction in the *hog1* and *pbs2* mutants (data not shown).

To investigate the participation of the HOG pathway in *HAL1* gene expression further, the putative STRE sequence (Kobayashi and McEntee, 1993; Marchler *et al*., 1993) present in the *HAL1* promoter was subjected to site-directed mutagenesis. When the CCCCT sequence at position –399 (see Figure 1) was changed to CTCGT, no change in *HAL1* gene expression under basal or induced conditions was observed (Figure 2B). However, these mutations have been reported to abolish stress-induced expression through STRE (Marchler *et al*., 1993). Furthermore, removal of the STRE-like sequence by 5′ deletion did not produce significant changes in *HAL1* gene expression (see below, Figure 4).

HAL1 expression is negatively modulated by protein kinase A

The induction of *CTT1* in response to a variety of stress conditions was shown to be modulated negatively by the levels of activity of PKA (Marchler *et al*., 1993). This effect has also been found in other HOG-dependent genes such as *HSP12* (Varela *et al*., 1995), *ALD2*, *PAI3*, *SIP18* (Miralles and Serrano, 1995) and *SSA3* (Boorstein and Craig, 1990). We have investigated whether the expression of the HOG-independent gene *HAL1* is altered in PKA mutants. As shown in Figure 3, salt induction of *HAL1* was abolished in the strain S13-58A (*bcy1 TPK1 tpk2 tpk3*) with constitutive high levels of PKA activity (Nikawa *et al*., 1987). Conversely, strain S13-58A-1 (*bcy1 tpk1^{w1} tpk2 tpk3*), displaying low levels of PKA activity, showed increased levels of *HAL1* mRNA in the absence of NaCl as compared with the wild-type strain. Interestingly this strain still displayed some induction of *HAL1* gene expression, in spite of the fact that it is not responsive to cAMP.

HAL1 gene expression is controlled through a negative promoter element

Our results suggested that *HAL1* expression is controlled through a mechanism different from the HOG–STRE pathway. In order to identify the promoter elements responsible for the osmotic induction of *HAL1*, 5′ serial deletions were generated in its upstream control region by PCR. The deleted promoters were then fused to the *LacZ* open reading frame (ORF) (Myers *et al*., 1986) (see Materials and methods), integrated into the yeast RS-16 strain and β-galactosidase activity was assayed under different growth conditions (Figure 4). As depicted in Figure 4, deletions from –401 to –231 did not produce significant changes in expression, but when the region between –231 and –156 was removed (pJAM138), the levels of expression in the absence of salt increased >10 times and no significant induction was obtained by the addition of NaCl. On the other hand, when this element was placed in a deregulated version of the *HAL1* gene promoter (pJAM139), it was able to repress transcription and thereby conferred responsiveness to osmotic stress (pJAM155). These results suggest that the region between –231 and –156 is involved in the osmotic induc-

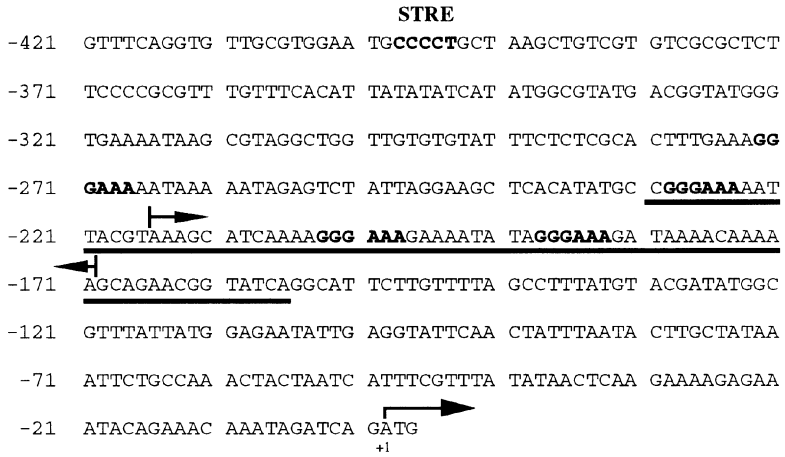

Fig. 1. *HAL1* gene promoter region. The *HAL1* promoter region is depicted from the ATG start codon (position +1) to position –421. URS_{HAL1}, spanning the region between –231 and –156, is underlined. The STRE-like sequence and the four repetitions of the motif GGGAAA are depicted in bold. The arrows in the central region of URS_{HAL1} indicate the 46 bp fragment utilized in pAPA3.

tion of *HAL1* and that it acts as an upstream repressing sequence. Therefore, we name this region URS_{HAL1}.

In order to test the ability of URS_{HAL1} to repress transcription from a heterologous promoter, a 75 bp fragment spanning this region (see Figure 1) was amplified by PCR, introducing *Sal*I flanking sites, and cloned into the *Sal*I site of pLGΔ312S (Guarente and Mason, 1983). This plasmid contains a promoter–reporter fusion between the wild-type *CYC1* gene promoter and the *LacZ* ORF, and displays high levels of β-galactosidase activity under normal growth conditions. This makes it suitable for the characterization of negative *cis* elements (Johnson and Herskowitz, 1985; Keleher *et al*., 1992). The activity of this reporter is slightly decreased under salt stress (Figure 5).

When URS_{HAL1} was inserted between the UAS and TATA elements of the *CYC1* gene (pJAM181), a strong decrease in expression was observed in normal medium (Figure 5). When two copies of the element were inserted, the effect was even stronger. The expression reverted to normal (pJAM181) or even higher (pJAM187) levels than the wild-type *CYC1* construct when the strains were assayed in medium with 1 M NaCl, indicating that the repression ability is salt dependent. Similar results were obtained by cloning URS_{HAL1} in the opposite orientation (pJAM184) or in the *Sma*I site upstream of *CYC1* UAS (data not shown).

The most conspicuous feature of URS_{HAL1} is the repetition of the motif GGGAAA. Particularly, two direct repeats of the GGGAAAGA motif lie in the central part of the element. However, a 46 bp fragment spanning this central region of the *HAL1* negative element (from –216 to –170, see Figure 1) was able neither to repress nor to confer salt-regulated transcription on the *CYC1* promoter (pAPA3, Figure 5).

