Repressors and Upstream Repressing Sequences of the Stress-Regulated *ENA1* Gene in *Saccharomyces cerevisiae*: bZIP Protein Sko1p Confers HOG-Dependent Osmotic Regulation

MARKUS PROFT* AND RAMÓN SERRANO

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, 46022 Valencia, Spain

The yeast *ENA1/PMR2A* gene encodes a cation extrusion ATPase in *Saccharomyces cerevisiae* which is essential for survival under salt stress conditions. One important mechanism of *ENA1* transcriptional regulation is based on repression under normal growth conditions, which is relieved by either osmotic induction or glucose starvation. Analysis of the *ENA1* promoter revealed a Mig1p-binding motif (-533 to -544) which was characterized as an upstream repressing sequence ($URS_{MIG-ENA1}$) regulated by carbon source. Its function was abolished in a *mig1 mig2* double-deletion strain as well as in either *ssn6* or *tup1* single mutants. A second URS at -502 to -513 is responsible for transcriptional repression regulated by osmotic stress and is similar to mammalian cyclic AMP response elements (CREs) that are recognized by CREB proteins. This $URS_{CRE-ENA1}$ element requires for its repression function the yeast CREB homolog Sko1p (Acr1p) as well as the integrity of the Ssn6p-Tup1p corepressor complex. When targeted to the *GAL1* promoter by fusing with the Gal4p DNA-binding domain, Sko1p acts as an Ssn6/Tup1p-dependent repressor regulated by osmotic stress. A glutathione *S*-transferase–Sko1 fusion protein binds specifically to the $URS_{CRE-ENA1}$ element. Furthermore, a *hog1* mitogen-activated protein kinase deletion strain could not counteract repression on $URS_{CRE-ENA1}$ during osmotic shock. The loss of *SKO1* completely restored *ENA1* expression in a *hog1* mutant and partially suppressed the osmotic stress sensitivity, qualifying Sko1p as a downstream effector of the HOG pathway. Our results indicate that different signalling pathways (HOG osmotic pathway and glucose repression pathway) use distinct promoter elements of *ENA1* ($URS_{CRE-ENA1}$ and $URS_{MIG-ENA1}$) via specific transcriptional repressors (Sko1p and Mig1/2p) and via the general Ssn6p-Tup1p complex. The physiological importance of the relief from repression during salt stress was also demonstrated by the increased tolerance of *sko1* or *ssn6* mutants to Na^+ or Li^+ stress.

The study of adaptation mechanisms during salinity stress in the yeast *Saccharomyces cerevisiae* has revealed several components of sensing and signal transduction pathways, as well as target genes whose expression is activated upon salt stress (for review see references 18, 45, and 46). Increased expression of the *ENA1* gene has been found to represent a crucial cellular response after salt challenge. The *ENA/PMR2* gene cluster of *S. cerevisiae* contains a tandem array of nearly identical genes encoding P-type ATPases involved in the extrusion of Na^+ and Li^+ ions from the cytoplasm (17, 59). Active export of these toxic ions is a crucial cellular process to avoid deleterious intracellular Na^+ and Li^+ concentrations. Mutants lacking the first *ENA* gene in the gene cluster (*ena1*) are hypersensitive to salt stress (17). The *ENA1* gene is highly regulated at the transcriptional level, and its expression is increased strongly in response to salt stress (12) and glucose starvation (1, 41).

Salt induction of *ENA1* expression depends on both the calcineurin pathway and the high-osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway (28). The first pathway is activated by high concentrations of either Na^+ or Ca^{2+} and is dependent on the phosphoprotein phosphatase calcineurin (28, 33). A calcium signalling pathway composed of calmodulin (7), the calcineurin heterodimeric enzyme (6, 23),

and the zinc finger transcriptional activator Crz1/Tcn1/Hal8p (31, 32, 50) has been reported to contribute to the resistance of yeast to elevated concentrations of several cations (Na^+, Li^+, and Mn^{2+}). Therefore, Ca^{2+}/calmodulin signalling may act, at least in part, through the transcriptional activation of ion transporter genes such as *ENA1*.

The HOG pathway responds to moderate concentrations of osmotic agents and rapidly activates via a multistep phosphorelay mechanism the Hog1p MAP kinase by Tyr phosphorylation (2, 26, 39). Although a great number of genes have been found to need HOG signalling for their osmotic up-regulation, the mechanism of gene activation through phosphorylated Hog1p kinase is still unknown. In *Schizosaccharomyces pombe*, the basic leucine zipper (bZIP) transcriptional activator Atf1p has been identified as a direct phosphorylation target of the Hog1p homolog MAP kinase Sty1p (49, 51, 60). Activated Atf1p, in turn, can bind directly to UASs (upstream activating sequences) located in various stress-regulated promoters and then trigger gene expression (60). In *S. cerevisiae*, stress response promoter elements (STREs) represent UASs that respond to a great variety of stresses (22, 27, 43) and are bound by the zinc finger activators Msn2p and Msn4p (30, 42). Recent work, however, indicates that osmotic induction of several genes including *ENA1* occurs by the release from transcriptional repression (29) and involves the general repressor complex Ssn6p-Tup1p. In the case of the *HAL1* gene, an upstream repressing sequence (URS) regulated by osmotic stress has been identified (29). This mechanism based on regulated re-

* Corresponding author. Mailing address: Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera s/n, 46022 Valencia, Spain. Phone: 34-96-3877860. Fax: 34-96-3877859. E-mail: mproft@ibmcp.upv.es.

TABLE 1. Strains of *S. cerevisiae* used in this work

Strain	Genotype	Reference
W303-1A	*MAT**a** can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 ade2-1*	57
MAP5	W303-1A with *tup1::loxp-KAN-loxp*	This work
MAP6	W303-1A with *ssn6::loxp-KAN-loxp*	This work
MAP12	W303-1A with *mig1::loxp-KAN-loxp*	This work
MAP19	W303-1A with *sko1::loxp-KAN-loxp*	This work
MAP20	W303-1A with *bcy1::LEU2*	This work
MAP21	W303-1A with *mig2::loxp-KAN-loxp*	This work
MAP24	W303-1A with *mig1::loxp mig2::loxp-KAN-loxp*	This work
MAP28	W303-1A with *mig1::loxp mig2::loxp sko1::loxp-KAN-loxp*	This work
MAP32	W303-1A with *hog1-Δ1::TRP1*	This work
MAP33	W303-1A with *hog1-Δ1::TRP1 sko1::loxp-KAN-loxp*	This work
YPH499	*MAT**a** ura3 leu2 his3 trp1 lys2 ade2*	2
JBY10	YPH499 with *hog1-Δ1::TRP1*	2
SKY683	W303-1A with *cnb1::LEU2*	11
SFY526	*MAT**a** ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 URA3::GAL1-lacZ*	1a
MAP34	SFY526 with *ssn6::loxp-KAN-loxp*	This work
MAP35	SFY526 with *tup1::loxp-KAN-loxp*	This work

pressors bound to URSs is similar to the one operating in carbon source regulation.

A great number of yeast genes, including *ENA1*, are derepressed under glucose starvation conditions, and for many of them the inactivation of the general repressor complex Mig1p-Ssn6p-Tup1p (21, 36, 54) through the protein kinase Snf1p (3, 53) has been reported as an important mechanism of glucose-regulated transcriptional control.