In vitro DNA–protein complex formation correlates with the repressing activity of URS_{HAL1}

In order to investigate whether the URS_{HAL1} sequence can bind protein factors, we performed band-shift experiments. The 75 bp –231/–156 fragment from the *HAL1* promoter was labelled and incubated with nuclear proteins extracted from unstressed and salt-treated cells (Figure 6). When the

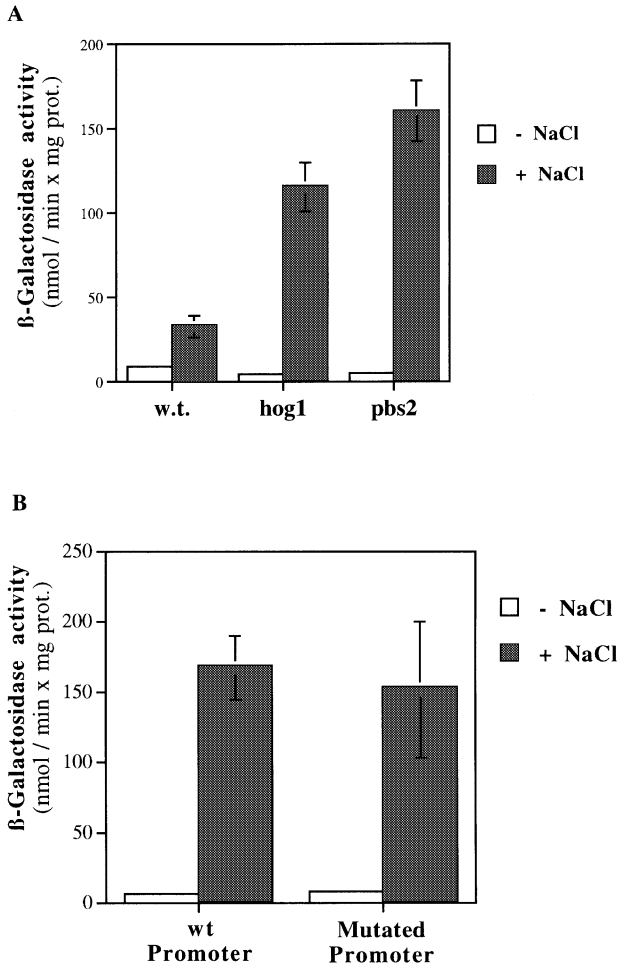

Fig. 2. Effects on *HAL1* expression of mutations in the HOG pathway. (**A**) Plasmid pRS-909, carrying a *HAL1–LacZ* fusion, was transformed into strains YPH499 (wild-type), JBY10 (*hog1*) and MAY1 (*pbs2*). β-Galactosidase activity was assayed in extracts from cells grown in the absence or presence of 0.5 M NaCl. (**B**) The pRS-909 plasmid was submitted to site-directed mutagenesis to change the putative STRE sequence of the *HAL1* promoter from CCCCT to CTCGT. β-Galactosidase activity was assayed in extracts from cells carrying the original pRS-909 plasmid, wild-type promoter or the mutated promoter in the presence or absence of 1 M NaCl.

probe was incubated with protein extracts from unstressed cells, a protein binding to URS_{HAL1} could be detected (marked with an arrow, lane 2 of Figure 6). The sequence specificity of this complex was proven by competition. The corresponding band disappeared when a 20-fold excess of the cold fragment was included in the assays (lane 3), but remained upon competition with the 46 bp fragment from pAPA3 (lane 4) that was not able to confer repression to the CYC1 promoter. Interestingly, the complex was not formed (or at least was modified) when nuclear proteins extracted from salt-treated cells were used (lanes 5–7). Thus, these experiments show that URS_{HAL1} binds protein(s) under normal growth conditions and that the formation of this complex correlates with the repression ability of the different DNA fragments and protein extracts.

HAL1 repression operates through the Ssn6–Tup1 general repressor

Many of the stress responses in yeast seem to operate through the combination of upstream activating sequences (UASs) and transcriptional activators, such as the Hsf1 and Msn2/Msn4 proteins involved in the heat shock response (Martínez-Pastor *et al*., 1996; Schmitt and McEntee, 1996; Piper, 1997) or the Yap1 and Yap2 activators involved in oxidative stress (Kuge and Jones, 1994). However, the *HAL1* gene is controlled through a different mechanism. The results described above suggest that *HAL1* gene transcription is regulated through a repressor whose function is inactivated by osmotic stress. Nevertheless, there is evidence that the expression of other stress genes is also controlled through transcriptional repression. This is the case for the *RNR 1*, *2* and *3* genes induced by DNA damage, or *ANB1* and *CYC7* induced during hypoxia. These genes contain negative promoter elements that repress transcription in the absence of stress (Lowry and Zitomer, 1988; Elledge and Davis, 1989; Hurd and Roberts, 1989; Deckert *et al*., 1995), and in both cases repression occurs through a general repressor complex encoded by *SSN6* and *TUP1* genes (Zhou and Elledge, 1992; Deckert *et al*., 1995). This information prompted us to study the expression of the *HAL1* gene in a *ssn6*-Δ9 mutant background lacking activity of the Ssn6–Tup1 general repressor (Schultz *et al*., 1990; Struhl, 1995).

As shown in Figure 7A, *HAL1* mRNA levels were strongly increased in the MCY1974 strain (*ssn6*-Δ*9*) as compared with the wild-type MCY829, although some induction (2-fold) was still achieved by the addition of salt. The Ssn6–Tup1 repressor is also required to regulate expression from plasmid pJAM181, in which URS_{HAL1} has been inserted between the UAS and TATA elements of the *CYC1* gene (Figure 7B). These results suggest that *HAL1* gene transcription is controlled by the Ssn6–Tup1 protein complex, and that this co-repressor operates through the URS_{HAL1} element.

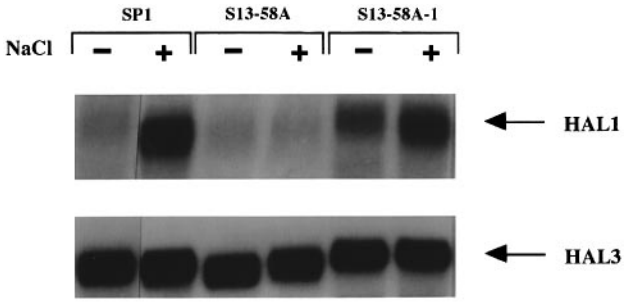

Fig. 3. *HAL1* expression is negatively modulated by protein kinase A. Northern analysis of total RNA from strains SP1 (wild-type), S13-58A (*bcy1 TPK1 tpk2 tpk3*) and S13-58A-1 (*bcy1 tpk1^{w1} tpk2 tpk3*) grown in the absence (–) or presence (+) of 1 M NaCl. The *HAL3* gene, showing constitutive expression (Ferrando *et al*., 1995), was used as loading control.

Repression through the Ssn6–Tup1 complex is a common feature of osmotic stress genes

Our results indicate that transcriptional repression is the major mechanism responsible for the osmotic induction of the *HAL1* gene. This situation is clearly distinct from the proposed regulation of HOG-dependent genes, where transcriptional activation occurs through STRE, which acts as a positive promoter element (Marchler *et al*., 1993; Schüller *et al*., 1994). In order to study whether repression

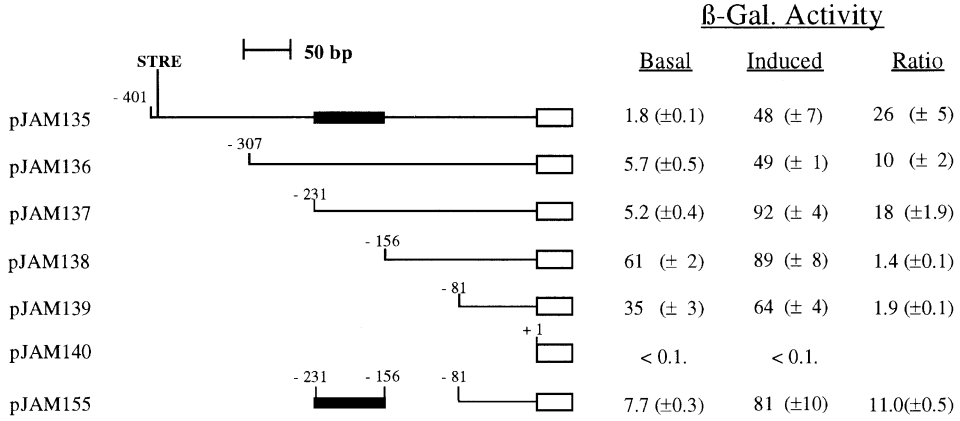

Fig. 4. Deletion analysis of the *HAL1* promoter. 5′ Serial deletions were generated in the promoter region of a *HAL1–LacZ* fusion and the resulting constructs were integrated into strain RS-16. A scheme of the different promoters is presented on the left. Numbers refer to nucleotide positions. Position +1 corresponds to the first nucleotide of the start codon (ATG) and position –1 corresponds to the nucleotide preceding it. The black box represents the negative element of the *HAL1* promoter identified in the present work (URS_{HAL1}, see Figure 1). The open box is the beginning of the reading frame. The position of the putative STRE sequence is indicated. β-Galactosidase activity was assayed in extracts from cells grown in the absence (basal) or presence (induced) of 1 M NaCl. Values represent the specific activity (nmol/min×mg protein) and are the average of three determinations, with standard deviations in parentheses.

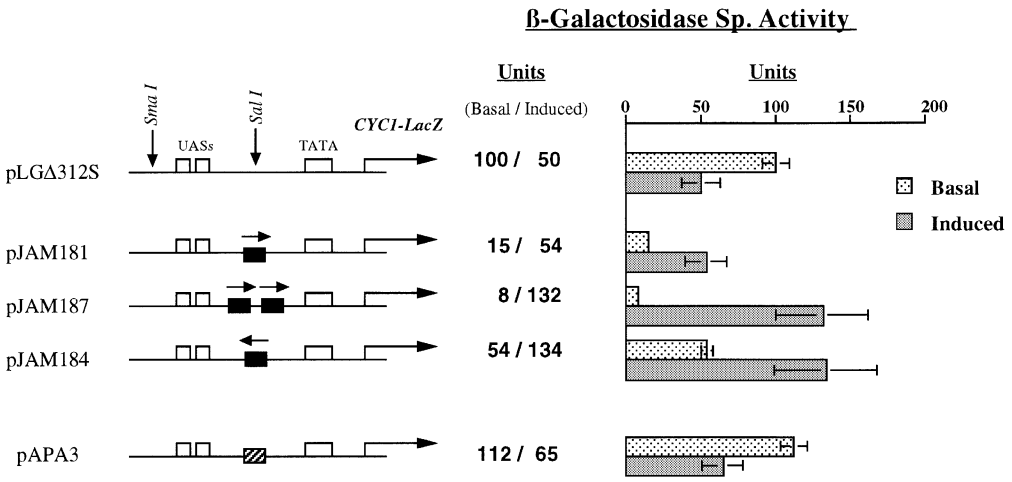

Fig. 5. URS_{HAL1} represses transcription in a heterologous promoter. URS_{HAL1} (black box) was cloned in the *Sal*I site of pLGΔ312S carrying a wild-type yeast *CYC1* gene promoter fused to the *E.coli LacZ* ORF in multicopy plasmids. The 46 bp fragment spanning the central region of URS_{HAL1} (hatched box) was also cloned in the *Sal*I site of pLGΔ312S. All the constructs were transformed into a RS-16 yeast background. The resulting strains were grown in the presence (induced) and absence (basal) of 1 M NaCl, and β-galactosidase activity was assayed. The activity in units (nmol/min×mg protein) refers to average values from at least three independent transformants.

could also take part in the control of expression of other salt-induced genes, we investigated the mRNA levels of several salt-induced genes in the *ssn6-Δ9* mutant strain by Northern blot.

As depicted in Figure 8, the HOG-dependent *GPD1*, *CTT1*, *ALD2* and *ENA1* genes as well as the HOG-independent *DDR48* gene (Miralles and Serrano, 1995) showed an increased basal expression in the *ssn6-Δ9* mutant. Interestingly, while *DDR48* and *ENA1* genes showed a completely deregulated pattern of expression in the *ssn6-Δ9* mutant, *GPD1*, *CTT1* and *ALD2* were induced to different extents by the addition of NaCl to the medium. The *SIP18* gene did not show increased expression in the *ssn6-Δ9* mutant in the absence of NaCl. However, its expression was strongly increased by the addition of salt in the *ssn6-Δ9* strain as compared with the wild-type. An identical pattern of expression was found in the case of the *PAI3* gene (data not shown), which is also HOG dependent (Miralles and Serrano, 1995).

We then investigated the tolerance of the *ssn6-Δ9* mutant to different stresses. Exponentially grown cultures of MCY829 (wild-type) and MCY1974 (*ssn6-Δ9*) strains were submitted to salt stress, oxidative stress and heat shock, and the rates of survival were determined (see Materials and methods). As depicted in Figure 9, the *ssn6-Δ9* mutant has an increased survival capability both in 1 M NaCl (16 ± 7% versus 37 ± 6%) and after oxidative stress (3 ± 2% versus 51 ± 9%), in accordance with the increased expression levels of defence genes such as *ENA1*, *HAL1* and *CTT1*. The *ssn6-Δ9* mutant, however, did not show increased tolerance to heat shock.