In this work, we analyzed the promoter of the *ENA1* gene and found that transcriptional regulation during osmotic stress as well as during glucose starvation occurs through a repression mechanism dependent on the Ssn6p-Tup1p general corepressor. Signalling through general glucose repression occurs through a Mig1/2p binding site (URS$_{MIG-ENA1}$), whereas osmotic stress signalling through the HOG pathway is mediated through a cyclic AMP (cAMP) response element (CRE)-like sequence (URS$_{CRE-ENA1}$) that is bound by the bZIP transcriptional factor Sko1p.

MATERIALS AND METHODS

Strains and growth conditions. The *S. cerevisiae* strains used in this work are listed in Table 1. Gene disruptions using the *loxp-KAN MX-loxp* cassette were carried out as described previously (16). All null mutations were verified by genomic PCR. YPD (or YPGal) contained 2% glucose (or 2% galactose), 2% peptone, and 1% yeast extract. Synthetic medium (SD) contained 2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), and the amino acids purine and pyrimidine bases required by the strain of interest. Yeast cells were transformed as described elsewhere (15). The growth of yeast strains under different osmotic and salt stress conditions was assayed by spotting dilutions of saturated cultures onto YPD plates containing the indicated concentrations of osmotic agents or salts.

Construction of plasmids. To analyze the *ENA1* promoter, different stretches from the 5' upstream region were amplified by PCR, generating *Pst*I and *Xho*I restriction sites at the ends. The fragments were then inserted into the *CYC1-lacZ* reporter construct pJS205 (44) that was digested with *Pst*I and *Xho*I. An empty vector control (pMP206) was generated by *Xho*I/*Sal*I digestion of pJS205 to remove the same *CYC1* promoter region as in all the insertion constructs and subsequent self-ligation. The URS$_{MIG-ENA1}$-*CYC1-lacZ* plasmid pMP222 was constructed by inserting the double-stranded oligonucleotide AGCTATTTTGCGGGGCATCGAT (giving *Hin*dIII-compatible ends after hybridization; the original *ENA1* sequence is underlined) into pMP206. Similarly, the URS$_{CRE-ENA1}$-*CYC1-lacZ* plasmid pMP224 was constructed by inserting the double-stranded oligonucleotide AGCTATCGATTATTTCCTACTTCTATGACGTTT (the original *ENA1* sequence is underlined) into pMP206 digested with *Hin*dIII. Constructs pMP226 (CRE-*CYC1-lacZ*) and pMP227 (mutant CRE [CRE*]-*CYC1-lacZ*) were obtained by insertion of double-stranded oligonucleotides ENACRE1/2 AGCTATCGATCTATGACGTTT (for pMP226; the original CRE sequence from *ENA1* is underlined) or ENACRE3/4 AGCTATCGATCTATGAT*GTTT (for pMP227; the point mutation within CRE is indicated by the asterisk) into pMP206 digested with *Hin*dIII. In all cases, the number and

orientation of inserted oligonucleotides were determined by sequencing. A *GAL4*$_{DBD}$-*SKO1* fusion plasmid (pMP235) was obtained by inserting a PCR fragment (*Sma*I/*Sal*I) containing nearly the entire *SKO1* gene (coding region for amino acids 4 to 647) in the two-hybrid vector pGBT9 (Clontech, Palo Alto, Calif.).

β-Galactosidase assay. Transformed yeast strains were grown selectively until saturation in the appropriate SD liquid media and were diluted into YPD. Logarithmically growing cells (optical density at 660 nm of 0.5 to 0.8) were then transferred to fresh YPD, YPGal, or YPD with NaCl, KCl, or sorbitol, and β-galactosidase activity was determined after 1 h (0.3 M NaCl and KCl, 0.5 M sorbitol) or 4 h (YPGal, YPD with 0.8 M NaCl or KCl). The enzyme assay was performed as described elsewhere (14). Results presented are mean values obtained from at least three independent transformants measured in duplicate.

Purification of GST-Sko1p and gel retardation. The entire reading frame of *SKO1* was obtained by PCR using genomic DNA as template generating a *Nco*I restriction site around the ATG start site and a *Sal*I restriction site after the stop site of *SKO1*. The fragment was inserted into the bacterial glutathione *S*-transferase (GST) expression vector pGEX-KG (Pharmacia Biotech) digested with *Nco*I and *Xho*I. Expression and purification by affinity chromatography of the full-length GST-Sko1 protein were performed as recommended by the manufacturer. For the protein-DNA binding studies, the double-stranded oligonucleotides ENACRE1/2 and ENACRE3/4 were ^{32}P-labeled by the Klenow fill-in reaction and purified by polyacrylamide gel electrophoresis. In the binding assays, approximately 1 μg of GST-Sko1p was incubated with 0.5 ng of labeled probe in the presence of 0.5 μg of poly(dI-dC)–10 mM HEPES (pH 7.4)–15% glycerol–0.1 mM EDTA–20 mM NaCl–4 mM MgCl$_2$–2 mM dithiothreitol at room temperature for 20 min. Binding reaction mixtures were directly loaded onto a 4% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA).

RESULTS

Deletion analysis of the *ENA1* promoter. A preliminary analysis of the *ENA1* promoter has shown that the region up to −752 (translational start point is +1) is responsible for the osmotically induced, calcineurin-independent expression of *ENA1* (1). To identify sequences in this upstream control region of *ENA1* that are important for its regulated expression, we investigated a set of PCR-generated segments of the *ENA1* promoter in a *CYC1-lacZ* test vector under basal (YPD) and induced (0.3 M NaCl) conditions (Fig. 1). The control construct pMP206 gave under both conditions high levels of β-galactosidase activity due to the *CYC1* TATA box-mediated expression. Insertion of a large upstream region of *ENA1* (−317 to −742) resulted in a strong decrease of expression under normal growth conditions, while β-galactosidase levels again reached control values after osmotic shock. This result indicated that regulation of the *ENA1* gene consists mainly of a derepression (rather than an activation) process. We also compared the induction of the artificial fusion of *ENA1* (−317 to
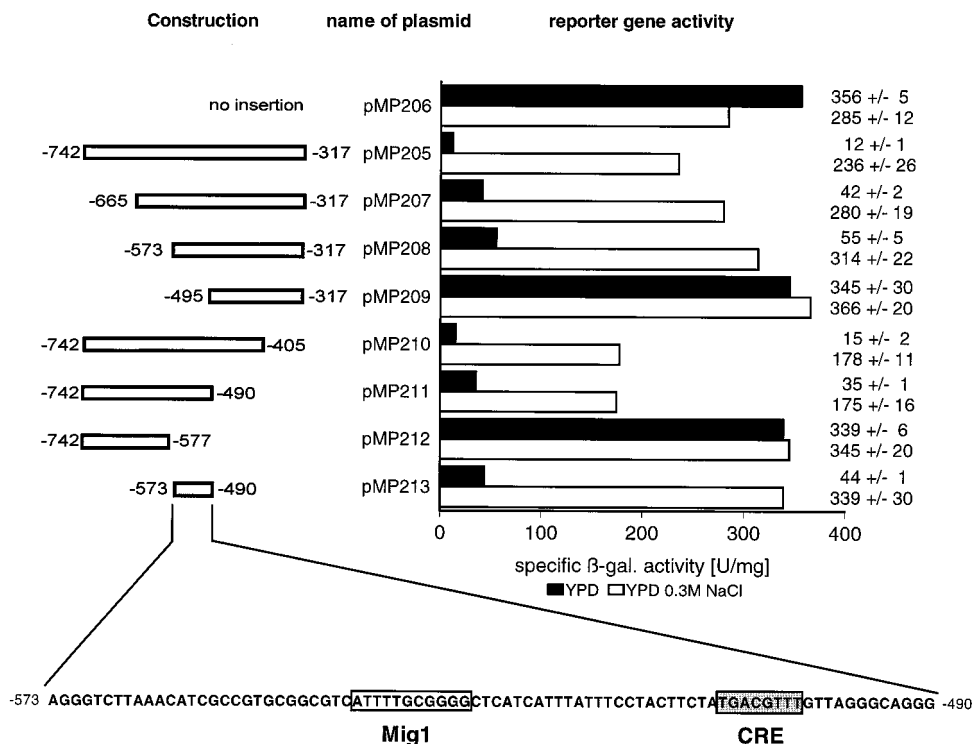
FIG. 1. Deletion analysis of the *ENA1* promoter. Segments from the *ENA1* upstream region indicated at the left were inserted into a *CYC1-lacZ* reporter. β-Galactosidase (β-gal.) specific activity (nanomoles per minute per milligram) was determined in transformed wild-type cells (W303-1A) after growth without (YPD) or with (YPD–0.3 M NaCl) salt. Absolute values including the standard deviation are given at the right. The sequence of URS$_{ENA1}$ (−490 to −573) is depicted at the bottom.