Ssn6 and Tup1 null mutations can suppress the osmotic sensitivity of HOG1 mutants

These results suggested that both HOG-dependent and HOG-independent genes are repressed by the Ssn6–Tup1 system under normal growth conditions. While in some cases, such as *ENA1* or *DDR48*, removal of repression seems to be sufficient to acquire maximal levels of transcription, in others, such as *GPD1*, *CTT1*, *ALD2*, *SIP18* and *PAI3* genes, osmotic stress can still increase

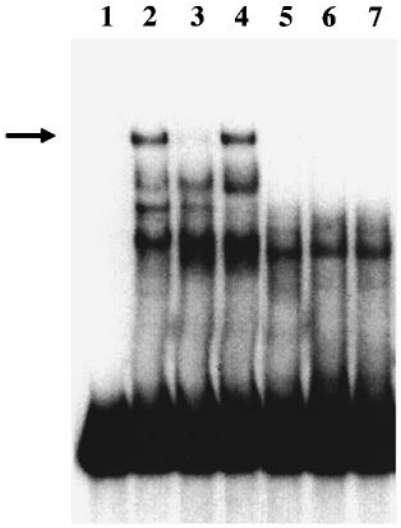

Fig. 6. URS_{HAL1} can bind protein(s) from cells under normal growth conditions, but not from cells under salt stress. Gel retardation experiments were carried out with the ^{32}P-labelled URS_{HAL1} fragment and protein extracts from the RS-16 strain. Lane 1, ^{32}P-labelled 75 bp URS_{HAL1}; lane 2, plus 15 μg of protein extract from cells grown in normal medium; lane 3, plus a 20-fold molar excess of unlabelled URS_{HAL1}; lane 4, as lane 2 plus a 20-fold molar excess of the unlabelled 46 bp fragment of pAPA3; lanes 5–7, ^{32}P-labelled 75 bp URS_{HAL1} plus 10, 20 and 30 μg, respectively of proteins extracted from cells grown in 1 M NaCl. The specific DNA–protein complex is indicated by an arrow.

transcription, suggesting the contribution of additional factors. It is noteworthy that, as depicted in Figure 8, some of the HOG-dependent genes are transcribed to high levels in a *ssn6-Δ9* mutant even in the absence of salt stress. Transcription of the HOG-dependent *ENA1* gene cannot be activated further by salt stress in the repression-deficient background. This means that the contribution of the HOG pathway, which is activated by osmotic stress, is not required for high levels of transcription of these genes in the *ssn6*$^-$ background, and suggests that at least part of the effects of the HOG pathway on gene transcription could be explained by the release of the Ssn6–

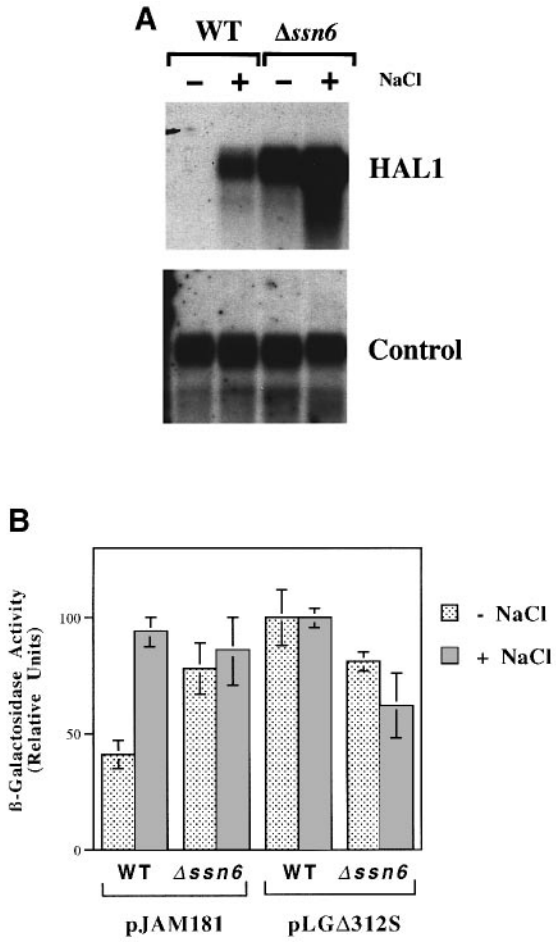

Fig. 7. The *HAL1* gene is repressed through the Ssn6–Tup1 system. (**A**) Total RNA was extracted from wild-type MCY829 (WT) and MCY1974 (Δ*ssn6*) strains grown in the presence (+) or absence (–) of 1 M NaCl. After electrophoresis and blotting, *HAL1* mRNA levels were assayed with specific radioactive probes. The constitutive *HAL3* gene was used as loading control. (**B**) The pJAM181 plasmid carrying URS_{HAL1} cloned in the *CYC1* promoter region and the control plasmid pLGΔ3125 were transformed into strains MCY829 (WT) and MCY1974 (Δ*ssn6*), and β-galactosidase activity was assayed after growth in normal and salt-containing (1 M NaCl) medium.

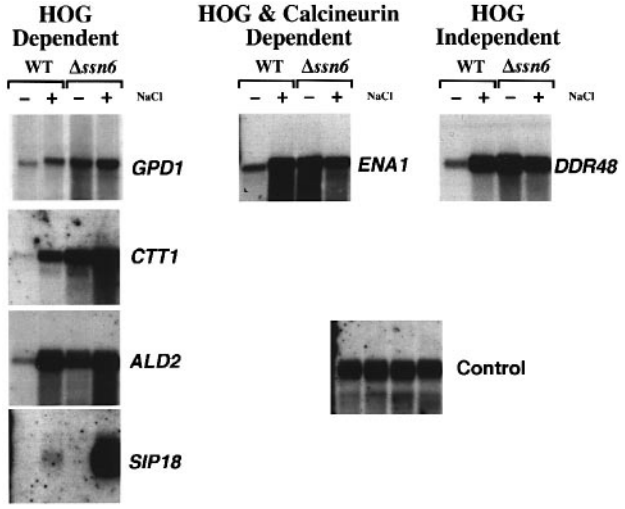

Fig. 8. Repression through the Ssn6–Tup1 system is a feature common to HOG-dependent and HOG-independent genes. Total RNA was extracted from wild-type MCY829 (WT) and MCY1974 (Δ*ssn6*) strains grown in the presence (+) or absence (–) of 1 M NaCl. After electrophoresis and blotting, mRNA levels of different genes were assayed with specific radioactive probes. The constitutive *HAL3* gene was used as loading control.