−742) with the *CYC1* TATA box in construct pMP205 with that of an entire *ENA1-lacZ* fusion (up to −1380), but neither the induction capacity nor the kinetics of induction by 0.3 M NaCl was found to be significantly different (data not shown). Therefore, the promoter region from −317 to −742 was subjected to further deletion analysis (Fig. 1, plasmids pMP207 to pMP213). Subsequent removal of 5′ or 3′ sequences resulted in a total loss of regulation when the region from −490 to −573 (referred to below as URS$_{ENA1}$) was affected (plasmids pMP209 and pMP212). Moreover, this region alone was able to change the constitutive control into a salt-regulated promoter by its function as a repressor under basal conditions (plasmid pMP213). Interestingly, the only STRE sequence found in the *ENA1* upstream region (AGGGG; −651 to −647) did not contribute to the regulation because its removal caused no loss of the responsiveness to stress (compare pMP207 and pMP208 in Fig. 1), and a promoter version containing STRE but not URS$_{ENA1}$ was no longer inducible by NaCl (pMP212). Assuming that URS$_{ENA1}$ contained the relevant protein-binding sites for the stress-regulated expression, we examined this 84-bp region by computer analysis using the MatInspector program (40). Within the URS element, two putative recognition sequences for transcription factors were found. Nucleotides −544 to −534 (ATTTTGCGGGG) perfectly match the consensus binding sequence of the yeast transcriptional repressor Mig1p (24), and nucleotides −509 to −502 (TGACGTTT) showed a similarity to mammalian CREs (4, 35).

The *ENA1* promoter contains functional Mig1p-binding and CRE sites. Preliminary characterization of the *ENA1* control region qualified the region from −490 to −573 as a URS element. Since we found that the repression effect through URS$_{ENA1}$ was counteracted by osmotic stress (0.3 and 0.8 M of

either NaCl or KCl) as well as by glucose starvation (galactose or ethanol as the carbon source [data not shown]), we now addressed the question of whether the response to these different environmental changes is triggered by distinct promoter motifs. By testing the two promoter elements separately, we found that both are efficiently repressing transcription under basal conditions (Fig. 2). However, they respond to completely different stimuli. A URS$_{MIG-ENA1}$-regulated reporter gene was exclusively derepressed by glucose starvation (pMP222 with YPGal) but not by osmotic induction, while a URS$_{CRE-ENA1}$-regulated reporter gene responded exclusively to osmotic stress (pMP224 with NaCl, KCl, and sorbitol) but not to glucose starvation. From these results, we conclude that glucose derepression and hyperosmotic shock induce *ENA1* expression independently via (at least) two distinct URS elements, URS$_{MIG-ENA1}$ and URS$_{CRE-ENA1}$, whose separation allowed us now to investigate the function of transcription factors and signalling components that would specifically affect these repression elements.

Repression through URS$_{MIG-ENA1}$ depends on the function of Mig1p and Mig2p. To characterize the roles of the two zinc finger repressors, Mig1p and Mig2p, that have been already reported to interact with the GC box motif (25, 36), we tested the repression effect of URS$_{MIG-ENA1}$ in Δ*mig1*, Δ*mig2*, and Δ*mig1* Δ*mig2* mutant strains. In the absence of either *MIG1* or *MIG2*, repression of a URS$_{MIG-ENA1}$-regulated reporter (pMP222) was only partially lost, while the absence of both genes caused nearly the complete loss of regulation occurring on this promoter element (Fig. 3). This result indicated that both homologous repressors, Mig1p and Mig2p, contribute nearly equally to glucose repression on the *ENA1* promoter. A similar effect of complete deregulation of URS$_{MIG-ENA1}$ as for
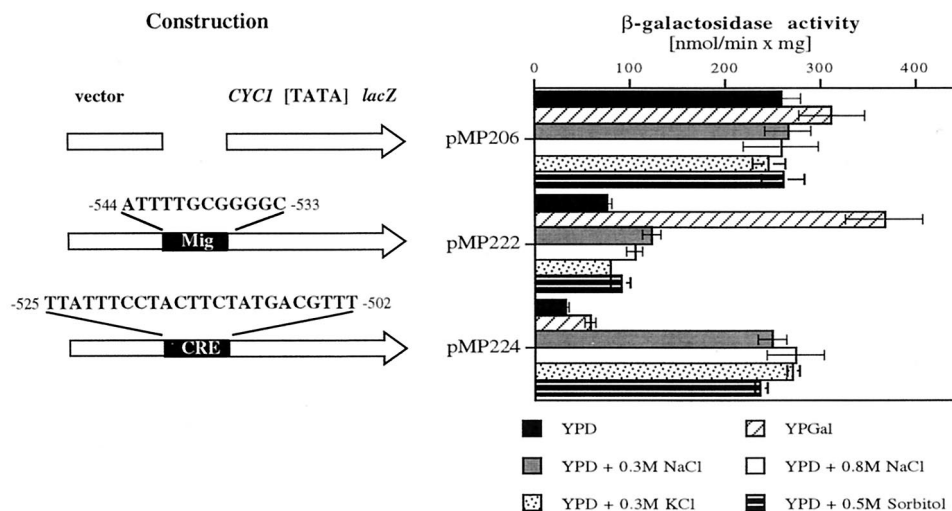
FIG. 2. URS$_{MIG-ENA1}$ and URS$_{CRE-ENA1}$ are functional repressor elements of the *ENA1* promoter. Oligonucleotides containing the indicated sequences of the *ENA1* promoter were inserted into a *CYC1*-[TATA]-driven *lacZ* reporter. β-Galactosidase activity was determined after growth of transformed cells (W303-1A) under basal (YPD), glucose-derepressed (YPGal), salt stress (YPD–0.3 M NaCl, YPD–0.8 M NaCl), or osmotic stress (YPD–0.3 M KCl or YPD–0.5 M sorbitol) conditions.

the Δ*mig1* Δ*mig2* mutant was observed in the absence of components of the general repressor complex *SSN6-TUP1* (Fig. 3).

Regulation through URS$_{CRE-ENA1}$ requires the functions of the bZIP repressor Sko1p (Acr1p) and the corepressors Ssn6p and Tup1p. The yeast *SKO1* (*ACR1*) gene has been found to encode a bZIP transcriptional repressor that binds to CRE sequences, although the physiological role of the protein remained undetermined (37, 56). We therefore examined whether the URS$_{CRE-ENA1}$ promoter element would need Sko1p for its repression function. Indeed, as depicted in Fig. 4, a Δ*sko1* mutant showed a complete loss of regulation through the CRE-like *ENA1* sequence, indicating that Sko1p is the CRE-interacting protein responsible for the osmotically regulated repression of *ENA1*. The URS$_{CRE-ENA1}$ motif, like the URS$_{MIG-ENA1}$ element, was dependent on a functional Ssn6p-

Tup1p general repressor complex since Δ*ssn6* or Δ*tup1* mutants were defective in repression of a URS$_{CRE-ENA1}$-*CYC1*-*lacZ* reporter plasmid (Fig. 4). Although both negative *cis* elements of the *ENA1* gene were dependent on Ssn6p-Tup1p, glucose and osmotic signalling were strictly separated on the level of the DNA-binding transcription factors, since the Δ*sko1* mutation did not affect URS$_{MIG-ENA1}$-regulation, nor were Δ*mig1* mutants defective in URS$_{CRE-ENA1}$ regulation (data not shown). Furthermore, we tested whether URS$_{CRE-ENA1}$ was able to repress activated transcription. We found that when placed upstream or downstream of UAS$_{Rap1}$ (binding site for the transcriptional activator Rap1p), the CRE of *ENA1* was a functional repressor regulated by osmotic stress and dependent on Sko1p and Ssn6p (data not shown).

Sko1p binds to the CRE-like sequence of *ENA1*. To test whether Sko1p can directly and specifically interact with the
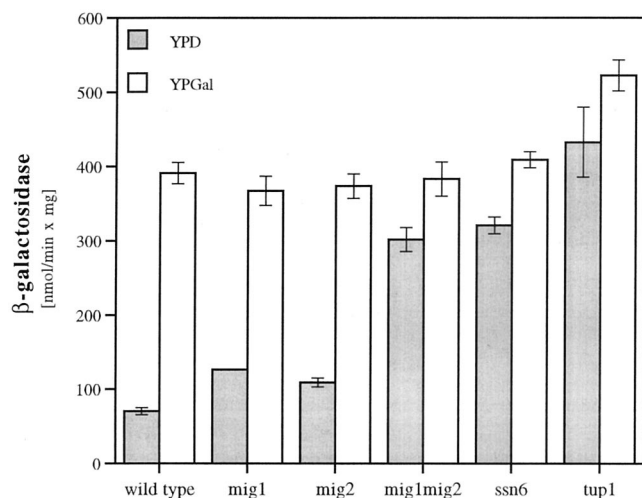

FIG. 3. URS$_{MIG-ENA1}$ function depends on *MIG1*, *MIG2*, *SSN6*, and *TUP1*. Repressed (growth in YPD) and derepressed (growth in YPGal) expression of a URS$_{MIG-ENA1}$-*CYC1*-*lacZ* fusion gene (pMP222) was measured in transformed wild-type (W303-1A) and various mutant (MAP12, Δ*mig1*; MAP21, Δ*mig2*; MAP24, Δ*mig1* Δ*mig2*; MAP6, Δ*ssn6*; MAP5, Δ*tup1*) strains.
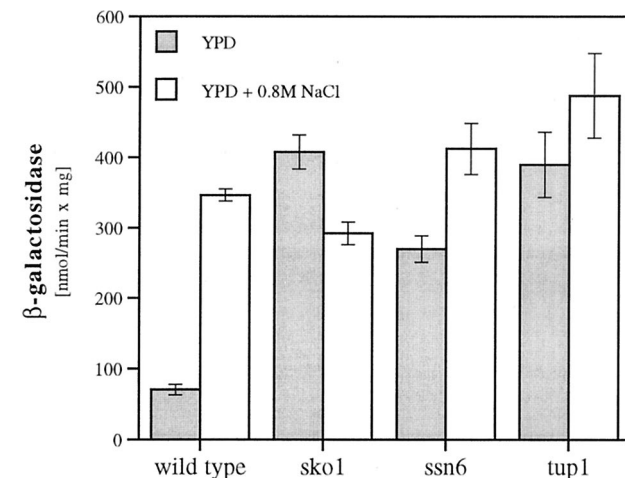

FIG. 4. URS$_{CRE-ENA1}$ function depends on *SKO1*, *SSN6*, and *TUP1*. Repressed (growth in YPD) and derepressed (growth in YPD–0.8 M NaCl) expression of a URS$_{CRE-ENA1}$-*CYC1*-*lacZ* fusion gene (pMP224) was measured in transformed wild-type (W303-1A) and various mutant (MAP19, Δ*sko1*; MAP6, Δ*ssn6*; MAP5, Δ*tup1*) strains.
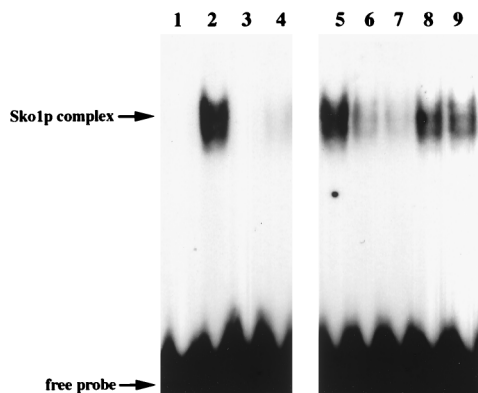
FIG. 5. Sko1p binds to CRE$_{ENA1}$ in vitro. Purified GST-Sko1p was incubated with labeled CRE (TGACGTTT) or CRE* (TGATGTTT). Lanes: 1, labeled CRE without added protein; 2, labeled CRE with added GST-Sko1p; 3, labeled CRE* without added protein; 4, labeled CRE* with added GST-Sko1p; 5, as lane 2; 6 (and 7), competition with 20× (and 50×) excess of CRE; 8 (and 9), competition with 20× (and 50×) excess of CRE*.

CRE motif of the *ENA1* promoter, we performed gel retardation assays using oligonucleotides containing the entire CRE sequence or a point-mutated version of the binding motif changing the core sequence ACGT to ATGT (designated CRE*). As shown in Fig. 5, Sko1p bound specifically to the CRE sequence (lane 2) but not to CRE* (lane 4). Moreover, the Sko1p complex was efficiently competed by the use of nonlabeled CRE but not the CRE* sequence (Fig. 5, lanes 6 to 9). The same oligonucleotides were also tested for transcriptional repression by insertion into the *CYC1-lacZ* reporter system. As shown in Fig. 6, the 12 nucleotides representing the original CRE$_{ENA1}$ motif repressed transcription under nonstress conditions independently of their orientation to the transcription start, while the CRE* sequence was not functional. The repression effect was counteracted by elevated concentrations of either NaCl (Fig. 6), KCl, or sorbitol (data not shown). Taken together, the results indicated that the binding of Sko1p to its CRE target sequence is responsible for *ENA1* repression that is relieved by osmotic shock.