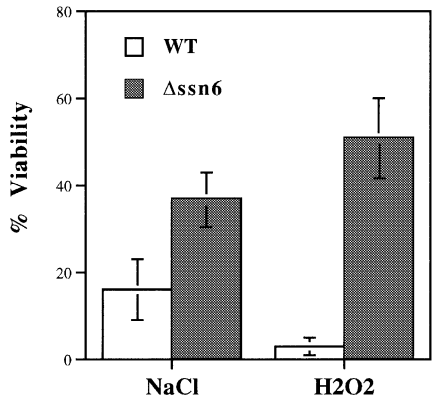

Fig. 9. The *ssn6* mutant has an increased tolerance to salt and oxidative stress. Wild-type MCY829 (WT) and MCY1974 (Δ*ssn6*) strains were grown to mid-exponential phase and different aliquots were then plated on YPD alone or on YPD plus 1 M NaCl, or treated with 3.5 mM H_2O_2 for 30 min and then plated on YPD. Survival rate was calculated by comparing the number of colony-forming units before and after the treatments.

Tup1-dependent repression. As a consequence, mutants lacking the Ssn6–Tup1 general repressor could have a potential ability to suppress the osmotic sensitivity of mutants in the HOG pathway. In order to investigate this possibility, *ssn6* and *tup1* null mutations were introduced in JBY10 (*hog1*) strains (Brewster *et al*., 1993), generating strains MAP-8 and MAP-7, respectively, corresponding to wild-type and *hog1* mutant, respectively. As presented in Figure 10, the *hog1* mutant, cannot grow in medium with high osmolarity. However, this growth defect is suppressed by the introduction of either *ssn6* or *tup1* null mutations. In order to rule out the possibility that the results in the complementation experiments were due to an indirect effect, Northern blot experiments were carried out to determine the level of expression of HOG-dependent genes during salt stress in wild-type, *hog1* single mutant and *hog1 ssn6* double mutant strains. As shown in Figure 11, transcription of *GPD1*, *CTT1* and *ENA1* genes during salt stress is strongly reduced in the *hog1* mutant. This decrease, however, is counteracted by the introduction of the *ssn6* null mutation. Similar results were obtained with a *hog1 tup1* double mutant.

Discussion

HAL1 is a major determinant of salt tolerance in yeast that co-operates together with *ENA1* and other genes in keeping low intracellular concentrations of Na^+ ions. *HAL1* and *ENA1* gene expression is activated during salt stress but, in spite of the fact that neither of the two genes can contribute to osmotic tolerance, both are also activated at the transcriptional level by solutes with no ionic toxicity, such as KCl or sorbitol (Gaxiola *et al*., 1992; Márquez and Serrano, 1996). *ENA1*, as well as other osmotically induced genes, is controlled by the HOG signalling pathway (Brewster *et al*., 1993; Márquez and Serrano, 1996). This MAP kinase pathway is activated rapidly during

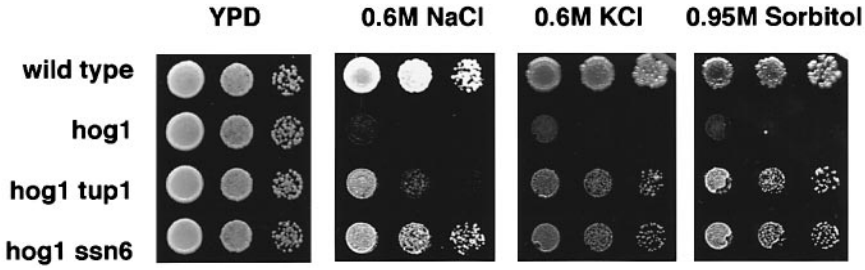

Fig. 10. Null mutations in the *SSN6* or *TUP1* gene suppress the osmotic sensitivity of the *hog1* mutant. *tup1* and *ssn6* null mutations were introduced in JBY10 (*hog1*) background. Growth of the strains JBY10 (*hog1*), MAP7 (*hog1 tup1*) and MAP8 (*hog1 ssn6*) in high osmolarity media is compared with growth of the wild-type YPH499 strain. Saturated cultures from each strain were diluted, spotted in different media and incubated at 30°C.

osmotic shock (Brewster *et al*., 1993; Maeda *et al.*, 1994) and it can activate transcription of a number of genes through the STRE *cis*-acting element (Marchler *et al*., 1993; Schüller *et al*., 1994). However, in the present work, we demonstrate that the osmotic induction of *HAL1* occurs through a different mechanism.

The osmotic induction of *HAL1* is HOG independent, as it can occur in *hog1* and *pbs2* null mutants. Accordingly, although the *HAL1* promoter contains a STRE-like element, it is totally dispensable for *HAL1* osmotic induction. This is the second gene reported to be induced by osmotic stress in a HOG-independent manner. In a recent work (Miralles and Serrano, 1995), a genomic locus containing a cluster of four genes, all of them inducible by osmotic stress, has been isolated. Three of them, *ALD2*, *PAI3* and *SIP18*, where found to be HOG dependent, while the fourth, *DDR48*, was found to be HOG independent. Remarkably, the HOG-dependent *ALD2* gene was expressed maximally at 0.3 M NaCl, corresponding to the maximal level of phosphorylation of the Hog1p MAP kinase (Brewster *et al.*, 1993), while both *HAL1* and *DDR48* require 1 M NaCl to be induced maximally. HOG-independent activation of the *ENA1* gene by high salt stress has been described to be mediated by the calcium- and calmodulin-dependent protein phosphatase calcineurin (Nakamura *et al*., 1993; Mendoza *et al*., 1994). This pathway, however, is activated specifically by Na^+, and not by osmotic stress (Márquez and Serrano, 1996). On the other hand, *HAL1* and *DDR48* induction is not altered in mutants lacking the regulatory subunit of calcineurin that prevents activity of the phosphatase (A.Ferrando and R.Serrano, unpublished). These observations might indicate the existence of an as yet unidentified signal transduction pathway controlling the expression of *HAL1* and *DDR48* genes in response to severe osmotic stress. Recently, it has been found that when yeast cells are treated with high osmotic stress (0.9 M NaCl or 1.4 M sorbitol) they rapidly accumulate high levels of phosphatidylinositol-3,5-biphosphate (Dove *et al*., 1997). Interestingly, the accumulation of this metabolite was HOG independent and did not occur at lower concentrations of salt.