Derepression of URS$_{CRE-ENA1}$ requires signalling through the Hog1p MAP kinase. Induction of *ENA1* expression during salt stress has been reported to be dependent on various sig-
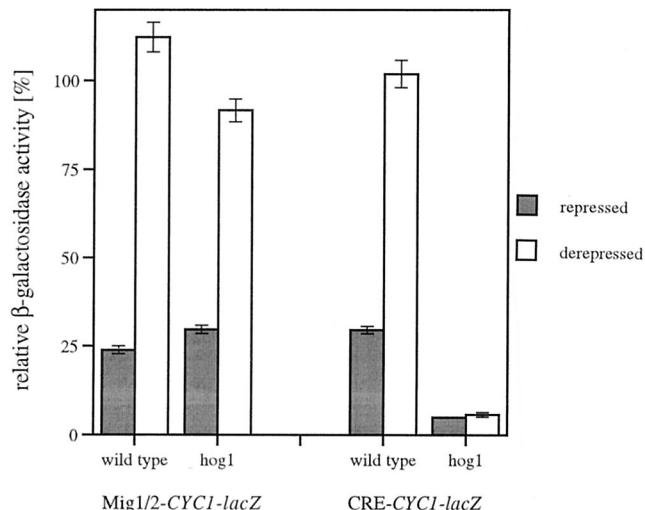

FIG. 7. Derepression of URS$_{CRE-ENA1}$ is dependent on the Hog1p MAP kinase. A URS$_{MIG-ENA1}$-*CYC1-lacZ* (pMP222) and a URS$_{CRE-ENA1}$-*CYC1-lacZ* (pMP224) reporter were assayed in wild-type (YPH499) and Δ*hog1* mutant (JBY10) cells under repressed (YPD) and derepressed (YPGal for pMP222; YPD–0.3 M NaCl for pMP224) conditions. The degree of repression is given as relative β-galactosidase activity compared to the nonregulated empty *CYC1-lacZ* vector.

nalling pathways (28). We therefore attempted to relate the CRE-mediated osmotic regulation to one (or more) of the known signal transduction pathways. We tested a variety of regulatory mutants for their effect on a URS$_{CRE-ENA1}$-*CYC1-lacZ* gene. No significant change in the repression/derepression behavior was found for mutants bearing Δ*cnb1*, defective in calcineurin phosphatase activity, or Δ*bcy1*, defective in signalling through protein kinase A (PKA) by constitutively activating PKAs (data not shown). However, a dramatic effect was observed with a Δ*hog1* mutant with impaired HOG MAP kinase signalling. As can be seen in Fig. 7, the Δ*hog1* strain repressed a CRE-regulated reporter gene even more strongly under basal conditions and was unable to remove repression during osmotic shock, while a Mig1p-binding site-regulated control was not affected. These results strongly suggested that the Sko1p-mediated repression on the CRE sequence is a target of osmotic sensing through the HOG pathway.
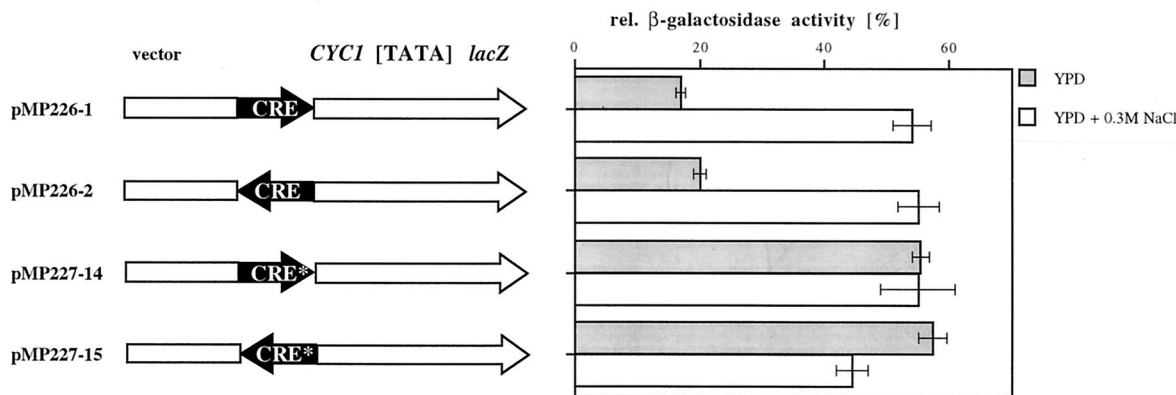

FIG. 6. Binding of Sko1p correlates with the repression of CREs. Oligonucleotides that were used for Sko1p-binding assays (Fig. 5) were tested for repression ability in a *CYC1-lacZ* reporter. Constructions indicating the orientation of oligonucleotide insertion are given at the left. The repression effect of each oligonucleotide is depicted at the right as a percentage of the activity of the control plasmid without any insertion.
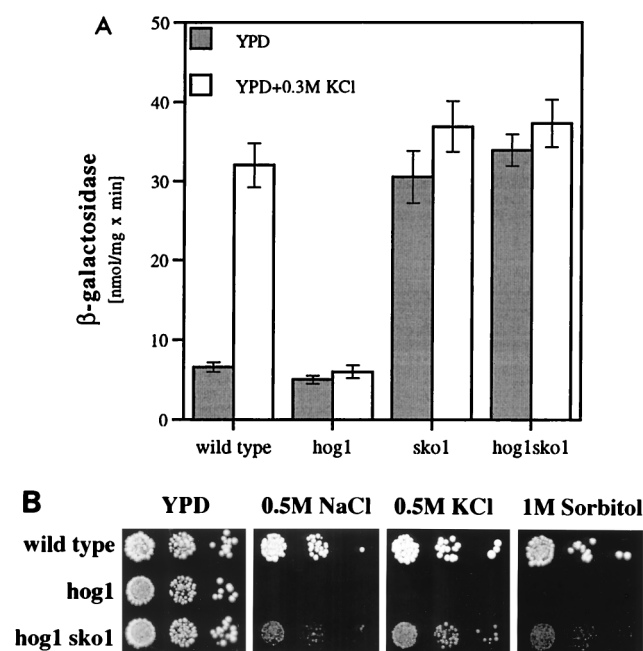
FIG. 8. Disruption of *SKO1* acts in an epistatic manner over the loss of Hog1p function. (A) Expression of an integrative *ENA1-lacZ* gene (pFR70i) was measured under repressed (YPD) and derepressed (YPD–0.3 M KCl) growth conditions in strains W303-1A (wild type), MAP32 (Δ*hog1*), MAP19 (Δ*sko1*), and MAP33 (Δ*hog1* Δ*sko1*). (B) Growth of wild-type strain W303-1A, MAP32 (Δ*hog1*), and MAP33 (Δ*hog1* Δ*sko1*) on high-osmolarity media.

TABLE 2. A Gal4$_{DBD}$-Sko1 fusion protein confers osmotic regulation to the heterologous *GAL1* promoter[a]

Strain	$GAL4_{DBD}$-$SKO1$	β-Galactosidase sp act (nmol/min/mg)		Induction factor
		YPD	0.3 M NaCl	
Wild type	−	1.08 ± 0.19	1.40 ± 0.10	1.3
	+	0.24 ± 0.06	2.39 ± 0.63	10
ssn6	−	1.51 ± 0.20	1.26 ± 0.11	0.8
	+	1.36 ± 0.16	2.74 ± 0.32	2.0
tup1	−	3.51 ± 0.49	2.93 ± 0.39	0.8
	+	4.86 ± 0.58	9.25 ± 0.86	1.9

[a] The $GAL4_{DBD}$-$SKO1$ fusion plasmid pMP235 was expressed in wild-type (SFY526) and in Δ*ssn6* (MAP34) and Δ*tup1* (MAP35) mutant strains. Expression of the integrated *GAL1-lacZ* reporter was assayed after growth in YPD and YPD–0.3 M NaCl. Strains without the *GAL4-SKO1* fusion contained the empty vector pGBT9.

Disruption of *SKO1* suppresses Δ*hog1* mutant phenotypes. To test whether the Hog1p kinase acts through the Sko1p repressor, we investigated epistatic effects of the loss of *SKO1* function over the *hog1* disruption. We examined the expression of an integrated *ENA1-lacZ* fusion gene in Δ*hog1* and Δ*sko1* single mutants and in Δ*hog1* Δ*sko1* double mutants. As shown in Fig. 8A, osmotic induction of *ENA1* by KCl is absent in *hog1* mutants. On the other hand, a strong increase in basal *ENA1* expression (YPD without salt) is observed in a Δ*sko1* strain. This elevated expression cannot be further induced by osmotic shock (0.3 M KCl). With respect to the expression of *ENA1*, the loss of *SKO1* completely compensates for the *hog1* deletion since the Δ*hog1* Δ*sko1* double mutant showed the same elevated expression levels as the Δ*sko1* strain. Therefore, we conclude that in the case of the osmotic up-regulation of *ENA1*, signalling through the HOG MAP kinase pathway acts on the Sko1p transcriptional repressor. This was also confirmed by the finding that additional deletion of *SKO1* in a Δ*hog1* background partially suppressed the osmotic sensitivity of Δ*hog1* mutants (Fig. 8B). Again, this result clearly qualified Sko1p as a downstream effector of the HOG pathway.

A Gal4$_{DBD}$-Sko1 fusion protein confers osmotic regulation to the *GAL1* promoter that depends on Ssn6p and Tup1p function. To prove that Sko1p contains transcriptional repression activity that is regulated by external osmolarity, we targeted the protein to the heterologous promoter of the *GAL1* gene by expressing a $GAL4_{DBD}$-$SKO1$ fusion. As depicted in Table 2 the fusion protein repressed the *GAL1-lacZ* reporter under normal growth conditions (YPD), while this down-regulation was counteracted by osmotic shock (0.3 M NaCl). This led to a strong osmotic regulation of the *GAL1* promoter (10-fold) that is normally not regulated by salt. Moreover, the differential transcriptional control through Gal4-Sko1p is

largely abolished in either *ssn6* or *tup1* mutants (Table 2), indicating that Sko1p function is dependent on the Ssn6-Tup1p corepressor complex.

***ENA1* expression is modulated by multiple repressors.** To test the extent to which the repressors Mig1/2p and Sko1p contribute to *ENA1* regulation, we measured the transcriptional regulation in the whole promoter context, using an integrative *ENA1-lacZ* fusion. In general, the loss of one of the three repressors caused a partial derepression of *ENA1* under normal growth conditions and concomitantly a drop in the level of induction observed upon glucose starvation or salt shock (Table 3). This was in agreement with the speculative role of all three repressors influencing the basal expression of *ENA1*. As was found for the specific regulation of URS$_{MIG-ENA1}$, the whole *ENA1* promoter was under the additive control of both repressors Mig1p and Mig2p since the double mutant showed an even higher degree of derepression than either of the single mutants. Although induction by both galactose and NaCl was reduced in a Δ*mig1* Δ*mig2* strain, specific involvement of Mig1/2p in salt induction is unlikely because a separate URS$_{MIG-ENA1}$ clearly was not derepressed by salinity. The loss of *SKO1* caused an increase in basal expression, and the inducibility of *ENA1* upon salt stress was severely diminished whereas induction upon glucose starvation was only slightly affected. This was in agreement with the previous finding that Sko1p is responsible for the part of repression that is susceptible to osmotic stress. However, Δ*sko1* mutants still showed a fourfold derepression upon severe Na$^+$ stress, and a Δ*sko1* Δ*mig1* Δ*mig2* strain, although dramatically impaired for *ENA1* transcriptional regulation, still responded to a high sodium shock, indicating that part(s) of the salt-regulated repression is not regulated by Sko1p. Therefore, we also derepressed *ENA1* transcription by osmotic stress by using 0.8 M KCl, which does not activate calcineurin signalling (28). Under these conditions, the wild type increases *ENA1* expression fivefold, while no derepression occurs in the *sko1* null mutant, which has derepressed *ENA1* expression levels under normal conditions (Table 3). Similar results were obtained with moderate concentrations of NaCl (0.3 M [data not shown]), indicating that Sko1p mediates osmotic induction whereas upon high-sodium challenge, the *ENA1* gene is additionally up-regulated by calcineurin-mediated activation. The most severe phenotype with respect to *ENA1* expression was exhibited by a Δ*ssn6* mutant strain, which lost all responsiveness due to the total derepression of the gene under nonstress conditions (Table 3). By applying severe salt stress under glucose derepression conditions (YPGal plus 0.8 M NaCl), the

TABLE 3. *ENA1* expression is modulated by multiple repressors[a]

Strain	β-Galactosidase sp act (nmol/min/mg of protein)				Derepression factor		
	YPD	YPGal	YPD–0.8 M NaCl	YPD–0.8 M KCl	Galactose	0.8 M NaCl	0.8 M KCl
Wild type	6.3 ± 0.6	40.9 ± 1.7	81.6 ± 4.1	32.0 ± 2.8	6.5	13.0	5.1
Δ*mig1*	15.4 ± 0.9	64.1 ± 7.6	130.7 ± 17.1	ND	4.2	8.5	ND
Δ*mig2*	19.8 ± 1.8	93.2 ± 10.5	192.4 ± 21.5	ND	4.7	9.7	ND
Δ*mig1* Δ*mig2*	64.8 ± 0.9	138.6 ± 13.9	287.5 ± 28.9	ND	2.1	4.4	ND
Δ*sko1*	30.5 ± 3.3	155.4 ± 17.6	126.3 ± 19.3	36.9 ± 3.2	5.1	4.1	1.2
Δ*sko1* Δ*mig1* Δ*mig2*	101.3 ± 11.3	185.9 ± 19.8	364.5 ± 42.8	ND	1.8	3.6	ND
Δ*ssn6*	316.7 ± 27.8	399.0 ± 46.1	423.7 ± 45.9	ND	1.2	1.3	ND

[a] Transcriptional regulation of *ENA1* was monitored by using an integrative *ENA1-lacZ* fusion (pFR70i) under repressed (YPD) and derepressed (YPGal, YPD–0.8 M NaCl, or YPD–0.8 M KCl) growth conditions. The strains used were W303-1A, MAP12, MAP21, MAP24, MAP19, MAP28, and MAP6 (see Table 1 for genotypes). ND, not determined.