A surprising observation is that *HAL1* gene induction at 0.5 M NaCl is higher in *hog1* and *pbs2* mutants than in the wild-type. This result apparently indicates that the HOG pathway negatively affects the activation of the pathway controlling *HAL1* expression. However, the biological significance of such a negative interaction is difficult to understand, since expression of both HOG-dependent and HOG-independent genes positively contribute to the adaptation to salt stress. A more likely explanation, however, comes from the realization that *hog1* and *pbs2* mutants are unable to make proper osmotic adjustment, as they have a reduced ability to synthesize glycerol. As a consequence, a relatively weak osmotic stress (0.5 M) might cause effects in the *hog1* mutant similar to those caused in the wild-type by higher concentrations of osmoticum, leading to increased expression of HOG-independent genes.

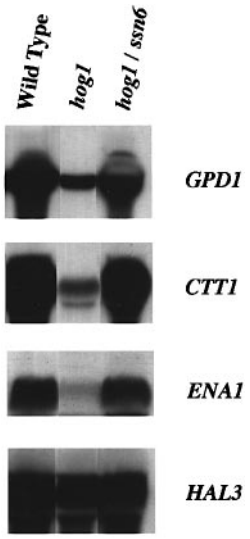

Fig. 11. A null mutation in the *ssn6* gene can restore high levels of stress gene transcription in a *hog1* mutant background. Total RNA was extracted from YPH499 (wild-type), JBY10 (*hog1*) and MAP8 (*hog1 ssn6*) strains after a 30 min shock with 0.5 M NaCl. After electrophoresis and blotting, the levels of several HOG-regulated genes were determined with specific radioactive probes. The constitutive *HAL3* gene was used as loading control.

Analysis of the *HAL1* promoter has revealed that the osmotic induction of the gene is controlled mainly through a negative promoter region located between –231 and –156. When this region is deleted, *HAL1* transcriptional activity is increased, and no further induction is obtained. This element is also able to repress transcription and to confer osmotic responsiveness to the *CYC1* promoter. Thus, the –231/–156 region qualifies as a URS (URS_{HAL1}). On the other hand, band-shift experiments indicate that URS_{HAL1} can form a specific DNA–protein complex in non-stressed cells, but this cannot be formed or is modified in salt-stressed cells. These results together strongly suggest that *HAL1* gene transcription is controlled through a

transcriptional repressor whose function is inactivated by osmotic stress.

Repression of the *HAL1* gene requires the general repressor complex Ssn6–Tup1, since *HAL1* gene transcription was derepressed in a *ssn6*-Δ*9* mutant background. The Ssn6–Tup1 general repressor is involved in transcriptional regulation of genes with different patterns of expression. It is required for repression of cell type-specific genes, glucose-repressed genes, hypoxic genes and also genes involved in flocculation (Johnson and Herskowitz, 1985; Keleher *et al*., 1992; Tzamarias and Struhl, 1995; Struhl, 1995). On the other hand, the Ssn6–Tup1 protein complex cannot bind DNA by itself. Instead it is recruited to different promoters through interaction with specific DNA-binding proteins, such as Mig1p in the case of glucose-repressed genes, Rox1p in the case of oxygen-regulated genes, and the α2/Mcm1 protein complex for the a-specific genes or the α2/a1 complex for the haploid-specific genes. These proteins bind to specific URSs in the promoter region of the regulated genes. While the repression activity of Ssn6–Tup1 seems to be constitutive, the expression or the activity of these DNA-binding proteins can be modulated in response to different stimuli, allowing in this way specific gene regulation through the same general repressor (Keleher *et al*., 1992; Tzamarias and Struhl, 1995).

Repression conferred by URS_{HAL1} seems to act through a similar mechanism, although the DNA-binding protein has not yet been identified. Computer analysis of this promoter region with MatInspector (Quandt *et al*., 1995) revealed no known DNA-binding motifs for yeast transcription factors. However, URS_{HAL1} contains three repeats of the motif GGGAAA. Another repeat of this motif is also present in the region immediately upstream of URS_{HAL1}. Nevertheless, a 46 bp promoter fragment containing two of these repeats was not able to confer repression. Footprinting experiments and genetic analysis are under way to identify the transcription factors binding to URS_{HAL1}.

The effect of the *ssn6*-Δ*9* mutation is not restricted to *HAL1*. The HOG-dependent *CTT1*, *ALD2* and *ENA1* genes are strongly derepressed in the mutant even in the absence of osmotic stress. The high levels of expression of stress genes correlate with an increased tolerance to salt and oxidative stress. Moreover, the fact that other stress responses such as the DNA damage response or the response to limiting oxygen have been described to be affected by the same co-repressor suggests that the Ssn6–Tup1 repression system might be a general modulator of stress responses.

Interestingly, in the *ssn6*-Δ*9* mutant, most of the HOG-dependent genes exhibited high levels of expression even in the absence of osmotic stress. This indicates that the contribution of the HOG pathway is not required for the transcription of these genes in the *ssn6*-Δ*9* mutant and suggest that at least part of the effect of the activation of the HOG pathway would be the release from repression by the Ssn6–Tup1 system. This seems to be the case for the *ENA1* gene. This gene is derepressed completely in the *ssn6*-Δ*9* mutant and salt stress cannot induce transcription further. Despite the fact that *ENA1* is a HOG-dependent gene, it contains no functional STRE elements in its promoter region. Also, we have found that the osmotic induction of *ENA1* is mediated by a URS. Repression through this URS requires the Ssn6–Tup1 complex and is abolished during osmotic stress in wild-type, but not in a *hog1* mutant (M.Proft and R.Serrano, in preparation).

The idea of the Ssn6–Tup1 repression system as a target for the HOG pathway is reinforced by the fact that *ssn6* and *tup1* null mutations can suppress the osmotic sensitivity of a *hog1* mutant. However, the HOG-dependent genes investigated exhibited different expression patterns. While the *ENA1* gene seems to be completely deregulated in the *ssn6*-Δ*9* mutant, *GPD1*, *CTT1* and *ALD2* expression is still induced by osmotic stress. The *SIP18* and *PAI3* genes are not derepressed under normal conditions in the *ssn6*-Δ*9* mutant, but their expression is increased greatly under osmotic stress. In our view, this multitude of regulatory patterns may arise from different combinations of positive and negative elements at the promoter level. Indeed, studies on the promoters of several stress-induced genes, e.g. *CTT1* and *HSP12*, revealed the presence of both positive (STRE) and negative elements in the same promoter (Belazzi *et al*., 1991; Varela *et al*., 1995). Positive and negative *cis*-acting elements have also been found in the promoter region of the *ENO1* gene (Cohen *et al*., 1987), which is also induced by osmotic stress, and sequences with homology to the *ENO1* negative element have been found in the promoter regions of the *GPD1*, *GPP2*, *GCY1* and *DAK1* genes (Norbeck and Blomberg, 1997). However, the contribution of these possible negative elements to osmotic induction has not been studied. Our results suggest that these negative elements might also be involved in the osmotic induction of the genes, and that the final levels of expression of the different genes may come from the contribution of both positive and negative promoter regions. For example, the induction by osmotic stress of the HOG-dependent genes in the *ssn6*-Δ*9* mutant is probably due to the contribution of the HOG-activated STRE elements.

As in the case of other stress genes, *HAL1* is also modulated negatively by high levels of cAMP-dependent protein kinase. The effect of PKA has been described to be mediated by the STRE element, suggesting that PKA could modify directly or indirectly the activity of the transcription factors binding the STRE. Nevertheless, we have shown that the STRE-like sequence in the *HAL1* promoter is not involved in osmotic activation. We have also found that a promoter deletion, pJAM136, lacking the STRE-like sequence but keeping full regulation, is still PKA regulated, suggesting that the STRE-like sequence in the *HAL1* promoter is not functional. Similarly, the *ENA1* gene, that is also modulated negatively by PKA (Márquez and Serrano, 1996), does not contain functional STREs (M.Proft and R.Serrano, in preparation). Hence, since the regulatory patterns and the architecture of the promoter regions of the stress genes seem to be rather diverse, PKA may have more than one target. Remarkably, although *ssn6* or *tup1* null mutations suppressed the osmotic sensitivity of the *hog1* mutant, these mutations were not able to affect any of the phenotypes of the *bcy1* mutant (data not shown). However, the fact that the *bcy1* mutant displays slow growth in normal medium indicates that its phenotype is not only due to an effect on stress gene transcription, and this makes it difficult to interpret these results.

As a final conclusion, our results indicate that the model of osmotic stress regulation in *S.cerevisiae* based on

Table I. List of yeast strains

Strain	Genotype	Source
RS-16	*MATa ura3-251,328,372 leu2-3,112*	Gaxiola *et al*. (1992)
W303-1A	*MATa can1-100 his3–11,15 leu2-3,112 trp1-1 ura3-1 GAL+ ade2-1*	Wallis *et al*. (1989)
YPH499	*MATa ura3 leu2 his3 trp1 lys2 ade2*	Brewster *et al*. (1993)
JBY10	YPH499 with *hog1-Δ1::TRP1*	Brewster *et al*. (1993)
MAY1	YPH499 with *pbs2-Δ2::LEU2*	Brewster *et al*. (1993)
MAP7	YPH499 with *hog1-Δ1::TRP1 tup1::kan^r*	this work
MAP8	YPH499 with *hog1-Δ1::TRP1 ssn6::kan^r*	this work
SP1	*MATa ura3 leu2 trp1 ade8 can1*	Nikawa *et al*. (1987)
S13-58A	SP1 with *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	Nikawa *et al*. (1987)
S13-58A-1	S13-58A with *tpk1^{w1}*	Nikawa *et al*. (1987)
MCY829	MATα *his3 lys2 ura3*	Schultz *et al*. (1990)
MCY1974	*MATα his3 lys2 ura3 trp1 ssn6-Δ9*	Schultz *et al*. (1990)

HOG-dependent activation of a positive promoter element (STRE) was clearly an oversimplification. The emerging picture includes a multitude of positive and negative promoter elements modulated by several signal transduction pathways, and many actors remain to be identified. The role of negative promoter elements and repressor proteins should be investigated in the stress signalling pathways of organisms such as *Schizosaccharomyces pombe* and mammalian cells (Derijard *et al*., 1994; Gupta *et al*., 1995; Raigenaud *et al*., 1995; Shiozaki and Russell, 1996; Wilkinson *et al*., 1996) where current models are based exclusively on activation by stress of transcription factors recognizing positive promoter elements.

Materials and methods

Culture media and growth conditions

YPD and synthetic medium were prepared according to Guthrie and Fink (1991). YPD contained 2% glucose, 1% yeast extract and 2% peptone. Synthetic medium contained 2% glucose, 0.7% yeast nitrogen base without amino acids (Difco), 50 mM MES [2-(*N*-morpholino) ethanesulfonic acid] adjusted to pH 6.0 with Tris, and the amino acids, purine and pyrimidine bases required by the strains. For selection of the Kan^r dominant marker, geneticin (Gibco BRL cat. 10131-019) was added to the YPD plates to a final concentration of 200 μg/ml.

Yeast strains

All yeast strains used in this work are listed in Table I. Strains MAP7 and MAP8 were generated by removing the whole *TUP1* and *SSN6* ORFs respectively in the *hog1* mutant strain JBY10 (Brewster *et al*., 1993). For this purpose, *TUP1::Kan^r* and *SSN6::Kan^r* disruption cassettes were generated by the use of the *LoxP–KanMX–Loxp* cassette according to Güldner *et al*. (1996) and transformed into yeast.

The JAM223 strain was obtained by transforming the W303-1A background with a *BCY1::URA3* disruption cassette (Toda *et al*., 1987). JAM224 and JAM225 were generated by transforming the W303-1A strain with the *SSN6::Kan^r* and *TUP1::Kan^r* disruption cassettes respectively and then introducing a *bcy1* null mutation by transforming with the *BCY1::URA3* disruption cassette.

Yeasts were transformed by the lithium acetate protocol according to Gietz *et al*. (1995). The disrupted genotypes were confirmed by carrying out genomic PCR.

HAL1 promoter analysis

The pRS-909 plasmid (Gaxiola *et al*., 1992) was constructed by fusing the *Bgl*II–*Pvu*II fragment from the *HAL1* locus to the *lacZ* gene in the YEp358R multicopy plasmid (Myers *et al*., 1986).