ENA1-lacZ gene was completely derepressed in the strains tested up to levels comparable to the expression observed in the Δ*ssn6* strain (data not shown). We also tested whether the elevated *ENA1* expression in the various mutants had consequences for the survival under osmotic and salt stress conditions. While a Δ*mig1* Δ*mig2* mutant strain grew only slightly better under conditions of elevated Li$^+$ concentrations, the Δ*sko1* and Δ*ssn6* mutant strains clearly showed a greater resistance to high concentrations of Na$^+$ and Li$^+$ (Fig. 9). Under osmotic stress conditions (1.5 M sorbitol and 1.5 M KCl), all strains grew similarly. According to their different degrees of derepression of the *ENA1* gene (Table 3), Δ*ssn6* mutants were more resistant to severe salt stress than Δ*sko1* mutants, as was particularly evident for Li$^+$ resistance. However, loss of the glucose-regulated repression in the Δ*mig1* Δ*mig2* double mutant did not give rise to a clear salt resistance despite activating *ENA1* expression even more strongly than the Δ*sko1* mutation. This result suggested that the Sko1p-Ssn6p/Tup1p-mediated regulation may also play an important role in the repression of other salt stress defense genes that are not affected by glucose repression.

DISCUSSION

Increased expression of the *ENA1* sodium and lithium extrusion ATPase in *S. cerevisiae* is a crucial adaptation to salt stress. We have shown that the underlying regulatory mechanism to adjust *ENA1* expression is based mainly on a negative control through multiple DNA-binding repressors that act together with the general corepressor complex Tup1p-Ssn6p. Deletion of *SSN6* has dramatic consequences with respect to the transcriptional regulation of *ENA1*, since the gene is nearly expressed to the maximal level under nonstress conditions and only a marginal up-regulation upon salt stress or glucose starvation is left. Similar results were obtained for a *tup1* null mutant (data not shown). This is in complete agreement with the recent finding that the Ssn6p-Tup1p complex is involved in

the osmotic regulation of stress-regulated genes (29). To different extents, transcriptional repression modulates the expression of other salt-inducible genes such as *GPD1*, *CTT1*, and *ALD2*, whose basal transcription is clearly increased in a Δ*ssn6* background (29). In the case of these genes, a further activation during osmotic shock can be observed. Therefore, the overall regulation on these promoters is composed of a negative component keeping transcript levels low during normal growth and a positive component that activates transcription only when cells are confronted with high salinity or other stresses. A positively acting STRE with the core AGGGG has been found to be responsible for transcriptional activation as a response to a great variety of stresses (22, 27, 43). STREs can be regarded as the promoter elements that trigger the cellular multistress response, as they can be activated by osmotic, oxidative, and heat stress, as well as by nutrient starvation. Osmotic induction of STRE is dependent on signalling through the HOG MAP kinase pathway (43), and generally STRE regulation is negatively affected by the RAS-cAMP pathway (27). The two zinc finger proteins Msn2p and Msn4p directly bind and activate STRE sequences and are important determinants of the multistress resistance of yeast cells (30, 42). Clearly the *ENA1* gene is not regulated by STREs. Only one sequence that matches the core sequence AGGGG (positions −651 to −647) can be found in the *ENA1* upstream region, but this promoter region is dispensable for stress induction (Fig. 1 and reference 1). Moreover, in a Δ*msn2*Δ*msn4* mutant background, *ENA1* transcriptional regulation upon salt shock and glucose starvation is not affected compared to the wild type (1, 39a). Together with the finding that in repression-deficient (Δ*ssn6* or Δ*tup1*) mutants *ENA1* transcription is nearly fully activated without any exposure to osmotic stress, it has to be concluded that stimulation of *ENA1* expression by osmotic stress acts through the inactivation of repression. According to this concept, we present the identification of two separated promoter elements that mediate negative regulation via two
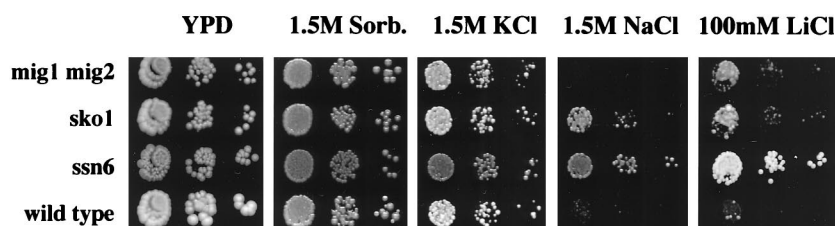

FIG. 9. Mutants Δ*sko1* and Δ*ssn6* are resistant to Na$^+$ and Li$^+$ stress. Growth of mutant strains MAP24 (Δ*mig1* Δ*mig2*), MAP19 (Δ*sko1*), and MAP6 (Δ*ssn6*) on high-osmolarity media is compared with that of the wild type (W303-1A).

different signalling systems, the general glucose repression pathway and the HOG MAP kinase pathway. The *ENA1* promoter is activated by carbon source starvation and osmotic stress independently, and both stimuli additively increase *ENA1* expression (1, 39a). The following results presented in this work demonstrate that repression by glucose is carried out by the binding of the zinc finger repressors Mig1p and Mig2p to the $URS_{MIG-ENA1}$ element (−533 to −544): (i) a separate $URS_{MIG-ENA1}$ element in single copy confers carbon source-regulated repression to a reporter gene; (ii) Δ*mig1* and Δ*mig2* single mutants (partially) and Δ*mig1*Δ*mig2* double mutants (totally) lose repression of a $URS_{MIG-ENA1}$-regulated reporter gene; and (iii) Δ*mig1*, Δ*mig2*, and Δ*mig1*Δ*mig2* mutants show increased basal levels of expression of an integrated *ENA1-lacZ* gene. Obviously the two homologous repressor proteins Mig1p and Mig2p can recognize the same promoter region within *ENA1*. The same observation, although with a minor contribution of Mig2p, has been made for the glucose-regulated *SUC2* gene (25). The mechanism by which Mig1p-mediated repression is removed upon carbon source starvation occurs through its phosphorylation by the Snf1p protein kinase (38, 38a, 53) and subsequent nuclear export (9). Accordingly, the induction of *ENA1* by glucose derepression is absent in an *snf1* null mutant (1).