Site-directed mutagenesis was performed on the *HAL1* promoter to remove the STRE-like sequence by the method of overlap extension using PCR (Ho *et al*., 1989). The two external primers were CCCTCTAGAATCCTCGCAGAAACAGTTT and CCCGGTACCGATGCATCTGATCTATTT at positions –450 and +7, respectively, which introduced *Xba*I and *Kpn*I sites (underlined). The internal primers CGTGGAATGCTCGTGCTAAGC and GCTTAGCACGAGCATTCCACG were designed to introduce two point mutations changing the STRE-like sequence from CCCCT to CTCGT. A wild-type (amplified only with external primers) and the mutagenized version of the promoter were cloned in YEp358R (Myers *et al*., 1986). Both constructions were checked by sequencing.

For the identification of *HAL1* upstream regulatory regions, six different fragments representing 5′ serial deletions of the *HAL1* promoter were obtained by PCR, with 5′ ends ranging from position –401 to position +1 with respect to the ATG codon and with the 3′ end at the *Pvu*II site in the *HAL1* ORF. All the fragments included an *Xba*I site at the 5′ end and a *Pvu*II site at the 3′ end, and were fused to the *Escherichia coli LacZ* ORF by cloning in YIp358R (Myers *et al*., 1986) with *Xba*I–*Sma*I digestion (pJAM135 to pJAM140). To generate pJAM155, the *HAL1* promoter region between –231 and –156 was amplified by PCR, introducing *Hin*dIII sites at both ends, and cloned in the *Hin*dIII site of pJAM139. Transformants with the correct orientation of the *Hin*dIII fragment (checked by sequencing) were selected. All the constructions were integrated in RS16 and at least three independent transformants from each construction were selected and assayed for reporter expression. The integrations were directed to the *URA3* locus by *Nco*I digestion.

To assay the repressing activity of URS_{HAL1} on a heterologous promoter, the 75 bp element was amplified by PCR, introducing *Sal*I sites at both ends, and cloned into the *Sal*I and *Sma*I sites of pLGΔ312S [wild-type yeast *CYC1* promoter fused to the *E.coli LacZ* ORF in a multicopy plasmid (Guarente and Mason, 1983)]. For the *Sma*I cloning, the PCR fragment was ligated directly to *Sma*I-digested pLGΔ312S. For the *Sal*I cloning, the PCR fragment was digested with *Sal*I and cloned into dephosphorylated *Sal*I-digested vector. The 46 bp sequence spanning the central region of the *HAL1* negative element (–216/–170) was also amplified by introducing *Sal*I sites at both ends and was cloned into the *Sal*I site of pLGΔ312S in the same way. In all cases, the resulting constructions were checked by sequencing.

Drop-tests and survival under different stress conditions

The drop-tests were performed by spotting ~2 μl of different dilutions of saturated yeast cultures in the indicated media and then incubating at 30°C for 2 (YPD medium) to 5 days (1 M NaCl-containing medium).

Survival rates after different stresses were determined in exponentially growing yeast cultures. Different aliquots of the cultures were diluted directly and plated in YPD and YPD plus 1 M NaCl, treated for 30 min with 3.5 mM H_2O_2 and then diluted and plated in YPD or heated to 55°C for 30, 60 or 90 min and then diluted and plated in YPD. The survival rate was calculated by comparing the number of colony-forming units before and after the treatments.

The ability of the *bcy1* mutants to grow on a non-fermentable carbon source was determined by plating the strains on potassium acetate-containing plates (2% potassium acetate, 1% yeast extract and 2% peptone).

Band-shift experiments

For the band-shift assays, crude protein extracts were obtained from yeasts grown in either YPD or YPD plus 1 M NaCl to mid-exponential phase (OD = 1.0). Then 50 ml of culture were pelleted and resuspended in 0.4 ml of lysis buffer [200 mM Tris pH 8, 400 mM $(NH_4)_2$ SO_4, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol] and the cells were broken with glass beads. The extracts were stored at –70°C and the protein concentration was determined by the Bradford assay.

The ^{32}P-labelled URS_{HAL1} fragment was obtained by digesting the plasmid pJAM181 with *Sal*I and filling the ends with Klenow and [α-^{32}P]dATP. The resulting 75 bp labelled double-stranded DNA probe was purified by polyacrylamide gel electrophoresis.

For the binding assays, the protein extracts were incubated at room temperature for 15 min with ~0.5 ng of labelled probe in the presence of 0.5 µg of poly(dIdC), 4 mM Tris–HCl pH 7.5, 4 mM $MgCl_2$, 10% glycerol in a final volume of 20 µl. DNA–protein complexes were resolved in 5% polyacrylamide gels with 0.5× TBE buffer (50 mM Tris–HCl pH 8, 50 mM boric acid, 1 mM EDTA). The electrophoresis were carried out for 2 h at 125 mV in a Bio-Rad Protean II vertical electrophoresis cell (Cat. No. 165-1852).

Northern blot and β-galactosidase assays

For gene expression assays, saturated pre-cultures were diluted 100 times in YPD or YPD with NaCl at the indicated concentration and grown at 30°C to mid-log phase (absorbance at 660 nm ~1.0). At this point, either RNA was extracted or β-galactosidase activity assayed.

Total RNA was isolated according to Carlson and Botstein (1982). Poly(A)$^+$ RNA was prepared from total RNA using the Dynal mRNA purification kit (Cat. No. 610.01). For Northern analysis, total RNA (30 µg per sample) or poly(A)$^+$ RNA (2 µg per sample) was separated by electrophoresis in formaldehyde gels (Ausubel *et al*., 1987), blotted to nylon membranes and hybridized to radioactive probes labelled by the random priming method (Feinberg and Vogelstein, 1983). The probes used were: a 0.9 kb *Nsi*I–*Nsi*I fragment containing the whole *HAL1* ORF, a 2.4 kb *Hin*dIII–*Bam*HI *CTT1* fragment from plasmid pBR322-5109 (Spevak *et al*., 1986) (a generous gift of Dr Ruis), a 1.5 kb PCR fragment spanning the whole *GPD1* ORF, a 310 bp *Sac*I–*Sac*I fragment from pVM22 comprising *DDR48* and *PAI3* genes (Miralles and Serrano, 1995), a 700 bp *Nde*I–*Nsi*I fragment for *SIP18 (*Miralles and Serrano, 1995) and a 2.1 kb *Kpn*I–*Kpn*I *HAL3* fragment (Ferrando *et al*., 1995).

β-Galactosidase activity was assayed as described by Gaxiola *et al*. (1992) and normalized to the protein concentration. Given values represent the average of at least three independent transformations.

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