Osmotic induction of many genes requires the HOG MAP kinase signalling cascade, and for *CTT1* (encoding cytosolic catalase) and *HSP12* (encoding a small heat shock protein), the importance of activated STREs has been demonstrated to be crucial for this process (43, 55). Nevertheless, it remains to be determined how the activated HOG pathway finally stimulates STRE-driven transcription via its binding factors Msn2p and Msn4p. Although *ENA1* is a HOG-dependent gene, its differential expression during osmotic shock is completely independent of Msn2p/4p and STREs. A very different mechanism must be proposed to explain how the active Hog1p MAP kinase can modulate *ENA1* expression. We provide several lines of evidence that this mechanism consists of the repressing activity of the bZIP transcription factor Sko1p, which recruits the Ssn6p-Tup1p corepressor by binding to the $URS_{CRE-ENA1}$ motif: (i) a single copy of the $URS_{CRE-ENA1}$ promoter element (−513 to −502) confers repression to a *CYC1-lacZ* reporter that is abolished under osmotic stress conditions; (ii) in the *sko1*, *ssn6*, and *tup1* null mutants the $URS_{CRE-ENA1}$-mediated repression is completely absent; (iii) a GST-Sko1 fusion protein binds specifically to the CRE_{ENA1} element in vitro; (iv) a $Gal4_{DBD}$-Sko1 fusion protein acts as a Ssn6p/Tup1p-dependent repressor of the *GAL1* promoter that is counteracted by osmotic stress; (v) in a *hog1* null mutant, repression occurring on $URS_{CRE-ENA1}$ is constitutive and cannot be overcome during salt treatment; (vi) osmotic stress-sensitive phenotypes of Δ*hog1* mutants can be rescued by additional deletion of *SKO1*; and (vii) Δ*sko1* mutants have increased basal *ENA1* expression and are hyperresistant to Na$^+$ and Li$^+$ stress. Therefore, we propose a model of osmotic gene induction that implies the inactivation of a Sko1p-Ssn6p/Tup1p repressor complex by the activity of the Hog1p kinase. This scenario has an interesting parallelism to stress signalling in fission yeast and higher eukaryotes, where transcription factors of the bZIP family are phosphorylation targets of MAP kinase cascades (19, 49, 52, 60). In the specific case of *S. pombe*, a signal transduction pathway that is activated by a broader spectrum of adverse environmental conditions (osmotic, oxidative, heat, and UV stress) than in the case of the *S. cerevisiae* HOG pathway has been identified. It contains the Hog1p-homologous MAP kinase Sty1p (Spc1p) (20, 34, 47) that is activated by the MAP kinase kinase Wis1p (48, 58). As a final signalling target in *S.*

pombe, the pleiotropic bZIP activator Atf1p has been identified (49, 51, 60). Activated Atf1p promotes the transcription from various target promoters, including those for stress response genes (*gpd1$^+$*, *fbp1$^+$*, and *ctt1$^+$*) and genes required for sexual differentiation (*ste11$^+$*), through binding to CRE motifs. Although the output of stress signalling in fission yeast was considered the primary Atf1p-mediated gene activation event, recent evidence suggests that transcriptional repression plays an important role in stress regulation of *S. pombe* (8).

Our genetic data strongly implicate the bZIP repressor Sko1p as a possible target of signalling through the HOG pathway of *S. cerevisiae*. Originally the *SKO1* (*ACR1*) gene was identified as a multicopy suppressor of lethal PKA overexpression (37) and by the loss of repression mediated through ATF/CREB sites in a *sko1* mutant (56). A contribution of Sko1p to the repression of *SUC2* (encoding invertase) has been reported (37), although this effect was much weaker than that exhibited by Mig1p. Although a putative PKA target motif (KRRMS) within Sko1p has been described, the relationship of this transcriptional repressor to cAMP signalling has not been described. The in vitro binding of Sko1p homodimers to the canonical CRE (TGACGTCA) and related sequences has been shown (37, 56), but the physiological function of Sko1p remained undetermined. We have demonstrated that Sko1p mediates repression to the *ENA1* gene that is counteracted upon osmotic shock by a mechanism dependent on Hog1p. Whether the Sko1p-CRE interaction also plays an important role in the regulation of other HOG-dependent genes should be investigated by identifying relevant CRE-like sequences in the various promoters. Although we demonstrate that Sko1p confers repression that is released by osmotic stress, a contribution of CRE-binding activators (56) to osmotic control cannot be excluded. We found no influence of signalling through PKA on the responsiveness of $URS_{CRE-ENA1}$ to osmotic shock. In a *bcy1* null mutant with constitutive PKA activity, derepression upon salt shock remained unaffected although in general the expression levels were markedly decreased both in the *CYC1-lacZ* control and in the $URS_{CRE-ENA1}$-regulated reporter (data not shown). This points to a more general modulation through PKA on both basal and stress-induced expression, as has been described for regulation of the whole *ENA1* promoter (28). However, the effect of a *hog1* null mutation on the Sko1p- and Ssn6p-Tup1p-regulated CRE was dramatic, and further experimental approaches will be focused on the likely interaction of Sko1p with the corepressor complex and the mechanism of signal transduction from activated Hog1p MAP kinase to Sko1p. In addition to the data presented in this work, a connection between HOG signalling and transcriptional repression has been established by the finding that also *ssn6* or *tup1* null mutations can partially suppress the osmotic stress-sensitive phenotype of Δ*hog1* mutant cells (29), a result very similar to those presented in this work for the *sko1* null mutation. Interestingly, the induction of the HOG-independent *HAL1* gene (14) by severe osmotic stress also occurs through the release from Ssn6p-Tup1p repression (29). In this case, a protein complex formed on a negative *HAL1* promoter element is abolished under stress conditions. Neither the DNA-binding protein nor the signal-transducing pathway operating in this case has been identified.

In addition to the negative mechanisms of regulation described in this work, *ENA1* is also subjected to positive control. A third signal transduction pathway involving Ca^{2+}/calmodulin-calcineurin contributes to the salt inducibility of the *ENA1* gene (28, 33). Signalling through calcineurin is activated by high Na$^+$ and Ca^{2+} concentrations and leads to the transcriptional activation of genes in addition to *ENA1* such as *PMC1*

and *PMR1*, encoding Ca^{2+}-ATPases (5), and *FKS2*, encoding a subunit of glucan synthase (10, 13). Very recently a calcineurin-dependent zinc-binding transcription factor, Crz1p (Tcn1p, Hal8p), that acts as an activator of transcription has been identified (31, 32, 50). The binding of Crz1p to a calcineurin-dependent UAS element in the *FKS2* promoter has been described (50). Δ*crz1* mutants show decreased *ENA1* expression and are hypersensitive to salt stress (32). Most likely, calcineurin signalling results in *ENA1* transcriptional activation through the binding of Crz1p. The UAS element responsible for this calcineurin-dependent activation in the *ENA1* upstream control region has not been identified. However, a promoter region (positions -752 to -853 in *ENA1*) that responds to high Ca^{2+} therefore could include a binding site for Crz1p has been described (1).

We have demonstrated that Sko1p-mediated repression explains the osmotic regulation of the *ENA1* gene since under conditions that do not activate the calcineurin pathway (0.8 M KCl or 0.3 M NaCl), the *sko1* null mutant cannot further increase *ENA1* expression. By additionally stimulating calcineurin signalling (0.8 M NaCl), the *ENA1* promoter is further activated independently of HOG- and Sko1p-mediated repression (Table 3). Taken together, these findings allow us to propose a model to explain how different environmental signals are integrated on the *ENA1* gene: (i) glucose starvation activates the Snf1p protein kinase that subsequently inhibits Mig1/2p-Ssn6p-Tup1p-mediated repression on the $URS_{MIG\text{-}ENA1}$ element; (ii) the high Na^+ or Ca^{2+} signal is triggered by the calcineurin phosphatase and subsequent activation of Crz1p (Tcn1p, Hal8p), which enhances transcription from a so far unidentified UAS_{ENA1}; (iii) osmotic induction through the HOG pathway operates by counteraction of Sko1p-Ssn6p-Tup1p-mediated repression on the $URS_{CRE\text{-}ENA1}$ element. The variety of regulatory events that influence transcription from the *ENA1* promoter region render this gene a very productive and complex model of stress signalling.

ACKNOWLEDGMENTS

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